Insights into human brain evolution from genomics and transcriptomics

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Bu tez, bana rüzgara karşı yürümeyi öğreten annem Selma Derman-Alagöz ve babam Oktay Alagöz'e ithaf edilmiştir.

This thesis is dedicated to my parents, Selma Derman-Alagöz and Oktay Alagöz, who taught me how to walk against the wind.

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General introduction

1. General introduction

1.1 Human-specific aspects of neuroanatomy and behaviour

Language and human evolution

Homo sapiens is the only species known to have the capacity to acquire, perceive, and produce complex language. Although various forms of acoustic communication have evolved in other species (e.g., bats, songbirds, parrots)[1], the syntactic complexity of human language and its centrality to our species' evolutionary success make it unique. Human infants can acquire language from an early age without formal education, if they are adequately exposed to language input[2], suggesting a biological foundation for this human-specific trait. Specifically, the genetic, cellular and neurobiological bases of language must have evolved in our lineage over the last ~7-9 million years, following the divergence of humans and chimpanzees from a common ancestral population[3]. The evolution of language coincides with substantial changes in human neuroanatomy (e.g., neocortical expansion, reorganisation of white-matter connections)[4], the increased expressive cognition evident in various forms of art[5,6], and remarkable technological advances[7]. This chronological overlap suggests a shared genetic and evolutionary basis for aspects of human language, cognition, and neuroanatomy. Hence, investigating the co-evolution and genetic links among these phenomena offers valuable insights into the neurobiological and evolutionary foundations of what has been referred to as the "*human condition*"[8].

Neuroanatomic differences between humans and non-human primates

Dramatic differences in brain size, neuron numbers, and cerebral convolution complexity have emerged across primates over the ~65-68 million years of their evolution[9], with humans possessing the largest brain among all extant primates[10]. On average, the human brain weighs approximately 1,500 grams and contains about 86 billion neurons, in stark contrast to the average chimpanzee brain, which weighs ~380 grams and contains 28 billion neurons[11,12] (Figure 1). These differences in brain size and neuron counts between humans and non-human primates (NHP) become even more pronounced in more evolutionarily distant species, such as Old and New World monkeys. For instance, the macaque brain weighs approximately 87 grams and contains around six billion neurons, while the marmoset brain weighs about eight grams and contains ~630 million neurons[10,12] (Figure 1).

The increase in human brain size is primarily driven by the expansion of the association cortex[13], regions implicated in higher-order behaviours. Comparative neuroimaging studies of human and chimpanzee brains have revealed that certain cortical regions, such as the anterior and posterior association areas, expanded disproportionately during human evolution[14,15]. Additionally, language-related regions of the human brain, including *Broca's* and *Wernicke's* areas[16,17], are larger and more strongly interconnected than their homologues in NHPs[18,19,20]. These regional neuroanatomic changes suggest that adaptive pressures and developmental

constraints over the course of human evolution shaped cortical areas implicated in specific functions and behaviours[21].

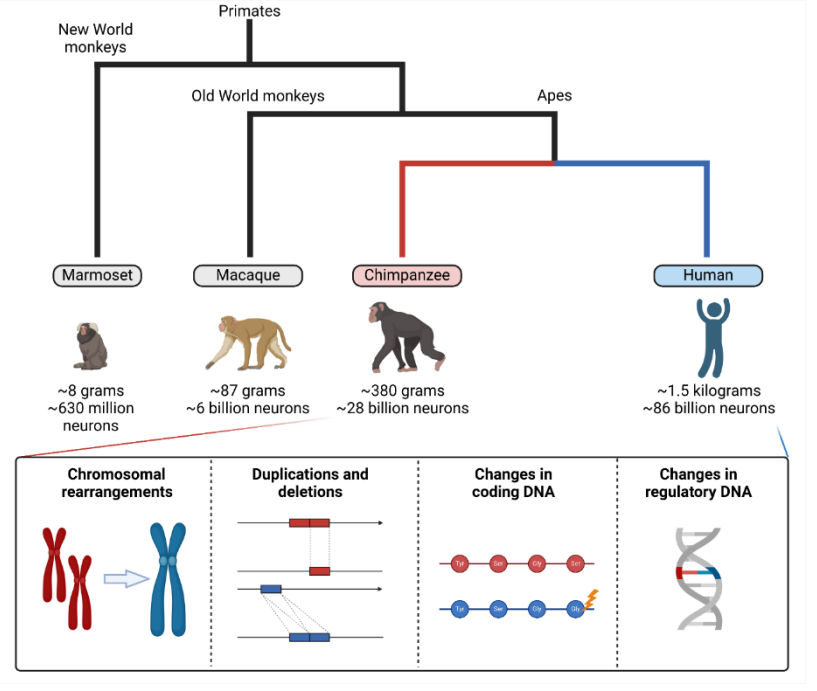

Figure 1: Phylogenetic tree of a selection of primates and examples of genetic mechanisms driving human brain evolution. Average brain weights and neuronal numbers of human and NHP brains are noted below. A subset of genetic factors driving human-chimpanzee differences in neuroanatomy are depicted in the bottom panel. Chimpanzee genomic state is represented by red, human genomic state by blue.

Another key specialisation of the human brain is its protracted maturation compared to NHPs[22]. The human brain reaches 28% of its adult size at birth, compared to 40% in chimpanzees and 38% in Australopithecines[23,24], an extinct hominin genus. Similarly, the development and myelination of white-matter connections in the human brain take longer than in NHPs, continuing until approximately 30 years of age[22]. This prolonged development of the human brain allows environmental factors to shape human neurodevelopment to a greater extent than in any other primates[25], contributing to increased behavioural plasticity[26]. Further evidence for delayed human neurodevelopment was provided by Sakai and colleagues, who tracked the development of two chimpanzee foetal brains *in utero* using three-dimensional ultrasound imaging[27]. The study demonstrated that chimpanzee brain growth velocity increases until the 22nd week of gestation, and then slows down until birth[27]. By contrast, the growth of the human foetal brain accelerates until at least the 32nd week of gestation[28], suggesting an evolutionary divergence in neurodevelopmental tempos between humans and chimpanzees at around the 22th week of gestation. Further genetic and cellular investigations will likely clarify the full array of diverse molecular changes underlying the evolution of neurodevelopment, such as chromosomal rearrangements, genomic duplications and deletions, protein-coding and non-coding single-nucleotide changes, as well as their impacts on the adult brain anatomy and behaviour (Figure 1).

Molecular and cellular variation across adult and developing primate brains

Despite the aforementioned differences in gross neuroanatomy between humans and NHPs, the six-layered basic architecture of the mammalian cortex[29] and the major cell types across primate cortices are notably conserved[30]. Similarly, consensus cell types and their proportions within cortical regions are nearly identical across humans and non-human great apes. However, there are substantial cross-species differences in gene expression programmes within homolog cell types and in cell type proportions across homologous cortical regions[30].

A recent comparative single-nucleus transcriptomic investigation of adult primary tissue from the posterior cingulate cortex of humans, chimpanzees, and rhesus macaques, revealed increased proportions of oligodendrocyte progenitor cells and the upregulation of *FOXP2*, a transcription factor implicated in speech apraxia, and expressive and receptive language disabilities[31], in two excitatory neuron subtypes in humans[32]. Similarly, a cross-species analysis of primary dorsolateral prefrontal cortex tissue from adult humans, chimpanzees, macaques and marmosets found human-specific upregulation of *FOXP2* in microglia cells[33]. Notably, compared to neuronal cells, glial cells (e.g., microglia, astrocytes, oligodendrocytes) have undergone faster transcriptomic divergence between humans and non-human great apes[30,34,35]. This highlights neuron-glia interactions as a potentially important area for future investigations of human brain specialisations. Another critical specialisation of the human brain is the increased number and variation of upper layer neurons[36], which may underlie enhanced cortico-cortical connections among association areas and contribute to the elevated cognitive skills of humans.

The protracted maturation of the human brain at the gross level is also reflected in molecular and cellular processes, which are thought to underpin human brain specialisations[37]. Despite the broadly conserved ontogeny of neurodevelopment across mammals[22], important human-, ape-, and primate-specific changes have been identified. These are often investigated using neuronal and organoid models, given the scarcity of NHP brain samples from early developmental stages. For instance, a recent study of telencephalic organoids revealed neuroepithelium cell shape differences between humans and non-human apes during the neuroepithelial-to-progenitor cell transition[38]. This study highlighted delayed maturation of human neuroepithelial cells compared to non-human apes, resulting in a larger founding progenitor pool during neurodevelopment[38]. Other studies have shown substantial differences in neurogenesis across primates, which continues for approximately four months during human foetal development, compared to ~2 months in macaques and ~1.5 weeks in mice[39,40,41]. Critical human-specific features of cortical progenitor cell behaviour were also identified using human and NHP organoid models. These include increased proliferative capacity of human neuronal progenitors[42], increased mTOR activation and broader cell fate potential in human outer radial glia[43], and altered cell cycle dynamics in human neuronal progenitors[44]. However, technical limitations of organoid models, such as the limited availability of primate induced pluripotent stem cell lines, make it challenging to distinguish between technical and cross-species variation[37,45]. Future studies on primary brain samples spanning the NHP phylogeny and ontogeny are needed to validate findings derived from two- and three-dimensional neuronal models.

1.2 Linking genetic variation to neuroanatomy and behaviour

Neuroimaging genomics reveals genetic factors shaping brain anatomy

Linking inter-individual genetic variability to phenotypic differences is challenging but essential for advancing evolutionary investigations of the human brain[46]. Fortunately, nature offers a wealth of genetic and phenotypic diversity across present-day humans, providing opportunities for genotype-phenotype mapping through genetic association studies. Over the last two decades, genome-wide association studies (GWAS) have emerged as a promising method for identifying sets of single-nucleotide polymorphisms (SNPs) associated with complex traits[47]. Neuroimaging genomics integrates brain imaging data with individual-level genotype information from tens of thousands of individuals, enabling the identification of genetic variants associated with phenotypic variability in brain anatomy[48]. First indications of genetic contributions to neuroimaging traits came from twin and family designs, demonstrating the heritable nature of neuroanatomic phenotypes such as brain volume[49] and white-matter connectivity[50]. However, the limited sample sizes of these early studies raised concerns about the robustness of their findings[51].

Recent advances in GWAS methodology[47,52] and the availability of large-scale datasets compiled by initiatives such as the Enhancing Neuro-Imaging through Meta-Analysis (ENIGMA) Consortium[53] and resources like the UK Biobank[54] have enabled statistically well-powered neuroimaging genomics investigations. Given the scarcity of human *post mortem* brain samples for neuroscientific research, the non-invasive collection of neuroanatomic data from tens of thousands of individuals via imaging modalities, including structural magnetic resonance imaging (MRI), diffusion-tensor imaging (DTI), computed tomography (CT), has become a valuable alternative to invasive and *post mortem* methods of neuroanatomical data sourcing.

Recent clinical and non-clinical neuroimaging genetics studies have utilised these large-scale databases to explore a wide range of features of the human brain. Clinical studies have identified putative genetic loci associated with neuroanatomical changes linked to brain disorders, such as Alzheimer's disease[55], schizophrenia[56] and Parkinson's disease[57], often employing case-control GWAS designs. On the non-clinical side, neuroimaging genetics studies have focussed on diverse aspects of brain anatomy, including morphometry, ageing, sex differences, and evolution[62]. A landmark study on brain morphometrics conducted a GWAS meta-analysis using structural MRI and genotype data from 60 cohorts (N=33,992), investigating the genetics of cortical surface area and thickness across 34 regions[63]. After replication and multiple testing correction, the study found that the total cortical surface area of the human brain has a SNP-heritability of 34% (standard error=3%) within the study sample, and identified 199 genetic variants significantly associated with cortical surface area and thickness of the different regions investigated. Similar studies of brain imaging phenotypes using UK Biobank have generated many datasets comprising GWAS summary data of an array of neuroanatomical measures, such as cortical surface area, white-matter connectivity and sulcal morphology[54,64,65]. The availability of such public datasets enabled post-GWAS functional and evolutionary investigations of a diverse set of brain structures, including some of the work presented in this thesis.

Inter-individual differences in language, reading, and musical rhythm skills

Similar to many other complex traits, the first evidence for the heritability of linguistic abilities emerged from twin and adoptee studies[66,67]. Numerous studies have shown that speech and reading abilities are influenced by both environmental and genetic factors[68,69]. Furthermore, clinical and genetic investigations into developmental speech disorders such as childhood apraxia of speech (CAS), have identified rare causal DNA variants with large effect sizes through family-based linkage studies[66] and recently through DNA sequencing studies of small cohorts and parent-offspring trios[70]. One prominent example is the KE family study: a linkage analysis in a large family in which nearly half of the family members were affected by CAS[71]. Genetic investigations revealed that affected family members carried a heterozygous missense mutation in the *FOXP2* gene that caused the speech disorder in the family[71,72,73]. This study provided the first genetic cause for CAS, and pathogenic variants of FOXP2 were since found in multiple other cases and families[74]. More recent DNA sequencing studies have implicated over 30 candidate genes in CAS susceptibility[70]. Rare-variant investigations have been less successful for other speech and language disorders, but have for example identified a missense variant in *GNPTAB* linked to persistent stuttering[75] and deletion of *CNTNAP2* associated with childhood-onset focal epilepsy and language regression[76]. Despite the clinical relevance of such rare variant studies, the polygenic nature of language-related skills necessitated genetic association investigations in large-scale population cohorts.

Leveraging genetic and phenotypic variation data from large sample sizes is essential for performing well-powered GWASs, ensuring minimised false-discovery rates while reliably identifying genetic associations. GWASs offer a number of possibilities for follow-up work that are less feasible for rare variant-based studies, including the ability to test for genetic correlations with other traits of interest, and to conduct follow-up functional and evolutionary analyses linking trait-associated genetic variants to sets of functionally annotated genes or evolutionarily annotated loci. Relevant recent GWAS efforts have focussed on exploring the inter-individual differences in language-related traits using behavioural and genetic data from over tens of thousands of individuals. A genome-wide association meta-analysis by the Genetics of Language (GenLang) Consortium[77] spearheaded the genetic investigations of language/reading-related traits by collating and analysing psychometric test data available from over 30,000 individuals. This study revealed moderate SNP-based heritability for language/reading traits (range: 13%-26%), and identified a first genome-wide significant locus associated with reading ability[77]. In parallel, personalized genomics companies such as 23andMe Inc. have collected genotype data along with self-report- and self-assessment-based behavioural data from over a million individuals, which have been contributed to several published GWASs. The availability of datasets of this scale has substantially boosted the statistical power of GWAS, enabling large-scale investigations into the genetics of dyslexia[78] and beat synchronisation skills[79]. These studies have identified many genome-wide significant loci associated with dyslexia and musical rhythm ability, as well as genetic links between various language/reading-, musicality-related and behavioural traits[78,79]. Moderate levels of SNP-based heritability were observed for dyslexia (~15%) and musical rhythm abilities (13-16%). Notably, reading is a recent human invention and did not evolve genetically, but through cultural innovation[80]. However, recent studies on genetics of reading-related traits, such as word reading abilities, spelling[77], and dyslexia[78], support previously established neurobiological links between reading and speech/language-related traits. The well-powered GWASs of reading- and

language-related traits and musical skills open up new avenues for investigating the shared genetics and co-evolution of pairs of these traits, such as language and musicality.

1.3 Using genetic association and transcriptomic methods to investigate molecular brain evolution

Post-GWAS investigations of complex trait evolution

Given the long causal chain spanning from genes to neuroanatomy and behaviour (Figure 2), using genetic association studies for evolutionary investigations requires innovative approaches. A promising approach towards understanding the evolution of complex human traits involves integrating genotype-phenotype maps identified by GWAS with evolutionary insights from ancient DNA, evolutionary genetics and comparative genomics fields. These disciplines provide annotations of the human genome by identifying regions and variants of evolutionary relevance, such as Human Accelerated Regions[81,82,83,84,85], Neandertal introgressed alleles[86,87], and human-gained enhancers[88]. Quantitative and qualitative investigations on how these evolutionary annotations overlap with trait-associated loci can illuminate the intricate links between evolutionary pressures and the genetic factors underlying complex human traits. One widely used method to explore these links is the partitioned SNP-heritability approach from the linkage-disequilibrium score regression (LDSC) software package[89]. This tool makes it possible to estimate the contribution of the SNPs located within a specific evolutionary annotation to a trait's total SNP-heritability, relative to the assumption of full polygenicity for the same trait. Other methods leverage the fact that the negative selection actively removes deleterious alleles from the gene pool, creating an inverse relationship between allele frequency and effect size[90,91,92]. By using this relationship as a predictor of negative selection, the level of genome-wide negative selection acting on a complex trait can be inferred[90,93].

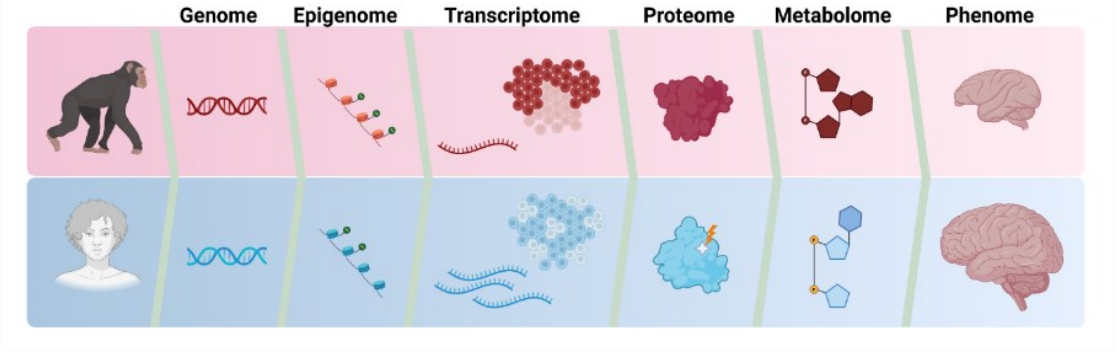

Figure 2: Overview of possible intermediate levels between genetics and complex traits. Cross-species genetic alterations can manifest themselves as phenotypic differences by influencing human biology at multiple levels, from genomes to phenomes.

A number of recent post-GWAS evolutionary investigations have focussed on various aspects of human brain and language-related traits, including cortical anatomy[94,95], endocranial globularity[95], language/reading-related traits[77,78], musical rhythm skills[79], and psychiatric disorders[97]. Among these, a particularly notable study is one examining the evolution of endocranial globularity[96], a unique feature of *Homo sapiens* anatomy[98]. In this study, the

researchers first derived a measure of endocranial globularity and obtained per-individual globularity scores using structural MRI data from a large number of healthy adults (N=33,996) from the UK Biobank. This was followed by conducting a GWAS of endocranial globularity, which identified 28 trait-associated loci and estimated the SNP-heritability as 32% (standard error=3.4%). Integration of post-GWAS evolutionary genetic analyses and fossil data-based species divergence time estimates[99] supported the hypothesis that the characteristic globular shape of the *Homo sapiens* endocranium emerged on our lineage between 300,000 and 120,000 years ago.

Comparative transcriptomics to probe molecular brain evolution

Inter- and intra-specific genetic variation in non-coding regions, identified through comparative genomics and GWAS, can have important implications for gene regulation. Such alterations in gene expression are thought to play a crucial role in the phenotypic divergence between closely related species[100,101,102], as they can influence the timing and spatial distribution of gene expression during foetal development and adulthood via different mechanisms in different species. Consequently, gene regulatory evolution can drive cellular brain evolution through three main mechanisms: i) the acquisition of new functions via changes in gene expression within existing cell types, ii) the relocation of existing cell types and alterations in their proportions, and iii) the emergence of entirely new cell types[103]. Comparative transcriptomics offers a particularly promising approach to explore cross-species differences in gene expression, cell type proportions and organisation. The recent availability and increasing cost-effectiveness of single cell/nucleus and spatial transcriptomic methodologies opens new avenues for exploring conserved and diverged gene regulatory programmes in developing and adult brain tissues across various phylogenies of interest.

The level of gene expression divergence across species is often correlated with phylogenetic distance, although there are exceptions such as the testis, which shows unusual cross-species divergence[103,104]. However, the extent of cross-species transcriptomic divergence may not be uniform across all regions of an organ. Comparative transcriptomics allows for the identification of gene expression differences specific to certain regions or cell types in a tissue. For instance, a study identified 261 differentially expressed genes in human radial glial cells located in the outer subventricular zone, compared to chimpanzee cerebral organoids and macaque cortical tissue[105]. Notably, these differentially expressed genes included a subset of genes involved in mTOR signaling[105]. Given the role of the mTOR pathway in the formation of radial glial cell morphology and migration patterns[106], this finding may point to human-specific alterations in neurodevelopment. As exemplified by this case, comparative transcriptomics proves to be a powerful instrument for investigating gene regulatory evolution in the context of human brain evolution. Furthermore, by combining comparative transcriptomics with methods such as comparative chromatin-accessibility assays, researchers can uncover the links between genetic and transcriptomic divergence between humans and NHPs.

1.4 Recent advances in genetic association and comparative genetics methods

Advances in GWAS

Over the last few decades, there has been growing recognition of the polygenic nature of complex traits[107,108], significantly enhancing the role of GWAS in investigating the genetics of various phenotypes. In the early days of GWAS, methodological limitations, such as difficulty in accounting for confounder effects and statistically underpowered small sample sizes, made replicability a substantial challenge. However, recent advances in genotyping methods, larger SNP panels, improvements in univariate and multivariate GWAS tools, and large-scale collaborative efforts to share and combine data from across different cohorts, have greatly enhanced the precision of GWAS effect size estimates[52]. These improvements are reflected in the increasing predictive accuracy of polygenic scores, particularly for certain diseases[52,109]. In parallel, the availability of large-scale genotype and phenotype databases from various initiatives and biobanks, such as the UK Biobank[110], BioBank Japan[111], the "All of Us" initiative[112], and 23andMe Inc., has substantially boosted GWAS sample sizes and thus the power of the approach to detect associations. In addition, the increase in sample sizes has improved the power to detect genetic variants with smaller allele frequencies. For example, (as noted above) recent work has revealed the most comprehensive genetic map of dyslexia-related genetic variants by leveraging data from over one million individuals, including tens of thousands of 23andMe customers who self-reported a dyslexia diagnosis[78]. On the methodological side, novel GWAS tools have been developed to address challenges arising from handling and analysing larger sample sizes, and to raise standards for quality control of genetic data[113]. Furthermore, the recent availability of robust statistical frameworks, such as Genomic Structural Equation Modelling (Genomic SEM)[114], has opened new avenues for investigating the shared genetic architecture and pleiotropic relationships across a set of complex traits, enabling researchers to formulate and empirically test novel research questions.

Advances in comparative genomics and ancient DNA

Groundbreaking advances in genome sequencing technologies since the early 2000s have enabled the generation of whole-genome assemblies for many species, including humans[115], chimpanzees[116], orang-utans[117], gorillas[118], and bonobos[119], in this chronological order. Despite considerable challenges in acquiring genetic samples from NHPs due to the global decline in primate populations[120], recent efforts to generate high-quality primate reference genomes fueled more accurate comparative genomic studies between humans and NHPs[3,9]. Using cost-effective short-read DNA sequencing, researchers can identify SNPs and cross-species single-nucleotide changes, providing valuable insights into the population structure and demography[121,122,123,124], as well as recent evolutionary history of a species[125,126]. Understanding these factors is crucial for the conservation of declining wild NHP populations. Moreover, as the sample sizes in NHP short-read DNA sequencing studies grow, the reliability of data on intra-specific genetic variation and cross-species derived alleles will improve. This will help refine existing human-derived allele lists[127], and contribute to ongoing research on the functional impacts of these alleles[128].

Recent advances in long-read sequencing technologies, driven by companies like the Pacific Biosciences and Oxford Nanopore Technologies, have enabled the scaffold-free and unbiased

generation of NHP genome assemblies[129]. These novel technologies allow for more precise investigation of structural variation within a population and across species[129]. Identifying structural variants in human and NHP genomes is crucial for a better understanding of their links to diseases[130] and evolution[131], as they can directly change the copy number of genes or affect gene regulation. Two notable examples of evolution through gene copy number changes are the *ARHGAP11B* and *NOTCH2NL* genes[132,133]. These genes emerged via gene duplications specifically in the human lineage, altering the rate and pace of neurogenesis and neuronal progenitor maturation, and have been argued to thereby contribute to human-specific aspects of neurodevelopment and neuroanatomy. Another investigation focussed on the evolution of the *NANOGP1* gene, a copy of the core pluripotency transcription factor *NANOG*, in primates[134]. *NANOGP1* emerged through a tandem duplication in the common ancestral population of Old World monkeys and apes, followed by the deactivation of this gene copy in lesser apes and monkeys via mechanisms such as partial exon deletions and missense mutations. In contrast, this gene copy showed some degree of genetic conservation in great apes, and partial functional conservation in humans[134]. These examples highlight the power of comparative genomic investigations across humans and NHPs to identify candidate genes and loci for functional validation assays, towards robust dissection of the molecular evolution of the human brain.

Finally, since the second half of 2000s, there has been a substantial increase in both the sample size and quality of ancient DNA data from extinct hominins, such as Neandertals and Denisovans[135,136,137,138], and ancient *Homo sapiens*[139,140,141]. While the first ancient DNA data was obtained from an extinct zebra species in 1984[142], the first next-generation sequencing-based ancient DNA data was generated from ancient mammoth specimens in 2006[143]. Since then, both data generation and analysis methods have greatly progressed, overcoming issues such as modern human DNA contamination in ancient samples, challenges with library preparation, and genomic mapping of fragmented ancient DNA sequences[144]. Today, genome-wide DNA sequencing data from over 10,000 ancient humans, primarily from Europe and Asia, are publicly available[145]. While the oldest nuclear DNA sequence from a genus *Homo* sample dates back to 430,000 years ago[138], the vast majority of ancient DNA samples were obtained from humans who lived within the last 10,000 years[146]. Furthermore, recent efforts to sequence ancient DNA from cave sediments have yielded mitochondrial and nuclear DNA from various mammals, including humans[147]. When combined with the "traditional" ancient DNA and comparative genomics datasets, sediment-derived ancient DNA presents an extremely innovative approach for investigating ancient human genomics.

Advances in comparative transcriptomics

Comprehensive gene expression quantification began with microarray technology, a method that uses a set of short oligonucleotide probes to capture fluorescently labelled RNA molecules[148]. Gene expression levels were quantified by measuring the intensity of light emitted by RNA molecules hybridised to these probes[148]. The first comparative transcriptomic studies on human and NHP brain tissues utilised microarray technology, providing valuable insights into inter- and intra-specific gene expression variation among primates[102]. Additionally, well-known reference databases, such as the Allen Human Brain Atlas[149], were generated using this technology, though these were based on human brain samples from a limited number of donors.

In the late 2000s, bulk RNA sequencing was developed and quickly became a cornerstone of molecular biology and functional genetics[150,151]. This method begins with RNA extraction from a tissue sample, after which RNA molecules are converted into double-stranded cDNA. Adapter-ligated sequencing libraries are then generated for high-throughput sequencing. Unlike microarray technology, bulk RNA sequencing does not rely on pre-designed single-stranded probes, allowing for unbiased and comprehensive gene expression quantification. The advent of bulk RNA sequencing provided a more precise, efficient, and cost-effective way to investigate gene expression at a transcriptome-wide scale[151]. In the following years, comparative studies leveraged this novel technology to study transcriptomic differences and similarities across species, including human vs. NHP comparisons[152]. These studies have identified a set of upregulated neuronal and glial marker genes in the adult human brain compared to those of chimpanzees, gorillas, and gibbons[152].

The resolution of gene expression measurement improved substantially with the increasing availability of single-cell RNA sequencing methods[153,154], which enabled researchers to test hypotheses about the transcriptomic aetiology of cell types and states for the first time. The first single-cell RNA sequencing study was performed on relatively large and easy-to-isolate oocytes[155]. By the mid-2010s, high-throughput and cost-effective single-cell sequencing technologies for all cell types became available[156,157,158]. Single-cell transcriptomics workflows typically start with single-cell sorting, using methods such as fluorescence-activated cell sorting (FACS), nanodroplets, or microfluidics. This is followed by RNA extraction, cDNA synthesis, and library preparation for sequencing[154,159]. Single-nucleus RNA sequencing is another well-established and widely used method, which specifically targets nuclear RNA. This method was shown to provide less biased cellular coverage, and is not affected by transcriptional artefacts related to single-cell sorting[160]. Given the challenges in single-cell sorting of neuronal cells due to their intricate cell morphologies, numerous comparative studies utilised single-nucleus RNA sequencing to investigate cell types and gene expression programs in human and NHP brain tissues[30,32,33].

Imaging-based techniques for spatial gene expression quantification, such as *in situ* hybridisation, have been available since the 1990s[161,162]. In fact, targeted sampling from brain regions of interest, followed by bulk or single-cell/nucleus RNA sequencing has already made it possible to obtain transcriptomic data with brain region-level spatial information. However, recent availability of high-resolution imaging- and sequencing-based spatial transcriptomics methods revolutionised the ability to investigate spatial gene expression with remarkable precision, down to the single cell and even subcellular resolutions[162,163]. Although spatial transcriptomics is becoming more widely used in clinical studies to elucidate the molecular and cellular microenvironment of various pathologies[164], its use in cross-species comparisons remains limited[165]. Expanding this line of research could provide novel insights into differences in cell type organisation and cell-cell interactions across primate brains, offering new perspectives on human brain evolution and function.

1.5 Aims of this thesis

As summarised above, various disciplines have significantly advanced our understanding of the genetic and biological foundations of human neuroanatomy and language-related skills. Yet, we are still far from fully grasping the evolutionary factors that shaped the genetic architecture

underlying the modern human brain and behaviour. We propose that conducting univariate and multivariate genome-wide analyses by harvesting the power of newly emerged biobanks and large-scale databases including genetic and phenotypic data, we can provide novel insights into the genetic factors underlying human specialisations in neuroanatomy and behaviour. Integrating evolutionary signals from the human genome, such as human-specific regulatory regions active in the foetal brain, with extensive genotype-phenotype maps, might help illuminate our evolutionary history. While genetic association studies combined with evolutionary analyses hold promise for understanding our evolutionary past, much remains to be uncovered.

Here, by integrating insights from neuroimaging genomics, behavioural genetics, and great ape brain spatial transcriptomics (Figure 3), this thesis aims to address the question of "*What are the physical bases of the human condition*?" Our goal is to contribute methodologically and scientifically to post-GWAS evolutionary analyses of cortical anatomy and human behaviour. Afterwards, we aim to move beyond genetic association studies to uncover the molecular and cellular mechanisms shaping the brains of our closest living relatives, chimpanzees, and pave the way for future comparative spatial transcriptomic investigations across primates.

I anticipate that understanding the tangible roots of what it means to be a human, and dissecting the fascinating yet fully biological processes that shaped the human brain and cognition over millions of years of human evolution might curb the anthropocentrism that is deeply embedded at the core of many cultures. In that sense, this thesis aims not only to illuminate our evolutionary past, but also contribute to fostering a humble and fully materialistic perspective on our insignificant place in the universe.

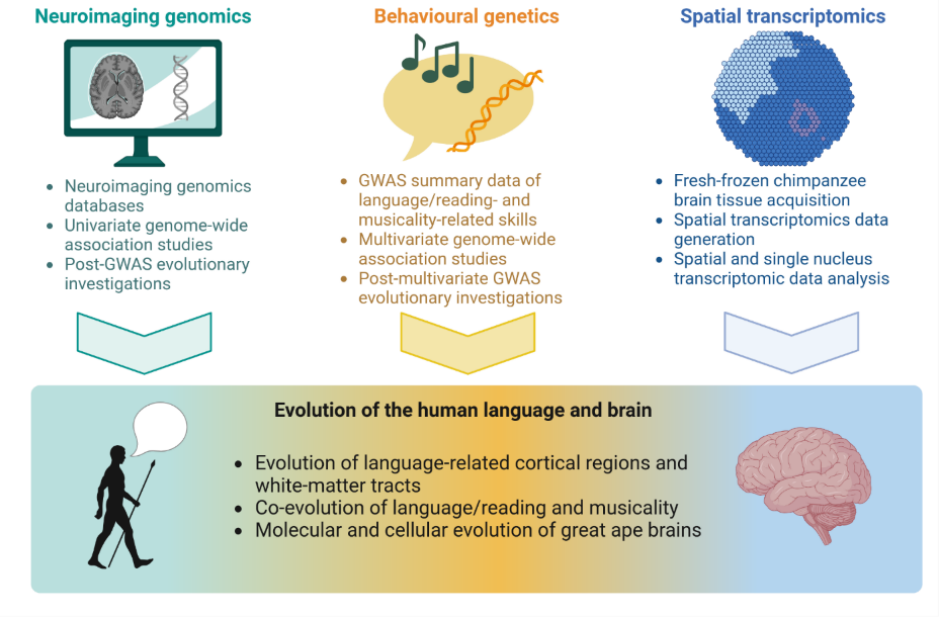

Figure 3: Three approaches to investigate the evolution of human language and brain. Above, an interdisciplinary framework to study human language and brain evolution by combining various computational and experimental methods. Below, the main research themes of this thesis.

1.6 Thesis outline

In ***Chapter 2***, we investigated the genetic underpinnings of human brain anatomy by integrating neuroimaging and genetic data from tens of thousands of individuals. Specifically, we focussed on regional and total cortical surface area, and white-matter connectivity measures across left and right hemispheres, using structural MRI and genotype data from the UK Biobank to perform GWASs for each brain imaging trait. We then followed up with SNP-heritability partitioning analysis using evolutionary annotations of the human genome and the LDSC method, and identified intriguing links between genetic variants, genes, cortical structures and the evolutionary past of our species.

We extended our efforts in neuroimaging genomics to investigate the evolution of human sulcal morphology in ***Chapter 3***. Using a set of publicly available GWAS summary statistics of four sulcal shape descriptors (sulcal depth, length, width, surface area) derived from structural MRI and genotype data from the UK Biobank, we identified sets of genomic loci with evolutionary significance. Following a similar workflow to that developed for ***Chapter 2***, we pinpointed sulci showing significant associations with a particular evolutionary annotation of the genome, and dissected exemplary loci that harbour trait-associated variants with evolutionary importance.

Given that human brain specialisations likely contribute to distinctive human behaviours, we changed gears from neuroimaging genomics to behavioural genetics in ***Chapter 4***, and investigated the shared genetics and evolutionary origins of language- and musical rhythm-related traits. Building on previous findings that showed significant correlations between language- and musical rhythm-related traits at the phenotypic level, we delved into the shared genetics and evolution of these uniquely human traits. Using two large-scale dyslexia and musical rhythm GWASs, we performed a multivariate GWAS and identified putative pleiotropic loci associated with both traits. Moreover, we examined the genetic relationship between dyslexia, musical rhythm impairment and the connectivity of a set of white-matter tracts with known links to language. Finally, we investigated the evolutionary factors shaping these traits, aiming to provide empirical evidence to inform long-standing debates on the co-evolution of language and musicality.

Identification of divergent and conserved gene expression programmes in human and NHP brains is crucial for advancing investigations of complex trait evolution. In ***Chapter 5***, we performed the first spatial transcriptomics study on great ape primary brain tissue, using a frontal pole sample from a freshly-frozen post-mortem chimpanzee brain. By integrating our chimpanzee brain spatial transcriptomics data with a publicly available single nucleus RNA sequencing dataset from the chimpanzee dorsolateral prefrontal cortex, we provided a first glimpse into the spatial organisation of chimpanzee brain cell types and gene expression patterns.

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Chapter 2

Using neuroimaging genomics to investigate the evolution of human brain structure

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Abstract

Alterations in brain size and organization represent some of the most distinctive changes in the emergence of our species. Yet, there is limited understanding of how genetic factors contributed to altered neuroanatomy during human evolution. Here, we analyze neuroimaging and genetic data from up to 30,000 people in the UK Biobank and integrate it with genomic annotations for different aspects of human evolution, including those based on ancient DNA and comparative genomics. We show that previously reported signals of recent polygenic selection for cortical anatomy are not replicable in a more ancestrally homogeneous sample. We then investigate relationships between evolutionary annotations and common genetic variants shaping cortical surface area and white-matter connectivity for each hemisphere. Our analyses identify single-nucleotide polymorphism heritability enrichment in human-gained regulatory elements that are active in early brain development, affecting surface areas of several parts of the cortex, including left-hemispheric speech-associated regions. We also detect heritability depletion in genomic regions with Neanderthal ancestry for connectivity of the uncinate fasciculus; this is a white-matter tract involved in memory, language, and socioemotional processing with relevance to neuropsychiatric disorders. Finally, we show that common genetic loci associated with left-hemispheric pars triangularis surface area overlap with a human-gained enhancer and affect regulation of *ZIC4*, a gene implicated in neurogenesis. This work demonstrates how genomic investigations of present-day neuroanatomical variation can help shed light on the complexities of our evolutionary past.

2.1 Introduction

The size and organization of the brain exhibit great variation across primates, with especially notable differences in our own species[1]. Comparative studies of ape neuroanatomy, along with endocranial fossil data from archaic hominins, indicate that there were dramatic expansions of cortical surface area on the lineage leading to Homo sapiens, as well as more recent shifts in brain shape[2,3,4]. Today, the human cerebral cortex has a surface area of ~1,843 ± 196 cm^2 per hemisphere, compared to ~599 cm^2 per hemisphere for our closest living relative, the chimpanzee[1]. It is thought that differences in brain size and shape were accompanied by altered architecture for a number of white matter tracts[5]. Given that changes in neuroanatomy coincided with the emergence of complex language and cognitive skills in humans[6], it has been suggested that an expanded cortex and/or associated alterations in connectivity and organization contributed to the evolution of complex behaviors[7,8,9]. Nonetheless, despite advances across a range of disciplines, we still have only a limited understanding of the molecular bases of these fundamental aspects of human brain evolution.

A promising way to address this gap is through large-scale neuroimaging genomic investigations in present-day humans, integrating with the latest data from the ancient DNA field and broader literature on comparative primate genetics. Datasets containing brain MRI data and genetic information on single-nucleotide polymorphisms (SNPs) for tens of thousands of individuals, such as those collected by the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium[10] and in population cohorts like the UK Biobank[11], have recently enabled the first well-powered genome-wide association studies (GWAS) of complex neuroanatomical traits. The last decade has also seen dramatic advances in ancient DNA research, including the discovery of putative Neanderthal introgressed alleles that can be found to varying degrees in the genomes of some present-day humans, resulting from admixture events ~50,000 to 60,000 y ago[12]. Such findings led to identification of long stretches of regions in the human genome that are significantly depleted of introgressed fragments—archaic deserts—with potential relevance for genetics of human-specific traits[13]. On a deeper timescale, enhancer elements that gained activity in the human lineage were identified by comparing genome-wide posttranslational histone modification profiles across human, macaque, and mouse cortical brain tissue[14]. Elements of this kind, referred to as human-gained enhancers (HGEs), can potentially shed light on the last ~30 My of brain evolution[15]. Here, we perform GWAS of structural neuroimaging measures in the UK Biobank (up to ~30,000 participants) and use the results, together with evolutionary annotations of the genome capturing different timescales along the human lineage, to gain insights into regional expansions of cortical surface area as well as effects on brain connectivity.

Prior work by Tilot et al.[16] investigated the evolution of hemisphere-averaged cortical surface area using a neuroimaging genetics meta-analysis comprising results from 33,992 participants of European ancestry (23,909 from 49 cohorts participating in the ENIGMA consortium and 10,083 from the UK Biobank)[10]. As well as directly using the available GWAS data to test for recent polygenic selection (i.e., involving effects at many different loci), the study took genomic regions with known relevance to human evolution and asked whether current genotypic variation in those regions contributes more than expected to interindividual differences in the neuroanatomical traits. There were two key findings: 1) evidence of recent polygenic selection based on singleton density scores (SDS)[17] for common variants associated with surface area and 2) enrichments of SNP-based heritability within HGEs active in fetal brain tissue, for a set of

cortical regions. One potential confound acknowledged by Tilot et al.[16] stemmed from the multicohort structure and residual population stratification of the ENIGMA dataset used for GWAS, factors which can adversely affect evolutionary analyses. In particular, with regard to the polygenic selection signals, SDS analysis is known to be susceptible to residual population stratification[18,19]. SNP-based heritability estimates are also prone to confounding effects of population stratification in admixed samples to a certain extent[20,21].

In the present study, we first performed a targeted replication analysis of the two main Tilot et al.[16] findings using a large independent cohort that is ancestrally homogenous (18,960 participants from UK Biobank, not included in the earlier analysis). The prior evolutionary investigations were limited by the use of measures averaged across hemispheres, but it is well established that certain cognitive specializations of our species, such as language, depend on lateralized circuits in the human brain. Thus, in the second stage of our study we went on to perform an analysis using genome-wide scans of 33 regional and global surface area measures for each hemisphere separately. We maximized power for this effort by using an even larger study sample (n = 30,332) from the UK Biobank, including a subset of individuals from the prior work on hemisphere averaged measures[16], allowing us to investigate evolutionary annotations for genetic variants affecting surface area of left and right-hemispheric regions. These analyses were enhanced over prior work by using updated sets of evolutionary annotations, including HGEs, Neanderthal introgressed alleles, and archaic deserts. In addition, we moved beyond surface area-based morphometrics to investigate brain connectivity. White-matter microstructure can be probed with diffusion MRI (dMRI) in vivo to explore structural connectivity patterns between different regions of the brain[22]. Therefore, we leveraged fractional anisotropy (FA) values of 48 standard space white-matter tracts in UK Biobank (n = 29,924) to identify genetic variants associated with white-matter connectivity within and across hemispheres then assessed links to evolution across the same annotations as those used for cortical surface area. We demonstrated the value of integrating neuroimaging genomics with data on human-specific regulatory elements, gene expression, and evolutionary history for identifying genetic pathways of interest, using the *ZIC4* gene as an illustration. Finally, we investigated human accelerated regions (HARs)[23,24,25,26,27] and differentially methylated regions between anatomically modern humans and archaic hominins (referred to as AMH-derived DMRs)[28], assessing overlaps with GWAS signals from all full and regional surface area measures. HARs and AMH-derived DMRs are two important evolutionary annotations that could not be robustly investigated using the partitioned heritability approach due to low SNP coverage (Materials and Methods). We anticipate that shedding light on present-day variation and evolution of human cortical anatomy will enhance our understanding of disorders affecting brain and cognition[29,30].

2.2 Results

Replication of Deep, but Not Recent, Evolutionary Findings for Hemisphere-Averaged Surface Area. Toward replication of the main Tilot et al.[16] findings, we first identified genetic loci associated with interindividual variation in hemisphere averaged cortical surface area using MRI and genome-wide genotype data of UK Biobank (accessed February 2020) in a set of individuals independent from those analyzed for the prior evolutionary study[16]. In particular, to avoid any overlap with data contributing to the initial discovery GWAS by Grasby et al.[10], participants from UK Biobank neuroimaging releases prior to 2018 were excluded from this part of the study. Moreover, our replication effort focused on individuals of White British ancestry to limit

potential population stratification. Anatomical measures were derived from structural MRI and the cortical regions were extracted from parcellations based on the Desikan–Killiany atlas[31]. Applying an additive model, we conducted GWAS for total surface area as well as 33 cortical regions averaged across both hemispheres in 18,960 individuals (SI Appendix, Table S1). In line with previous work, common genetic variants accounted for 40.22% (SEM = 4%) of the phenotypic variation in total surface area (SI Appendix, Table S2). We observed very high genetic correlations between our independent UK Biobank subset of White British ancestry and the earlier GWAS data [global surface area r_g(SEM) = 1.03(0.05), $P_{rG} = 4.52 \times 10^{-91}$] (SI Appendix, Fig. S1 and Table S3), suggesting consistent genetic underpinnings of cortical anatomy in Grasby et al.[10] and the independent sample that we analyzed here, as also illustrated by the similarity in Manhattan plots (Fig. 1A).

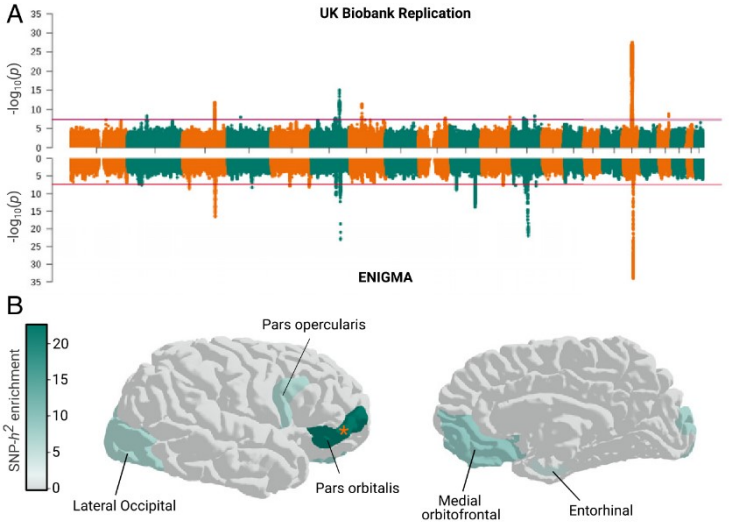

Fig. 1. Replication of deep evolutionary findings for hemisphere-averaged surface area. (A) Manhattan plot of loci associated with total surface area in UK Biobank replication sample (*Top*, this study) and ENIGMA (*Bottom*) from Grasby et al.[10] GWAS. Red horizontal lines indicate genome-wide significant multiple testing correction threshold. (B) SNP heritability of pars orbitalis is enriched in HGEs active at the seventh postconception week in fetal human brain, both in Tilot et al.[16] and our UK Biobank replication. SNP-heritability enrichments were estimated with LD-Score Regression. For this replication analysis, we tested only the five regions showing significance in the prior study, applying FDR correction accordingly. Regions that were not tested are in gray.

To further minimize potential confounding effects of residual population stratification on GWAS effect size estimates, we applied the ancestry regression procedure implemented by Tilot et al.[16]. As in that study, we then analyzed the ancestry regressed-GWAS summary statistics using SDS[17], a method for detecting signs of polygenic selection acting over the past ~2,000 to 3,000 y. First, we performed a targeted replication analysis for the full cortical surface and the eight cortical regions that had been reported as being under recent selection in the Tilot et al. study[16] and found no evidence of recent selection on the cortical area of any of these brain regions in this independent homogenous sample (SI Appendix, Table S4). Next, we correlated the SDS results of all brain regions with results from Tilot et al.[16] but found no significant correlation (SI Appendix, Fig. S2).

We went on to study the second major finding of Tilot et al.[16], concerning the role of genetic variants located in HGEs active in fetal brain[14]. As in the prior study, we used LD-score regression (LDSC) partitioned heritability analysis[32], a method that can assess whether common variation in particular regions of the genome contributes disproportionately to the total SNP heritability of a trait. Specifically, we tested in our independent homogenous sample whether we could replicate the finding that HGEs active in fetal human brain at the seventh week postconception impact hemisphere-averaged cortical surface area. We robustly replicated the SNP-heritability enrichment signal for pars orbitalis [enrichment(SEM) = 22.82(8.02), P_{FDR} = 0.004] (Fig. 1B). This brain region showed the strongest evidence for SNP-heritability enrichment in Tilot et al.[16] and is involved in aspects of language processing[33,34]. Here, it should be noted that both the current and previous studies controlled for global surface area when performing GWAS for different cortical regions. The consistency of the findings also confirmed the robustness of LDSC heritability partitioning as a tool for investigating evolutionary annotations.

Hemispheric Surface Area–Associated SNPs Are Enriched in Fetal Brain HGEs, Including Regions Involved in Speech, Language, and Visual Processing. Given that cognitive specializations of our species, such as language, depend on lateralized circuits in the cortex[35], we went on to perform GWAS of 33 regional and global surface area measures for each hemisphere separately (68 measures in total). Since this part of our work was toward novel evolutionary analyses that had not been run in the prior study, we here used all the available participants of European ancestry with genetic/neuroimaging data (n = 30,332) in the UK Biobank (SI Appendix, Table S5). GWAS results for cortical regions were controlled for the matching total hemispheric surface area and may thus be considered independent of the total measures. Common variation accounted for 39.02% (SEM = 3.14%) of total left-hemispheric and 39.37% (SEM = 3.21%) of total right-hemispheric surface area variation (SI Appendix, Table S6). Because our targeted follow-up of Tilot et al.[16] showed a lack of replicability for SDS results (elaborated on in Discussion), in line with other work showing that this polygenic selection method can be easily confounded by residual population stratification[18,19], our new analyses focused only on the partitioned heritability approach[32]. In addition, partitioned heritability allowed us to analyze all individuals of European ancestry (not just the White British subsample), increasing the statistical power of the GWAS.

To enhance reliability of the LDSC-based partitioned heritability approach, we curated refined sets of evolutionary annotations that encompass at least 1% of the well-imputed and quality-controlled 1000 Genomes Phase 3 reference panel SNPs[36]. Further, we combined biologically related annotations and removed SNPs that overlapped in opposing annotations (i.e., in archaic deserts and Neanderthal introgressed alleles). Three distinct sets of annotations passed our quality control (Materials and Methods) and were taken forward for the evolutionary analysis: 1) genomic regions covering fetal brain HGEs active at 7^{th}, 8.5^{th}, and 12^{th} postconception weeks, 2) Neanderthal introgressed alleles, and 3) archaic deserts.

After false discovery rate (FDR) correction for 43 independent GWAS traits [estimated with PhenoSpD[37] using a genetic correlation matrix of the 68 cortical area traits], there were six left-hemisphere regions and four right-hemisphere regions that showed significant SNP-heritability enrichment in fetal brain HGEs (Fig. 2A and SI Appendix, Tables S7 and S8). Significant enrichments were identified for brain regions with average to high SNP-heritability estimates; it is possible that some positive findings may have been missed for brain regions with low SNPheritability estimates due to lack of power (Fig. 2B and SI Appendix, Fig. S3). Left-

hemisphere cortical regions that reached significance, pars triangularis [enrichment(SEM) = 9.65(3.04), P_{FDR} = 0.015] and pars opercularis [enrichment(SEM) = 9.61(3.47), P_{FDR} = 0.044], together form Broca's area, well known for its roles in speech-associated functions, among others[38]. The other significant left-hemispheric regions are involved in functions such as word processing, visual processing, and object recognition[39]. Right-hemispheric cortical regions that showed significant SNP-heritability enrichment in fetal brain HGEs have been linked to visual processing and language perception[40,41]. One region reached significance for heritability enrichment in fetal brain HGEs on both hemispheres: lingual gyrus, a region related to vision and word recognition[41]. We did not find a significant enrichment or depletion for any surface area phenotype with respect to Neanderthal introgressed alleles. However, significant SNP-heritability depletion was seen within archaic deserts for total left- and right-hemispheric, as well as left-hemispheric pars opercularis, surface area [total left: enrichment(SEM) = 0.52(0.1), P_{FDR} = 0.0006; total right: enrichment(SEM) = 0.57(0.11), P_{FDR} = 0.005; left pars opercularis: enrichment(SEM) = 0.03(0.3), P_{FDR} = 0.008] (SI Appendix, Fig. S4).

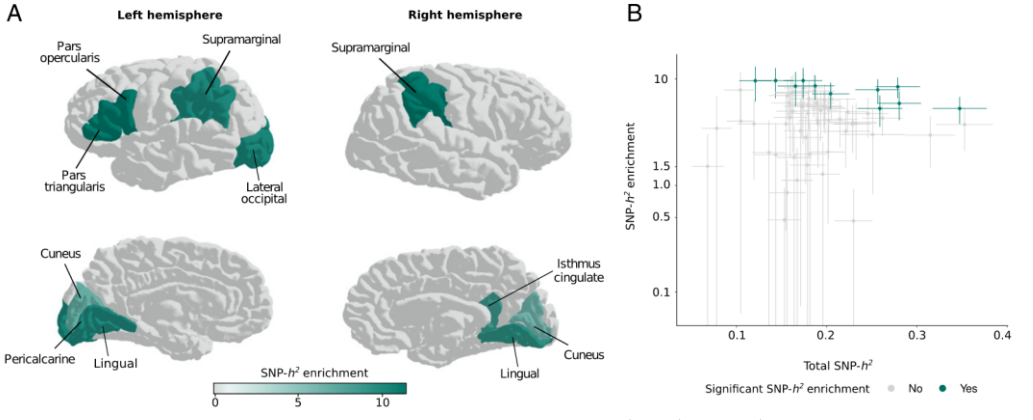

Fig. 2. SNP heritability explained by genetic variants in HGEs active at 7th, 8.5th, and 12th postconception weeks in fetal brain reached significance for various regions in each hemisphere. (A) Brain plots showing SNP-heritability enrichment levels for left and right hemisphere regions that reached significance. (B) The relation of total SNP-heritability to SNP-heritability enrichment in HGEs. Data points represent regional hemispheric surface areas. Regions that showed significant heritability enrichment (P_{FDR} < 0.05) are indicated in green and others in gray. FDR correction was applied for 43 independent cortical regions (*Materials and Methods*). Error bars represent SEs.

Left-Hemispheric Uncinate Fasciculus Heritability Is Depleted in Neanderthal Introgressed Fragments. We next moved beyond cortical surface areas to investigate measures of structural connectivity between different regions. Here, we leveraged FA metrics of 48 white-matter tracts in UK Biobank (n = 29,924) to identify genetic variants associated with white-matter integrity within and across hemispheres, using protocols that closely matched those used for our surface area GWASs (SI Appendix, Table S9). Similar to our findings for hemispheric surface area, white-matter connectivity GWAS identified large SNP-heritability levels for all 48 tracts (SI Appendix, Table S10), allowing assessment of links to evolution using LDSC partitioned heritability across the same annotations that we had used for cortical surface area. Genetic correlations (SI Appendix, Fig. S5 and Table S11) showed high consistency with relevant publicly available GWAS summary statistics obtained via the Oxford Brain Imaging Genetics Server[42].

Our analysis detected a heritability depletion in Neanderthal introgressed alleles for left-hemispheric uncinate fasciculus [enrichment(SEM) = 0.01(0.28), P_{FDR} = 0.022] (Fig. 3), after correction for 25 independent traits [estimated with PhenoSpD[37] using a genetic correlation matrix of the 48 tracts]. Although the tract is highly conserved, surface projections of the uncinate fasciculus show differences between humans and macaques[43], consistent with divergence of fascicular anatomy between Old World Monkeys and apes. No other significant enrichments or depletions were found for any of the white-matter tracts with the evolutionary annotations that we tested (SI Appendix, Table S12).

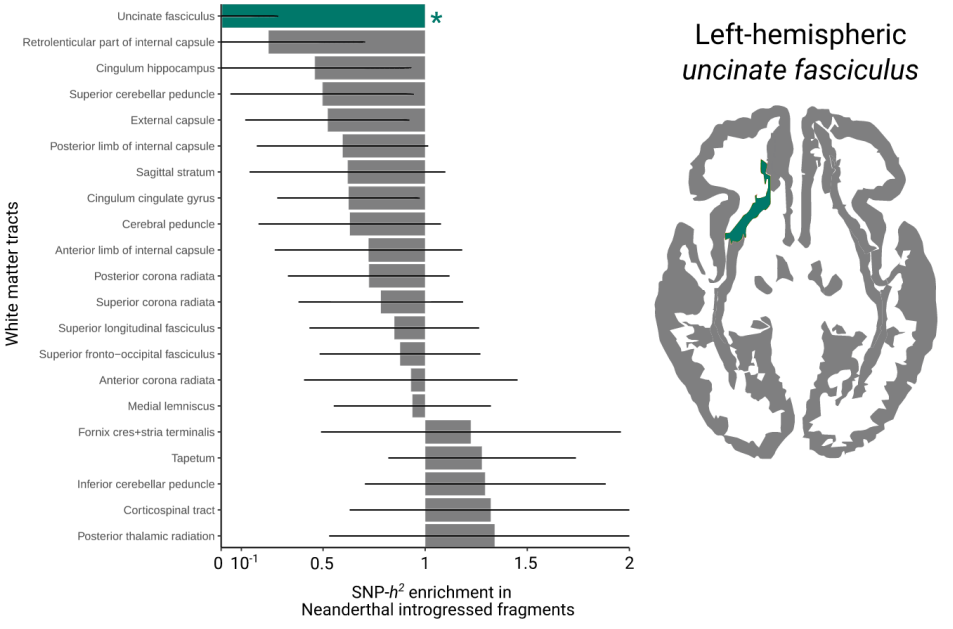

Fig. 3. Strong SNP-heritability depletion of left-hemispheric uncinate fasciculus is in regions of Neanderthal introgression. Depletion and enrichment of heritability for left-hemispheric white-matter tracts. FDR correction was applied for 25 independent white-matter phenotypes (*Materials and Methods*). Lefthemispheric uncinate fasciculus is the only tract that was significant after FDR correction. *$P < 0.05$.

Surface Area–Associated Loci in HGE Regions Impact Expression of the ZIC4 Gene. In the last part of the study, we zeroed in on loci of potential interest to more specifically evaluate the degree of convergence of trait, functional, and evolutionary associations. As an illustration, given the involvement of left hemispheric pars triangularis in functions related to speech and semantic processing (44), as well as the significant heritability enrichment for fetal brain HGEs, we examined SNPs associated with this region in further detail. Among the 13 independent genome-wide significant ($P < 5 \times 10^{-8}$) left-hemispheric pars triangularis–associated loci (LD blocks harboring genome-wide significant SNPs that are in LD r2 > 0.6), there are two that overlap with a fetal brain HGE. This includes the chromosome3 locus near *ZIC4*, which has the lowest P value for association with this brain region (rs2279829; $P = 1.49 \times 10^{-30}$, Beta = 17.37) and contains a genome-wide significant SNP (rs1875748; $P = 1.54 \times 10^{-13}$, Beta = 9.84) that falls in an HGE element located adjacent to *ZIC4* (chr3:147,101,875-147,103,850) (Fig. 4A). We annotated the locus with expression quantitative trait locus (eQTL) and chromatin interaction data, to further investigate functional consequences of the variants in this enhancer element.

Chromatin interaction data from neural progenitor cells and fetal brain tissue indicated that the fetal brain HGE interacts with the promoter region of *ZIC4* and *ZIC1* (Fig. 4A). SNPs in the locus, including rs1875748, are annotated as *ZIC4* eQTLs in adult brain tissue, and there is evidence to suggest that the SNPs also function as *ZIC1* eQTLs (SI Appendix, Table S13). The minor allele (T) of rs1875748 is associated both with increased surface area of the left-hemispheric pars triangularis and with increased *ZIC4* expression in adult brain cortical tissue (Fig. 4B). Comparative genomics analysis indicated that this minor allele corresponds to the ancestral allele, and we detect the same polymorphism at the site in the available ancient DNA data from Neanderthals and Denisovans (Fig. 4C). The Human Genome Dating Atlas estimates rs1875748 polymorphism to be 47,675 generations old (95% confidence interval), corresponding to ∼1.2 Ma assuming 25 y per generation[45]. Given the association between the human-derived allele at rs1875748 and *ZIC4* expression in adult brain tissue, we further investigated the genetic architecture of the *ZIC4* locus (which also harbors the *ZIC1* gene). We looked for human-specific regional deletions and insertions in the last ∼6 My of human evolution by aligning human and chimpanzee sequences for a genomic locus flanking *ZIC4* 45 kb upstream and downstream (SI Appendix, Fig. S6) but found no major genomic rearrangements. Finally, we generated a multiple sequence alignment for *ZIC4* coding sequence across 36 mammalian species to run phylogenetic analysis by maximum likelihood (PAML)[46] to test for selection. Comparison between PAML site models for *ZIC4* coding sequence with the likelihood ratio test shows that 13.5% of the codons evolve under positive selection (χ^2 $P < 0.001$) (SI Appendix, Table S14). Finally, we applied our overlap analysis to HARs and AMHderived DMRs (two interesting evolutionary annotations that could not be reliably investigated with partitioned heritability) using an extended list of genome-wide significant loci ($P < 5 \times 10^{-8}$ and SNPs that are in LD $r^2 > 0.6$, comprising 2,596 loci in total) from all full and regional left- and right-hemisphere surface area GWASs. There were 10 loci coinciding with HARs and 23 loci located within AMH-derived DMRs (SI Appendix, Fig. S7 and Table S15). Intriguingly, 5 of the 10 loci that overlap with HARs are significantly associated with left or right hemisphere lateral orbitofrontal surface area, while loci coinciding with AMH-derived DMRs are associated with a range of cortical regions including superior parietal, lateral occipital, and pars orbitalis.

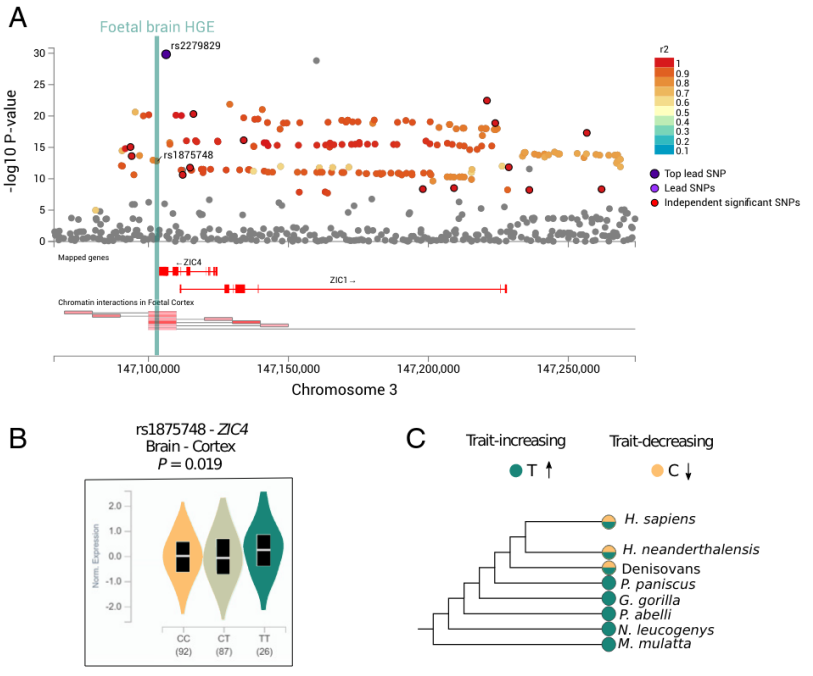

Fig. 4. Functional and evolutionary annotation of the pars triangularis–associated *ZIC4* locus. (A) The LocusZoom plot (hg19) shows the SNPs in the locus genome-wide significantly ($P < 5 \times 10^{-8}$) associated with the pars triangularis that overlaps with the *ZIC4* and *ZIC1* genes. Colors represent linkage disequilibrium with the most significant SNP rs2279829 based on the 1000 Genomes Project reference data. A fetal brain HGE is located adjacent to *ZIC4* and contains the genome-wide significant SNP rs1875748. Chromatin interaction data from fetal brain that overlaps the locus and originates in the fetal brain HGE is shown below the LocusZoom plot from FUMA. (B) eQTL plot showing impact of rs1875748 on *ZIC4* expression in adult cortical brain tissue. Data and figures were taken from the GTEx database. (C) Ancestral and derived allele states in five nonhuman primates, Denisovans, Neanderthals, and *H. sapiens*. Effect directions of alleles are shown with arrows.

2.3 Discussion

Recent availability of large-scale neuroimaging and genetics data in tens of thousands of individuals enabled us to investigate the evolution of complex neuroanatomical traits through common variation in a present-day population. By integrating genome-wide scans for cortical surface area and white-matter connectivity with relevant genomic annotations, we map current genetic variation with respect to annotations reflecting different aspects of human evolution, over diverse timescales.

The first part of our study attempted targeted replication of evolutionary findings from Tilot et al.[16] concerning hemisphere-averaged cortical surface area in a multisite sample that had combined many cohorts. We found that the signals of recent polygenic selection identified by Tilot et al.[16] could not be replicated in an independent ancestrally homogeneous cohort of a size similar to that in the earlier work. Our results suggest that, despite the use of an ancestry regression procedure, Tilot et al.[16] had not been able to fully exclude the effects of residual population stratification on GWAS effect size estimates, a known confound of the SDS method. Indeed, recent studies using this method have shown that polygenic selection signals for height based on SNPs below genome-wide significance are extremely sensitive to population

stratification[18,19]. On the other hand, following the partitioned heritability approach of Tilot et al.[16] we could successfully replicate findings related to deeper evolutionary timescales. In particular, in our independent sample we robustly replicated the heritability enrichment signal for pars orbitalis surface area in fetal brain HGEs active at the seventh postconception week. Thus, the findings confirm the hypothesis that genetic variation in fetal brain HGEs can significantly impact neuroanatomical features as measured in living adults. Moreover, given the demonstrated replicability of the approach, we went on to apply partitioned heritability of evolutionary annotations to novel brain traits that had not been investigated in the previous work. The extreme sensitivity of the polygenic selection methods to residual population stratification stems from the fact that GWAS effect sizes are stratified across European populations. In contrast, Sohail et al.[19] suggested that LD-score regression is less likely to be affected by this issue, as residual stratification affects high- and low-LD SNPs to a similar extent, and so should not have a large impact on the parameter estimates. Sohail et al. also compared bivariate LD-score regression estimates from separate datasets to confirm its robustness.

Analyzing regions of evolutionary significance in genome screens of each hemisphere separately, we observed enriched heritability in fetal brain HGEs for surface area of left-hemispheric regions implicated in speech and language and of right hemispheric regions involved in vision and self-projection, among others. Given the documented differences in inferior frontal gyrus anatomy in humans and macaques[47], and the importance of this part of the brain for speech and language in health and disease, the enrichment of genetic variants in humanspecific enhancer elements is intriguing. In addition, findings showing heritability enrichment of visual-cortical regions in the same regulatory elements are consistent with the existence of substantial differences in the organization of primary visual cortex between humans and nonhuman primates[48]. Moving beyond partitioned heritability, our work demonstrates how convergence of neuroimaging genomics with evolutionary data and information on gene function can shed light on contributions of potential loci of interest. As an example, we zeroed in on a locus on chromosome 3, using eQTL data to show that the same variants that are associated with the anatomy of the pars triangularis affect the neural expression of *ZIC4*. Of note, one of the eQTLs is located in the fetal brain HGE adjacent to *ZIC4*. Our analyses indicate that the *ZIC4*/*ZIC1* promoter region interacts with this enhancer element, suggesting further relevance for these two genes in cortical evolution, and we additionally show that the *ZIC4* coding sequence has been under positive selection in mammals. Both *ZIC4* and its colocalized paralog *ZIC1* are known to be involved in neurogenesis[49]. Overlap analysis between GWAS signals from all full and regional surface-area GWASs and two evolutionary annotations (HARs and AMH-derived DMRs) pointed to a set of putative evolutionarily interesting loci and genes which can be candidates for further research in this domain. We note that this overlap analysis is a qualitative approach that does not involve evaluation of statistical significance but rather generates hypotheses for future targeted investigations of loci of interest.

Lack of significant heritability enrichments for cortical surface area within Neanderthal introgressed alleles and archaic deserts may suggest that variants influencing these traits tend to concentrate at genomic regions shaped in earlier periods of human evolution, consistent with the more prominent neuroanatomical differences between H. sapiens and nonhuman primates compared to Neanderthals. Nonetheless, we observed a significant heritability depletion for total surface area and left hemispheric pars opercularis in archaic deserts, findings that are difficult to interpret. The depletion does not indicate an overall lack of common variants in the archaic deserts, as heritability partitioning is performed per variant, and all annotations contain

at least 1% of SNPs in the reference panel. Archaic deserts are known to be enriched for genes expressed in certain brain regions including developing cortex and adult striatum[13], as well as for enhancer regions active in brain tissue[50]. Neanderthal introgressed alleles in these brain enhancers have been subject to unusually stringent purifying selection[50]. Thus, we hypothesize that the significant SNP-heritability depletion for full and regional cortical surface areas in archaic deserts could be a result of the strong purifying selection on the Neanderthal introgressed variants in brain enhancers. In other words, Neanderthal introgressed alleles might have initially replaced key variants in brain enhancers for brain structure, and the subsequent negative selection on these enhancers might have resulted in the identified depletion of SNP heritability observed in current living populations. Another explanation would be that archaic deserts coincide with regions of the genome that lack genes and regulatory elements involved in the early neurodevelopmental processes that establish cortical surface area. While it is known that archaic deserts are enriched for regulatory regions active in the brain, these may conceivably affect other aspects of neural structure/function, or different developmental periods.

Analyzing white-matter measures, we identified another relationship with ancient DNA data—a substantial SNP-heritability depletion within the Neanderthal introgressed alleles annotation, for connectivity of the left-hemispheric uncinate fasciculus. In other words, Neanderthal alleles that were introduced into the human genome through ancient admixture events and that have persisted to the present-day explain substantially less variability in uncinate fasciculus structure than expected under the null hypothesis of complete polygenicity, perhaps in relation to conservation of H. sapiens–specific features of neuroanatomy. A caveat of our dMRI based analysis is that we were not able to here investigate all tracts of potential evolutionary interest in the human brain. In particular, white-matter connectivity data for the arcuate fasciculus, a tract with especially compelling links to language processing and well-documented differences in comparative primate neuroanatomy, are not available in the UK Biobank and so could not be included in the present study. It has been hypothesized that language processing involves two streams; the dorsal stream connects Broca's and Wernicke's regions via the arcuate fasciculus and is involved in mapping sound to articulation, whereas the ventral language stream connects the anterior and lateral temporal lobe via the uncinate fasciculus and is involved in mapping sound to meaning[51]. On the other hand, it is also known that the uncinate is highly conserved across humans and macaques[40]. Thus, despite its critical role in the so-called language-relevant connectome[52], the evolutionary relevance of our heritability depletion finding for this trait is unclear. However, we note that heritability depletion in Neanderthal introgressed fragments has been observed for a number of other complex human traits[53]. In addition to its involvement in language, the uncinate fasciculus has been linked to cognitive functions such as episodic memory, and aspects of socioemotional processing, and implicated in a range of neurodevelopmental and psychiatric disorders[54].

Notably, a lack of fossilized brain tissue from archaic hominins means that most prior knowledge of the neuroanatomical specializations of our species concerns gross levels such as overall brain size and shape. Hence, for the main part of this project it was necessary to adopt a systematic objective screening approach that considered all the regions of cortex and whitematter tracts available, yielding a higher multiple-testing burden and a consequent reduction in power. There are several other limitations of our study. First, for data availability reasons, the results were all derived from a population with European ancestry. Therefore, the scope of work is necessarily restricted to investigation of detectable polymorphic sites in a limited population. To

consolidate findings, further research will be needed using neuroimaging genetics datasets from populations with diverse ancestral backgrounds, once sufficiently large-scale cohorts have been assembled. Second, it is likely that many of the critical genetic changes that affected key aspects of cortical anatomy in the course of hominin evolution have become fixed in all living humans and so cannot be directly studied in GWAS data from extant populations. Here, we capture the existing genetic and phenotypic variation in the UK Biobank and assess contributions of different evolutionary genomic annotations to heritabilities of neuroanatomical traits, as an indirect way to probe the relevance of those annotations for different aspects of human brain structure. Third, the relationship between heritability and evolution is far from straightforward, especially in light of the complex history of archaic hominins, involving multiple admixture events. Contributions of common genetic variants to heritability are shaped by selection and demography, and heritable variation is maintained by mutation–selection balance[55]. Combining our approach with additional ancient DNA data that robustly track allele frequencies over the last ~40,000 y or so could be valuable for enhancing analyses of the impacts of Neanderthal introgression. Fourth, although we used the largest homogenous sample currently available, we acknowledge that for those neuroimaging traits with modest SNP-based heritability estimates, larger GWAS sample sizes may be required to detect enrichments and/or depletions. As indicated by the fairly large error bars of the partitioned heritability results and varying levels of total SNP-heritability per trait, a larger GWAS sample size would have potential to reveal further enrichment and/or depletion signals, especially for the traits with lower SNP-heritability estimates. Although we are confident that we have been able to detect true heritability enrichment and depletion signals using the current cohort, it remains likely that the power was not sufficient to capture all the positive signals. Finally, there are other annotations of the human genome that are of interest for understanding cortical evolution beyond those included in the current study. However, given that such annotations are related to particular evolutionary events (e.g., selective sweeps, interspecific differences in chromatin accessibility), they often cover a relatively small number of SNPs, which complicates analyses based on SNP heritability. Thus, development of alternative methods beyond heritability partitioning will be important to derive more comprehensive accounts of the genetic bases of human brain evolution.

Overall, through analyses of large-scale neuroimaging genetics data, we showed that patterns of common variation in HGE elements, Neanderthal introgressed alleles, and archaic deserts influence the surface area and white-matter connectivity of our brains at global, regional, hemispheric, and microstructural levels in complex ways. Applying similar approaches to other measures of human brain structure and function, and in further large samples, holds promise for gaining further insights into evolution of our species.

2.4 Materials and Methods

Dataset. All data for neuroimaging genetics were obtained from the UK Biobank under the research application 16066 with C.F. as the principal investigator. The present study involved two samples using surface-based morphometric data, further referred to as replication and hemisphere-specific sample, and one dMRI sample. Data availability and sample-specific preprocessing and quality control are described in detail in SI Appendix. Informed consent was obtained for all participants by UK Biobank with details about data collection and ethical procedures described elsewhere[56,57].

Neuroimaging Phenotypes. This study made use of imaging-derived phenotypes generated by an imaging-processing pipeline developed and run on behalf of the UK Biobank[58,59]. We used imaging-derived structural measures (UK Biobank category 192) where cortical surface was parcellated for each individual according to the Desikan–Killiany atlas[31] and a standard preprocessed dMRI measure (UK Biobank category 134). All relevant preprocessing steps are described in full in the UK Biobank imaging documentation (https://biobank. ctsu.ox.ac.uk/crystal/crystal/docs/brain_mri.pdf).

Genome-Wide Association Analysis. For all individuals, imputed variant genotype data (UK Biobank Category 263, bgen files; imputed data v3, released March 2018) were extracted and variant-level quality control and SNP statistics were computed for each dataset using qctool v2.0.2 (https://www.well.ox.ac.uk/ ~gav/qctool_v2/). In each of the three study samples, variants with a minor allele frequency <0.1%, Hardy–Weinberg equilibrium P value $<1 \times 10^{-6}$, imputation quality INFO score <0.7 (included in the imputed UK Biobank data) and multiallelic SNPs were excluded.

This procedure resulted in 14,537,705 biallelic variants in the replication sample, 14,532,493 in the hemisphere-specific sample, and 14,531,866 in the dMRI sample. For the replication sample, following the approach of Tilot et al.[16] and Grasby et al.[10], univariate association analyses were conducted for total surface area and 33 cortical regions, where both metrics were averaged across hemispheres, resulting in 34 traits in total. For the hemisphere-specific sample, these same traits were analyzed separately for each hemisphere, resulting in 68 traits in total. For the dMRI sample, FA values for all 48 standard-space tracts were used as traits. Associations of phenotypes with sample-specific imputed genotype dosages were tested applying an additive model in BGENIE (v1.2)[56].

Estimating Number of Independent Neuroimaging Traits. For the hemisphere-specific surfaced-based and the dMRI analysis streams we accommodated the multiple-testing burden while considering the correlation structure of the traits under investigation. In each case, we used spectral decomposition of matrices (SpD) implemented in the R package PhenoSpD[37] to calculate the effective number of independent variables (VeffLi), resulting in 43.07 and 25.34 independent traits for hemisphere-specific and dMRI samples, respectively.

Recent Polygenic Selection Replication Analysis. For the hemisphere averaged replication sample, to most closely match the Tilot et al.[16] study design we first assessed the impact of population stratification on effect sizes of GWAS summary statistics by applying a block jackknife correlation test between the top 20 ancestry principal components (PCs) and GWAS beta values. We then applied the ancestry regression method as implemented in Tilot et al.[16]. Summary statistics of each hemisphere-averaged GWAS were fitted to the top 20 ancestry PCs using a regression model. The LDSC[60] intercept for each set of summary statistics was estimated as an additional measure of population stratification, following the guidelines given in the LDSC website (https://github.com/ bulik/ldsc/wiki/LD-Score-Estimation-Tutorial). SDS[17] analysis as implemented in Tilot et al.[16] was then applied, following instructions in https://bitbucket. org/jasonlouisstein/enigmaevolma6/src/master. We first downloaded SDS values per genomic variant from https://datadryad.org/stash/dataset/doi:10.5061/dryad. kd58f and merged with ancestry-regressed summary statistics by SNP IDs, while SNPs lacking an SDS value were removed from the analysis. In case of mismatching alleles, the SDS values were flipped to match the correct allele. Variants in merged files were sorted based on genomic position, and a block

jackknife Spearman's correlation test (100 blocks) between trait-increasing SDS values and GWAS Z-scores was applied. This analysis was applied for 33 cortical regions segmented with FreeSurfer[22] and full cortical surface area. In this case, to match the evaluation procedure adopted by Tilot et al.[16], P values were conservatively corrected for multiple testing (without taking into account nonindependence of traits) using Benjamini–Hochberg FDR[61]. We assessed consistency with the Tilot et al. findings in two stages: 1) a targeted replication of only the nine significant traits from the prior report and 2) correlation analysis of all 34 traits across the two studies.

Partitioned Heritability Analysis with Custom Evolutionary Annotations. Surface area SNP-heritability contributions of each evolutionary annotation were computed using LDSC partitioned heritability analysis[32] following the guidelines in the Wiki page (https://github.com/bulik/ldsc/wiki/PartitionedHeritability). For the replication study, proportion of heritability, heritability enrichment, and SNP proportions within fetal cortical HGEs active at the seventh postconception week[14] were computed. Fetal HGEs annotation was controlled for both the baselineLD model v2.2 and fetal brain active regulatory elements from the Roadmap Epigenomics Consortium database[62]. Before partitioning heritabilities for hemispheric surface area and whitematter tract SNP-heritabilities, three newly curated enhanced evolutionary annotations were established. We made sure that these three annotations cover at least 1% of the total number of well-imputed SNPs in 1000 Genomes Phase 3 reference panel (only HapMap3 SNPs without the MHC region) (https://github.com/bulik/ldsc) to enhance reliability of heritability estimates. For HGEs, we merged HGEs active in human fetal cortical tissue[14] at three consecutive developmental stages adopted by Tilot et al.[16], creating a single annotation covering all fetal cortical HGEs, increasing the SNP proportion of fetal brain enhancers annotation to above 1%. For Neanderthal introgressed alleles[63], we made use of a list covering Neanderthal introgressed SNPs and SNPs in perfect LD with them ($r^2 = 1$). The archaic deserts[13] annotation was also refined, by removing Neanderthal introgressed SNPs[12] within this annotation. Vernot et al.[13] used a sliding window approach to describe genomic regions that are significantly depleted of Neanderthal sequences in Europeans, East Asians, South Asians, and Melanesians, with average introgression percent $<10^{-3.5}$. This analysis yielded six genomic regions that are significantly depleted for Neanderthal sequence, spanning 85.3 Mb in total. We removed 128 Neanderthal introgressed SNPs in these desert regions, corresponding to 10^{-4} of the total length of Neanderthal deserts, and removed the haplotypes that these introgressed SNPs were in LD with ($r^2 > 0.6$). HGEs active in fetal cortical tissue, archaic deserts and Neanderthal introgressed alleles were controlled for the baselineLD v2.2 model provided by the Alkes Price group[32].

Functional and Evolutionary Annotation of Associated Loci. The GWAS results for left pars triangularis were annotated using Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA; https://fuma.ctglab.nl; version 1.3.6a)[64]. Using the SNP2GENE function, genome-wide significant loci were annotated with eQTL data from four databases with gene expression data of fetal and adult brain samples: GTEx V8 (brain samples only; www.gtexportal.org), BRAINEAC (www.braineac.org), CommonMind consortium[65], and psychENCODE[66]. Loci were also annotated with chromatin interaction data of fetal and adult brain samples from three sources: psychENCODE, GiustiRodriguez et al.[67] and Schmitt et al.[68]. GTEx V8 data assessed through the GTEx Portal (https://www.gtexportal.org) was used to visualize the association between rs1875748 and *ZIC4* expression in adult cortical brain tissue. Neanderthal and Denisovan allele states for SNPs of interest were derived from the ancient DNA

genotype data provided by the Max Planck Institute for Evolutionary Anthropology (cdna.eva.mpg.de/neandertal/)[69]. Sequence alignment for the *ZIC4* locus was performed using sequence data from GRCh37 and panTro3, DOTTER[70] for alignment and plotting. Multiple sequence alignment of 36 mammalian species was downloaded from the Ensembl phylogenetic context server[71]. We used the codeml program from the PAML package[46] to detect selection on the *ZIC4* coding sequence. To identify genome-wide significant loci associated with any of our neuroimaging traits, we performed clumping with PLINK[72], and selected SNPs that are in LD (r^2 > 0.6) with clumped GWAS SNPs. We then identified the loci that overlap with HAR or AMH-derived DMRs by using the findOverlaps function from the GenomicRanges R package[73].

Data Analysis and Visualization. Genetic and evolutionary analyses were conducted on the computing cluster of the Max Planck Institute for Psycholinguistics. Results were parsed and organized with bash scripts. Statistical analyses were conducted with R, Python, and Linux Shell. Plots were generated using R packages plotly and ggplot2.

Data, Materials, and Software Availability. Neuroimaging and genotype data used for GWAS are available from UK Biobank (https://www.ukbiobank.ac.uk). GWAS summary statistics for hemispheric and hemisphere-averaged surface area metrics, as well as for dMRI metrics, are deposited at The Language Archive, a public data archive hosted by the Max Planck Institute for Psycholinguistics (https://archive.mpi.nl/mpi/islandora/object/mpi:1839_4f0e197a_d3cc_4bf7_a5ef_dbe956f59691?asOfDateTime=2022-05-25T12:03:02.720Z)[74]. All scripts used for the analysis are available on the project GitHub repository (https://github. com/galagoz/cortical-evo)[75]. Previously published data were used for this work[10,16,42].

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Supplementary materials

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Chapter 3

Neuroimaging genomics as a window into the evolution of human sulcal organization

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Abstract

Primate brain evolution has involved prominent expansions of the cerebral cortex, with largest effects observed in the human lineage. Such expansions were accompanied by fine-grained anatomical alterations, including increased cortical folding. However, the molecular bases of evolutionary alterations in human sulcal organization are not yet well understood. Here, we integrated data from recently completed large-scale neuroimaging genetic analyses with annotations of the human genome relevant to various periods and events in our evolutionary history. These analyses identified single-nucleotide polymorphism (SNP) heritability enrichments in fetal brain human-gained enhancer (HGE) elements for a number of sulcal structures, including the central sulcus, which is implicated in human hand dexterity. We zeroed in on a genomic region that harbors DNA variants associated with left central sulcus shape, an HGE element, and genetic loci involved in neurogenesis including *ZIC4*, to illustrate the value of this approach for probing the complex factors contributing to human sulcal evolution.

3.1 Introduction

The evolution of mammals led to considerable interspecific diversity in neuroanatomy, including substantial cortical expansions in the primate order[1]. These expansions were accompanied by an array of changes at different anatomical levels, such as the emergence of primate-specific cell types[2], overall increases in cortical surface area[3], and reorganizations of gyral and sulcal morphology[4]. As humans have the highest gyrification index among primates[5], the gyral and sulcal organization of the human cortex stands out as a trait of particular evolutionary interest, with potential relevance for aspects of cognition and behavior[6,7]. Although the anatomical basis of sulcal morphology in primates is rather well studied[8], we are only just beginning to gain insights into genetic pathways that contribute to overall degree of gyrification and regional distribution and morphology of individual sulci. Moreover, the ways in which such genetic pathways changed in hominin evolution are poorly understood.

While comparative approaches have revealed critical differences in gyral and sulcal organization across primates[5,9,10], findings are mainly limited to gross anatomical features and do not provide insights into their biological bases. On the other hand, much of our understanding of molecular genetic contributions to primate brain evolution comes from in vitro neuronal and organoid models and in silico comparative genomic and transcriptomic studies, which have identified primate-specific brain cell-types and human-specific molecular changes[2,11]. Yet, such findings often require validation in primary tissue, which is strictly limited by the scarcity of high-quality nonhuman primate brain samples. Thus, there remains a large gap between the comparative neuroimaging and experimental approaches, hindering a comprehensive understanding of human and nonhuman primate brain evolution.

Advances in large-scale neuroimaging genomics investigations in present-day humans have lately proven useful to bridge this gap, as they combine some aspects of neuroanatomical and molecular approaches. Datasets encompassing both brain magnetic resonance imaging (MRI) and single-nucleotide polymorphism (SNP) information from tens of thousands of people, such as the UK Biobank[12], and the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) Consortium[13], have allowed the first well-powered genome-wide association studies (GWASs) of interindividual differences in a variety of neuroanatomical traits including cortical surface area, white-matter connectivity, and sulcal morphology[14,15,16]. In parallel, the fields of ancient DNA and comparative primate genomics have been generating considerable amounts of genomic and functional data, which highlight regions of the genome that may have been important in human evolution. Availability of multiple high-quality Neandertal genomes[17,18,19] led to the identification of Neandertal introgressed alleles in presentday humans as a result of the admixture events from ∼50,000 to 60,000 years ago (Vernot and Akey 2014). This enabled genomic annotations not only of Neandertal introgressed fragments but also of regions that are significantly depleted of Neandertal ancestry, archaic deserts, in present-day humans[20]. On the other hand, comparing histone modification profiles of fetal human, macaque and mouse brain tissues revealed regions of the genome that are active solely in the fetal human brain, referred to as human-gained enhancers (HGEs), with potential relevance within the last ∼30 million years of evolution along the lineage that led to *Homo sapiens*[21].

Initial work by Lemaitre et al.[22] investigated the evolution of sulcal opening and depth using summary statistics from 18,000 individuals of European ancestry from UK Biobank, in combination with evolutionary annotations of the human genome compiled by Tilot et al.[23].

While they found significant SNP-heritability enrichment for fetal brain HGEs in left and right calloso-marginal posterior fissures and the right central sulcus, their approach also showed a number of significant partitioned SNP-heritability estimates that had a negative sign, a pattern of findings that is biologically implausible. They highlighted that these might ref lect limitations of the study related to (i) low SNP proportions covered by evolutionary annotations relative to the reference panel and (ii) the sample size of the early UK Biobank neuroimaging data release[24], which was relatively modest for contemporary GWAS efforts. In this study, we adopted an improved analytic pipeline to address these limitations by using three newly curated evolutionary annotations with sufficient SNP coverage[25], two additional sulcal morphology measures that were not investigated by Lemaitre et al.[22] (sulcal length and surface area), and an even larger sample size.

Here, we made use of results from the latest genome and exome wide association study on regional sulcal morphology in around 26,530 individuals in UK Biobank, which incorporated four regional shape parameters (length, mean depth, surface area, and width) from each sulcus, comprising in total 450 sulcal parameters across 58 sulci[26]. We integrated the summary statistics of this study with an enhanced set of evolutionary annotations, and split our analytic pipeline into two parallel streams: (i) a hypothesis-based targeted study of four sulci with high evolutionary relevance based on prior literature and (ii) a hypothesis-generating exploratory study of 45 sulci, with both streamlines focusing on regional hemispheric sulcal anatomy measures [see Figure S1 for an overview of the trait selection workflow]. For the targeted study, we hypothesized that the common genetic variation underlying presentday anatomic variation in these sulci would be enriched in fetal brain HGEs, as the sulci are phenotypically divergent between humans and Old World monkeys (OWMs). In addition, given the variable organization of such sulci across apes, we might expect enrichment or depletion signals in evolutionary annotations that tag more recent timescales, such as those related to Neandertal ancestry.

The four sulci of interest in our targeted approach (the central sulcus, the paracentral sulcus, the parieto-occipital sulcus, and the superior temporal sulcus), are important and relevant for human brain evolution for a number of reasons. The central sulcus was shown to exhibit changes in surface area, and in its folding pattern, particularly during the evolution of OWMs by a comparative study using magnetic resonance imaging of OWM, apes, and humans[27]. The paracentral sulcus[9], the parieto-occipital sulcus[28], and the superior temporal sulcus[29] show the most marked morphological changes when comparing OWMs to apes and humans. For the targeted approach, we aimed to exploit all four available sulcal shape descriptors and identified significant SNP-heritability enrichment signals in HGEs for the width of both the right and left central sulcus, extending prior righthemispheric findings[26]. The exploratory stream involved analyses of 45 sulci and revealed that common genetic variants associated with the right hemisphere olfactory sulcus depth are significantly enriched in HGE elements. We also discovered a set of genetic variants associated with the right orbital sulcus depth that fall into two human accelerated region (HAR) elements. Overall, we demonstrate that combining neuroimaging genetics with heritability-based post-GWAS evolutionary analyses can shed light on aspects of human brain evolution by identifying links between brain structures and evolutionary annotations of the human genome and highlighting genes of particular interest for future experimental follow-up.

3.2 Materials and methods

GWAS summary statistics

Sulcal morphology GWASs were performed by Sun et al.[26] using a recent release of the UK Biobank brain imaging and genotype data set (n = 26,530). Specifically, 62 sulcal folding traits per brain hemisphere and four sulcal shape descriptors were examined, resulting in 450 sulcal parameter phenotypes after excluding shape descriptors with >75% missingness, including 58 sulci and four sulcal shape parameters. The brain imaging dataset contains reliable quantifications of four sulcal shape descriptors: sulcal depth, length, width, and surface area, each representing a different aspect of sulcal morphology. Imaging phenotypes were inverse-rank-normalized in order to approximate a standard normal distribution and minimize outlier effects. The total sample of 40,169 individuals was split into discovery ($N_{Discovery}$ = 26,530) and replication ($N_{Replication}$ = 13,639) cohorts, with the discovery cohort including data from only European ancestry individuals and the replication cohort including admixed ancestries, which would likely confound any heritability-based evolutionary analyses[30]. Sample sizes differed depending on the specific sulcal parameters and are listed in Tables S2 and S3. The online browser for GWAS summary statistics is publicly available at https://enigma-brain.org/sulci-browser [see Sun et al.[26] for details].

SNP-heritability thresholding

We performed univariate linkage-disequilibrium score regression (LDSC)[31] to estimate total SNP-heritability of 450 sulcal parameters obtained from Sun et al.[26] GWAS summary statistics to optimize our partitioned heritability analysis. We then filtered sulcal parameters based on these SNP-heritability estimates using R (v4.0.3), prior to performing LDSC partitioned heritability analysis. Our filtering process consisted of two stages: (i) exclusion of traits with SNP-heritability < 10% and (ii) exclusion of traits with non-significant ($\alpha_{Bonferroni}$ = 0.05/163) SNP-heritability estimates. We performed the significance test by generating the cumulative probability distribution of the total SNP-heritability estimates of 163 preselected sulcal parameters, using the p.norm function of R-base (v4.0.3), yielding 153 sulcal parameters with significant SNP-heritability estimates for the subsequent partitioned heritability analysis.

Estimating number of independent neuroimaging traits

For the targeted and exploratory analysis streams, we reduced the multiple-testing burden by considering the genetic correlations across investigated traits. We used spectral decomposition of matrices (SpD) implemented in the R package PhenoSpD[32] to estimate the effective number of independent variables (VeffLi). This resulted in 9.48 and 73.59 independent traits for the targeted and exploratory analysis subsets, respectively.

Partitioned heritability analysis

Contributions of evolutionary annotations to the total SNPheritability of each sulcal morphology measure were computed using the LDSC partitioned heritability tool[33], following the guideline and tutorials in the LDSC Github Wiki website (https://github.com/bulik/ldsc/wiki/PartitionedHeritability). We exploited three evolutionary annotations prepared by Alagöz et al.[25], with increased SNP-coverage ratios covering at least 1% of the SNPs in the 1000 Genomes reference panel[34] (HapMap3 SNPs, the MHC region SNPs were excluded): fetal brain HGEs[21], Neandertal introgressed alleles[35], and archaic deserts[20]. All

annotations were controlled for the baselineLD v2.2 model provided by the Alkes Price group, whereas the fetal brain HGE annotation was additionally controlled for the active regulatory elements from the Roadmap Epigenomics Consortium database[36]. The fetal brain HGE annotation covers the enhancer elements that are active in human fetal cortical brain tissue at 7^{th}, 8.5^{th} and 12^{th} postconception weeks, while inactive in macaque and mouse brains during the corresponding developmental stages. The Neandertal introgression annotation is a list of SNPs including the genomic coordinates of the introgressed SNPs, and their "LD-friends" [SNPs that are in perfect LD ($r^2 = 1$) with the introgressed allele]. The archaic deserts annotation encompasses large stretches of the genome that are significantly depleted for Neandertal introgressed alleles. These deserts were identified by Vernot et al.[20] by using a sliding window approach, which detects regions that are 10 Mb or larger and significantly depleted of Neandertal DNA in various populations including Europeans, Asians, and Melanesians (average introgression percent per region < $10^{-3.5}$, see Vernot et al.[20] for details).

Functional and evolutionary annotation of GWAS loci

For left hemisphere central sulcus width, we performed a qualitative analysis to identify overlaps between fetal brain HGE elements and genome-wide significant ($P < 5 \times 10^{-8}$) loci ($r^2 > 0.6$), which we identified using the clump function of PLINK[37]. After detecting the genome-wide significant SNPs associated with width of the left central sulcus that fall into the fetal brain HGE annotation, we focused on an example region harboring a GWAS hit, an HGE element, and *ZIC4*/*ZIC1* genes. We used the SNP2GENE function of Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA, version 1.3.6a)[38] to assess the chromatin interaction profile of the ZIC4 locus with data from fetal and adult brain samples[39,40]. We investigated the cisexpression quantitative trait loci (eQTL) properties of the GWAS hit lead SNP using the MetaBrain brain tissue gene expression database[41]. For the comparative genomic analysis, we derived the Neandertal and Denisovan allelic states for our SNP of interest using the publicly available ancient genotype dataset from the Max Planck Institute for Evolutionary Anthropology (cdna.eva.mpg.de/neandertal/) and used the Ensembl phylogenetic context server[42] to fetch the allelic states of nonhuman primates. We performed an overlap analysis between genome-wide significant loci (SNPs with $P < 5 \times 10^{-8}$ and SNPs that are in LD $r^2 > 0.6$ with such SNPs) associated with any sulcal parameter and HARs by using PLINK[37] and the findOverlaps function from the GenomicRanges R package[43]. Overlapping loci were visually inspected in more detail by making LocusZoom plots[44], and the exact SNPs that fall into HAR elements were extracted from the summary statistics. Gene expression trajectories of *ZIC1* and *ZIC4* in anterior cingulate cortex, dorsolateral prefrontal cortex, inferolateral temporal cortex, ventrolateral prefrontal cortex, dorsolateral prefrontal cortex, occipital neocortex, orbital frontal cortex, posterior superior temporal cortex, parietal neocortex, posteroventral parietal cortex, primary auditory cortex, primary visual cortex, primary somatosensory cortex, temporal neocortex, and ventrolateral prefrontal cortex were extracted from the BrainSpan Atlas of the Developing Human Brain dataset[45], which comprises gene expression data of a period from the 8^{th} post-conception week up to 40 years of age.

3.3 Results

SNP-heritability estimation and establishment of the targeted and exploratory analytic streams

We used publicly available sulcal morphology GWAS summary statistics (UK Biobank, n = 26,530) of 450 sulcal parameters [see Sun et al.[26] for the details]. These GWASs were performed on 58 sulci across brain hemispheres and four sulcal shape descriptors (mean depth, surface area, width, length). We estimated the total SNP-heritability of each sulcal parameter using univariate LDSC[31] and showed that the SNP-heritability estimates varied across sulci and sulcal shape descriptors ranging between 0.07% and 31%, with a median SNP-heritability of 7% (SE = 0.03) (Fig. 1A, Table S1).

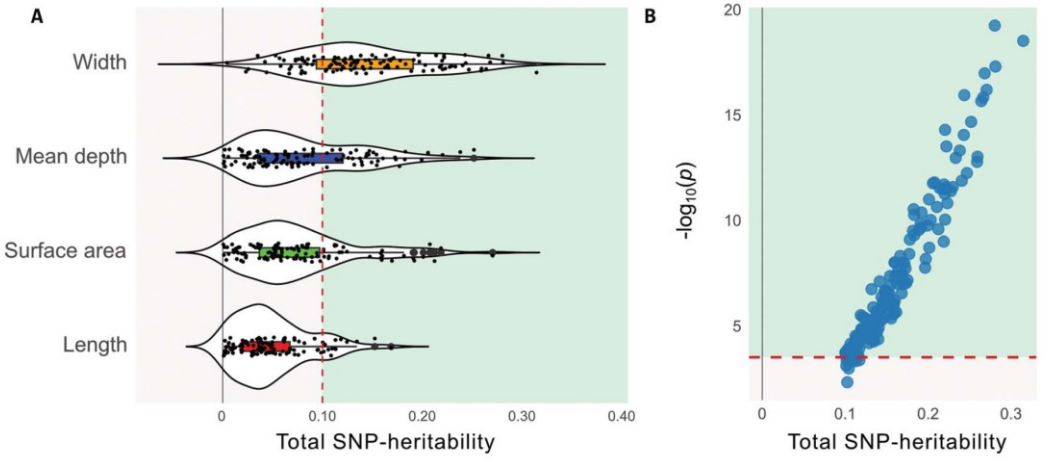

Fig. 1. Univariate SNP-heritability estimates and heritability-based filtering of sulcal parameters. A) Total SNP-heritabilities of the 450 sulcal parameters estimated using the GWAS summary statistics from Sun et al.[26]. The mean total SNP-heritability estimates varied across sulcal shape descriptors. Traits with >10% heritability were kept for the second step of filtering. The dashed line indicates the 10% cut-off. Traits that are kept for subsequent filtering are highlighted in green (to the right of the dashed line); filtered ones are depicted in gray (to the left of the dashed line). B) $-\log_{10}$(P-values) ref lecting SNP-heritability significance of each sulcal parameter. The dashed line indicates the significance threshold [$-\log_{10}(0.05/163)$]. Traits that are kept for partitioned heritability analysis are highlighted in green (above the dashed line); filtered ones are depicted in gray (below the dashed line).

Given the importance of trait heritability for obtaining statistically reliable partitioned heritability estimates[33], we optimized our analytic pipeline for the subsequent partitioned heritability analysis (i.e. to minimize negative partitioned heritability estimates, reduce the standard errors, and minimize the multiple-testing burden). For this purpose, we applied a twostep heritability-based filtering, before performing any enrichment tests. We first filtered out traits with a SNP-heritability estimate of less than 10%[46] and obtained 163 sulcal parameters (52 sulci, four sulcal shape descriptors) (Fig. 1A). Secondly, we excluded any trait for which the SNP-heritability estimate did not meet significance ($\alpha_{Bonferroni}$ = 0.05/163, significance test against zero), yielding 153 sulcal parameters (49 sulci, four sulcal shape descriptors) (Fig. 1B). These traits were used to curate two subsets for our targeted and exploratory analysis streams. For the targeted analysis, we included the central sulcus, the paracentral sulcus, the parieto-occipital sulcus, and the superior temporal sulcus and three sulcal shape descriptors. These criteria yielded, across the two hemispheres, a total of 14 sulcal parameters with sufficient SNP-heritability (range: 12% to 31%, four sulci, three remaining sulcal shape descriptors) (Table S2). Importantly, none of the sulcal length parameters survived our heritability thresholding for the

targeted trait selection. For the exploratory stream, we did not preselect any sulci and included all four shape descriptors again. Consequently, 139 sulcal parameters with sufficient SNP-heritability estimates were identified for the exploratory analysis stream (range: 10% to 28%, 45 sulci, four sulcal shape descriptors) (Table S3).

As shown by Sun et al.[26], some of the sulcal parameters are phenotypically and genetically correlated across and within brain hemispheres. Hence, we estimated the effective number of independent traits within targeted and exploratory stream subsets using PhenoSpD (v1.0.0)[32] and two genetic correlation matrices of 14 and 139 sulcal traits, which identified 9 and 74 independent variables, respectively. The number of independent variables was subsequently used for multiple testing correction.

Central sulcus width–associated genetic variants are enriched in fetal brain HGEs

In the targeted study, we performed LDSC-based partitioned heritability[33] analysis of the 14 preselected sulcal parameter GWAS summary statistics from UK Biobank (n = 26,530)[26] that showed sufficient heritability estimates (according to filtering criteria described above), with a median of 16% (Table S2). For each of these sulcal parameters, we tested SNP-heritability enrichment/depletion in three evolutionary annotations covering different evolutionary timescales: fetal brain HGEs, Neandertal introgressed alleles, and archaic deserts. After false discovery rate (FDR) correction for nine independent sulcal parameters, we identified significant SNP-heritability enrichments in fetal brain HGEs for the width of left and right central sulci [left: Enrichment(SE) = 7.75(2.52), P_{FDR} = 0.04, right: Enrichment(SE) = 7.57(2.44), P_{FDR} = 0.04] (Fig. 2, Table S4), as well as right parieto-occipital sulcus surface area [Enrichment(SE) = 13.03(3.86), P_{FDR} = 0.01] and right superior temporal sulcus width [Enrichment(SE) = 5.66(1.94), P_{FDR} = 0.04] (Fig. 2, Table S4). No other significant positive enrichments or depletions were found in Neandertal introgressed alleles and archaic deserts annotations for the remaining sulcal parameters in our targeted analysis (Tables S5 and S6).

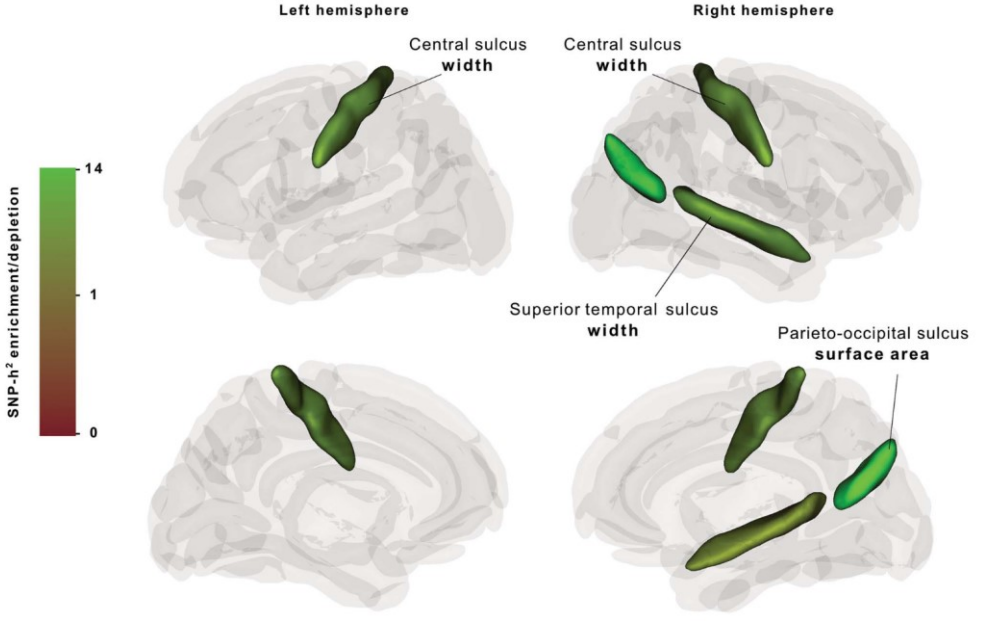

Fig. 2. Three-dimensional visualization of brain sulcal structure and SNP-heritability enrichment/depletion levels in fetal brain HGEs (covering active enhancer elements at 7^{th}, 8.5^{th}, and 12^{th} pcw) for the targeted sulci in each hemisphere. Non-significant results for targeted sulci are shown in dark gray and other sulci in light gray.

In addition to the three evolutionary annotations tested here, we estimated SNP-heritability enrichment in fetal brain active regulatory elements to see whether the SNP-heritability enrichment signals in fetal brain HGEs are a common property of brain active regulatory annotations. Interestingly, left [Enrichment(SE) = 9.02(2.88), P_{FDR} = 0.01] and right [Enrichment(SE) = 12.83(3.89), P_{FDR} = 0.0002] superior temporal sulcus mean depth yielded significant SNP-heritability enrichment in fetal brain active regulatory elements (Table S7), yet these sulcal parameters do not overlap with the ones for which we identified significant enrichment in fetal brain HGE analysis.

Right hemisphere olfactory sulcus width SNP-heritability is enriched in fetal brain HGEs

For the exploratory stream, we widened the scope of our analysis to examine sulcal morphology across the whole brain. We tested the 139 sulcal trait GWAS summary statistics from UK Biobank (n = 26,530)[26] with a median SNP-heritability estimate of 15% (SE = 0.03) for SNP-heritability enrichment and depletion in the same three evolutionary annotations as the targeted approach. After FDR correction for 74 independent sulcal parameters, we identified a significant heritability enrichment in fetal brain HGEs for the mean depth of the right olfactory sulcus [Enrichment(SE) = 11.26(2.91), P_{FDR} = 0.02] (Fig. 3, Table S7). We also identified significant SNP-heritability depletions in the archaic deserts for the width measures of five sulcal parameters: The left internal frontal sulcus [Enrichment(SE) = 0.11(0.25), P_{FDR} = 0.03], the left inferior frontal sulcus [Enrichment(SE) = 0.07(0.32), P_{FDR} = 0.04], the left posterior inferior temporal sulcus [Enrichment(SE) = 0.13(0.30), P_{FDR} = 0.04], as well as the right insula [Enrichment(SE) = 0.28(0.24), P_{FDR} = 0.04], and the right posterior terminal ascending branch of the superior temporal sulcus [Enrichment(SE) = 0.14(0.29), P_{FDR} = 0.04] (Fig. 3, Table S8). We did not detect any significant positive enrichment or depletion signals for the Neandertal introgressed alleles annotation (Table S9).

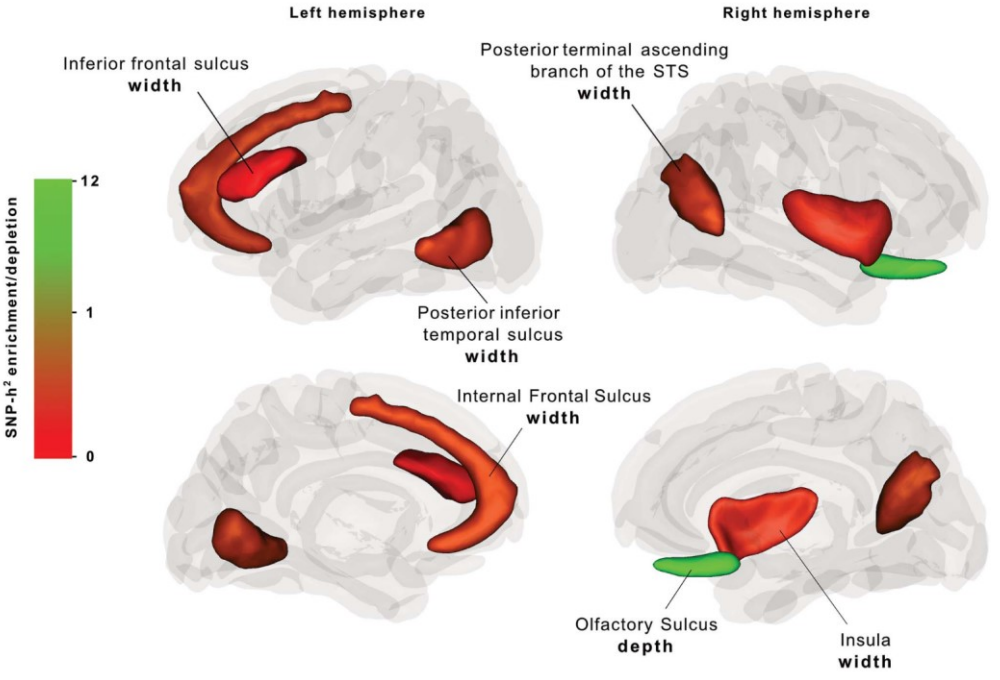

Fig. 3. Three-dimensional visualization of brain sulcal structure and SNP-heritability enrichment/depletion levels in fetal brain HGEs and archaic deserts for the sulci analyzed in the exploratory analysis stream. Non-significant results for targeted sulci are shown in dark gray and other sulci in light gray.

We then estimated SNP-heritability enrichment in fetal brain active regulatory elements to see whether SNP-heritability enrichment in fetal brain HGEs is a common property of brain active regulatory regions. We identified six sulcal parameters with significant SNP-heritability enrichment in fetal brain active regulatory elements (Table S11). There are not any parameters that overlap with the ones identified from fetal brain HGE analysis.

Left central sulcus width–associated variants overlap with an HGE colocalized with *ZIC4* and *ZIC1* genes

Finally, we aimed to pin down putative loci and genes implicated in the evolution of human sulcal organization by evaluating the links among sulcal morphology–associated common genetic variation, HGEs, and chromatin interaction patterns in fetal human brain tissue. Given the importance of the central sulcus in manual motor skills and dexterity, which are critical human adaptations[41,47], and the fact that it yielded two robust significant SNP-heritability enrichment signals (Table S4), we followed up with evolutionary and functional annotations of the common genetic variants associated with this sulcus. Out of 11 independent genome-wide significant ($P < 5 \times 10^{-8}$) loci (blocks of LD covering lead SNPs and adjacent SNPs in linkage disequilibrium, $r^2 > 0.6$) associated with left central sulcus width, we detected five LD blocks that overlap with fetal brain HGE elements (Table S10). Intriguingly, one of these five loci is tagged by the independent genome-wide significant SNP rs884370 [$P = 1.39 \times 10^{-8}$, Beta(SE) = −0.05(0.009)], which is located in a fetal brain HGE element adjacent to the *ZIC4* gene (chr3:147,101,875-147,103,850) (Fig. 4A). We further annotated the locus using the chromatin interaction data from fetal brain tissue to test the regulatory relevance of this enhancer element

and the SNP rs884370. FUMA functional annotation[38] revealed that the HGE element interacts with the promoter regions of *ZIC4* and *ZIC1* (Fig. 4A).

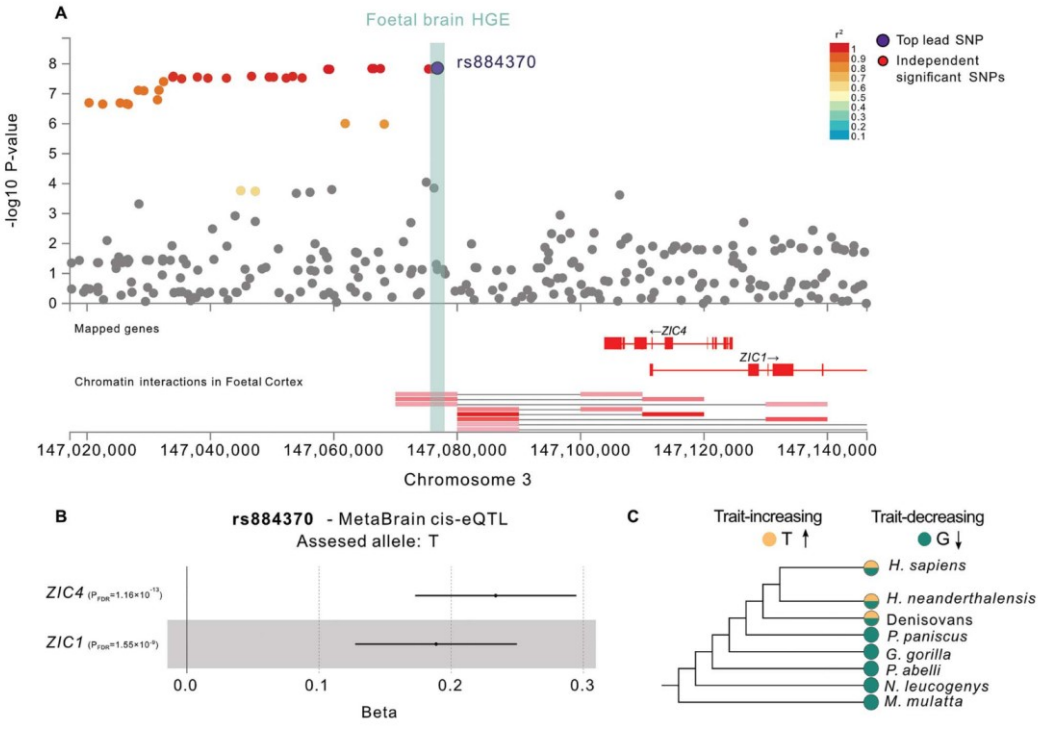

Fig. 4. Functional-evolutionary annotation of the locus around rs884370, which is associated with the width of the left-hemisphere central sulcus. A) The LocusZoom plot obtained by FUMA shows SNPs with genome-wide significant association ($P < 5 \times 10^{-8}$). The fetal brain HGE element that overlaps with rs884370 is shown as a light green bar. Colors represent linkage disequilibrium with the lead SNP rs884370. The chromatin interaction profile of the locus demonstrates the overlap between the HGE element and the interaction region. hg19 coordinates are shown. B) cis-eQTL plot of rs884370, showing the impact of this SNP on *ZIC4* and *ZIC1* expression in adult brain tissue for the T allele. Error bars represent the standard error. C) Derived and ancestral allelic states for rs884370 are shown for *H. sapiens*, Neandertals, Denisovans, and five nonhuman primates. Color coding indicates the derived and ancestral states.

We further scrutinized the regulatory and evolutionary relevance of the SNP rs884370 by leveraging the MetaBrain eQTL dataset[48], a publicly available ancient DNA genotype dataset, and non-human primate genome assemblies. Our analyses revealed that rs884370 is annotated as a cis-eQTL of *ZIC4* [$P = 5.81 \times 10^{-14}$, Beta(SE) = 0.23(0.03)] and *ZIC1* [$P = 1.55 \times 10^{-14}$, Beta(SE) = 0.23(0.03)] in adult brain tissue (Fig. 4B, Table S11). The assessed (alternative) allele of rs884370 (T) is associated with increased expression of both *ZIC4* and *ZIC1* in adult brain tissue, as well as with increased width of the left hemisphere central sulcus (Fig. 4B). To understand the role of *ZIC1* and *ZIC4* in human brain development, we extracted the gene expression profiles of these genes in cortical regions over a large time period from 8th post-conception weeks (pcw) up to adulthood by using the BrainSpan dataset[45] (Fig. S3). *ZIC1* expression in the prenatal cortex peaks at early stages of brain development around 8–10 pcw, descends to lower levels at 19–24 pcw. Interestingly, the *ZIC1* expression averaged across 15 cortical regions and 11 developmental periods is higher than *ZIC4* expression for all time points from development to adulthood ($ZIC1_{RPKM} = 2.96$, $ZIC4_{RPKM} = 0.40$). Our comparative genomics analysis reveals that

the trait-increasing allele (T) is a hominin-derived allele. We detect the same polymorphism at the site in the available ancient DNA data from Neandertals and Denisovans (Fig. 4C). Finally, we used The Human Genome Dating Atlas (HGD)[49] to estimate the age of the rs884370 polymorphism. HGD estimates that it is 54,171 generations old (95% confidence interval), corresponding to ∼1.35 mya assuming 25 years per generation.

Finally, we performed another overlap analysis between targeted and exploratory stream sulcal traits and HARs, representing an evolutionary annotation that we could not investigate using partitioned heritability due to its relatively small SNP coverage. Hence, we extracted the list of genome-wide significant loci (SNPs with $P < 5 \times 10^{-8}$ and SNPs that are in LD $r^2 > 0.6$, comprising 136 loci in targeted and 481 loci in exploratory stream) from the GWAS summary statistics of the targeted and exploratory stream sulcal traits. We discovered four SNPs overlapping with two adjacent HAR elements (Fig. S2 and Table S14).

3.4 Discussion

We exploited summary statistics of a large-scale genome-wide scan for regional bilateral sulcal anatomy measures by Sun et al.[26], which leveraged a dataset encompassing structural brain MRI and genotype information from over 26,530 individuals. We investigated the links between genetic variation underlying variability in sulcal organization and the marks that various evolutionary events have left in our genomes during human evolution. This noninvasive neuroimaging genetics strategy allowed us to reveal aspects of brain evolution by investigating the standing genomic and neuroanatomical variation in present-day humans, which has been largely unexplored by prior comparative primate neuroanatomical and neuronal model–based experimental work. Our study has three main advantages compared to prior similar studies: (1) larger sample size yielding higher power, (2) two previously unexplored sulcal measures (surface area and length), and (3) three enhanced evolutionary annotations of suitable SNP coverage.

Our targeted approach tested 14 evolutionarily relevant sulcal measures and three annotations for SNP-heritability enrichment/depletion, but note that we could not predict whether to expect an enrichment or depletion signal in the Neandertal ancestryrelated annotations, since the sulcal anatomy of Neandertals is largely unknown, due to absence of fossilized brain tissue. Our partitioned heritability analysis revealed significant enrichment signals in fetal brain HGEs for left and right hemispheric central sulcus width, which overlap with the findings of Lemaitre et al.[22] showing right central sulcus heritability enrichment in fetal brain HGEs active in 7th pcw. The central sulcus divides pre- and postcentral gyri, and the precentral gyrus is involved in motor-hand control in a contralateral manner[28]. The morphological differentiation of this sulcus among primates does not show a lateralized pattern[28], which is in line with our robust enrichment findings for both hemispheres. We believe that the larger sample size of the current study (n = 26,530) and the fact that our enhanced fetal brain HGE annotation covers a wider neurodevelopmental window of the fetal brain, as well as encompassing more SNPs, enabled us to identify a global (i.e. bilateral) enrichment signal for the central sulcus, as opposed to the right-hemispheric finding of Lemaitre et al.[22]. Significant enrichment signals for the left and right calloso-marginal posterior fissure in the aforementioned study are absent in our results, possibly because our fetal brain HGE annotation minimizes the false-positive enrichment estimates. The significant heritability enrichment for the surface area of right parieto-occipital in fetal brain HGEs further supports our central sulcus finding, as this sulcus separates parietal and occipital cortex[29] regions hypothesized to show human specialization for body–tool

coordination[50,51]. Additionally, we identified significant heritability enrichment in fetal brain HGEs for the width of the superior temporal sulcus, which is known for its involvement in social perception and cognition and deemed to have a key role in the convergence of spoken and written language[52,53]. Interestingly, this enrichment was detected in the right hemisphere, which might highlight the prominent rightward asymmetries in the central regions of the superior temporal sulcus, with particular differences in depth across hemispheres, which are less pronounced in chimpanzees[54,55]. Importantly, none of these enrichment signals were evident in our control analysis with the fetal brain active regulatory elements annotation, indicating that the relative contribution of genetic variants in fetal brain HGEs to sulcal morphology formation is not a general property of human brain enhancers. Altogether, the enrichment signals in fetal brain HGEs for the entirety of the central sulcus, the right parietooccipital sulcus, and the superior temporal sulcus may indicate the involvement of human-gained gene regulatory elements in the evolution of human hand dexterity, social cognition, and speech. Here, we note that in addition to the four sulci that we tested, there are other sulci that are relevant for human brain evolution. For example, paracingulate sulcus, the intralimbic sulcus, anterior and posterior vertical paracingulate sulcus, and dorso- and ventromedial-polar sulci were associated with the expansion of the medial frontal cortex in humans compared to chimpanzees[10]. However, these sulci were not part of the sulcus atlas used by Sun et al.[26] and therefore could not be included in our targeted approach. In summary, the targeted analysis stream identified significant heritability enrichment signals in fetal brain HGEs for four sulcal parameters across brain hemispheres, showing HGEs play a substantial role in the formation and evolution of these particular sulci.

The exploratory analysis on the remaining 139 sulcal parameters revealed a robust significant heritability enrichment signal in the HGEs for the depth of the right olfactory sulcus. The olfactory sulcus divides the medial orbitofrontal gyrus and the rectus gyrus, harboring the olfactory bulb and tract[56], suggesting a close link between the anatomies of the olfactory sulcus and the bulb. Reduced olfactory sulcus depth has been linked to congenital anosmia, which is a disorder identified by the inability of smell[57]. On the other hand, it has been reported in the primate comparative neuroanatomy literature that a reduction in olfactory bulb size was followed by a shift toward visual system reliance, evolving independently in OWMs and apes[58]. This is in line with our enrichment finding in fetal brain HGEs for the right olfactory sulcus, suggesting involvement of human-gained regulatory regions in the evolution of human olfactory structure. The fact that we detect this signal only for the right hemispheric olfactory sulcus is difficult to interpret. The right olfactory sulcus has been reported to be deeper compared to the left, showing an anatomic lateralization for this sulcus. Intriguingly, olfactory regions have been shown to be functionally lateralized as well, with the left hemisphere being involved in emotional processing of odors and the right side in the process of recognition memory[59]. Combining our heritability enrichment finding for the right hemisphere olfactory sulcus with the anatomical and functional lateralization of this sulcus suggests involvement of HGEs in odor-based recognition memory differences between humans and OWMs.

Interpretation of the heritability depletion in archaic deserts for the width of left hemisphere internal frontal sulcus, inferior frontal sulcus, and posterior inferior temporal sulcus, as well as for the width of right hemisphere insula and the posterior terminal ascending branch of superior temporal sulcus, is more speculative. Heritability depletion in archaic deserts might indicate that these genomic regions lack genes and regulatory elements involved in the aspects of fetal brain development that shape this particular sulcal structure. In line with our results, Alagöz et al.[25]

reported significant heritability depletion in archaic deserts for surface area of the left pars opercularis, which is topologically in close proximity to both the internal frontal sulcus and inferior frontal sulcus and is associated with speech processing, articulation, and phonological processing[60].

Finally, we focused on an example locus to show the intricate links between common genetic variants associated with sulcal morphology, functional context, and their evolutionary past. We chose a genome-wide significant locus ($P < 5 \times 10^{-8}$) on chromosome 3 that is associated with the left hemisphere central sulcus width, tagged by the lead SNP rs884370. Our analysis showed that the rs884370 is located within a fetal brain HGE element near *ZIC4* and regulates the neural expression of *ZIC1* and *ZIC4*, which are adjacent paralogue genes known to be involved in neurogenesis[61]. Our gene expression trajectory look-ups in the BrainSpan dataset[45] showed that both genes are expressed at varying levels in preand postnatal human cortex, with *ZIC1* expression surpassing *ZIC4* expression at all developmental stages and adulthood, which may suggest a more important role for *ZIC1* in neurodevelopment and formation of human sulcal anatomy. We further demonstrate that the *ZIC4* promoter region interacts with this HGE element, providing evidence for the involvement of HGEs in the evolution of neurodevelopment through differential regulation of *ZIC4* across primates. A previous study[25] identified common genetic variants associated with the surface area of the left hemisphere pars triangularis, a cortical region involved in speech and language, located in the same fetal brain HGE element that is an eQTL for *ZIC4* expression in human cortical tissue. The findings here converge with this previous finding, showing that the standing genetic variation on HGE elements impacts various aspects of human cortical anatomy from surface area to sulcal morphology. In the exploratory stream of the study, we did not identify significant heritability enrichments or depletions within Neandertal introgressed alleles for any sulcal traits. The absence of such signals may indicate that the common genetic variants associated with sulcal organization do not concentrate at or are diminished from the Neandertal introgressed regions. This is consistent with larger neuroanatomical differences across primates, rather than between H. sapiens and Neandertals. Our comparative genomic findings for rs884370 further support this by showing that this polymorphism is shared across present-day humans, Neandertals, and Denisovans, but is not identified in nonhuman primates. Overlap analysis between HARs and GWAS signals from our targeted and exploratory analysis streams yielded four SNPs associated with right orbital sulcus located in two adjacent HAR elements. The same HAR elements were previously found to be associated with right lateral orbitofrontal cortex surface area[25], which, together with our findings, might suggest a putative role for these two HAR elements in the evolution of right orbitofrontal cortex arealization and gyrification. Yet, we note that the overlap analysis is a qualitative method, which is useful for discovering genes and loci of interest for future experimental investigations.

Our study has a number of limitations. First, the comparative neuroanatomy field has investigated sulcal structures at various levels, with higher priority on the sulci with clinical importance or colocalization with cortical regions such as Broca's area. Thus, the breadth of prior information on sulcal anatomy is skewed toward a small subset of sulci. This makes it challenging to formulate testable hypotheses for the genetic and molecular evolution of sulcal organization at a global level. Hence, we had to split our analytic pipeline into targeted and exploratory branches, each focusing on different sets of sulci with different levels of prior available knowledge. Second, this work is based on GWAS summary statistics[26] derived from a European ancestry population, hindering a full understanding of the link between human

genetic variation and phenotypic variability in sulcal morphology. Third, even though we used the largest available GWAS of sulcal morphology with data from 26,530 people, we acknowledge that even larger sample sizes will be required to capture all true positive heritability enrichment and depletion signals in the tested annotations. Fourth, as with most large scale neuroimaging studies, the quality of the MRI-based sulcal parameter estimates was not checked manually, due to practical challenges of doing this across tens of thousands of individuals. Fifth, in addition to three evolutionary annotations of the human genome that we tested here, there are other interesting sets of genomic regions that would be relevant for studying human brain evolution. However, as previously reported[25], annotations often cover a relatively small percentage of the SNPs available in population genetics reference panels, which can lead to spurious enrichment/depletion signals and significantly negative partitioned heritability estimates, beyond biological plausibility. We thus preferred to adopt a more conservative approach by using evolutionary annotations with at least 1% SNP coverage, to minimize significant negative heritability estimates, and made no attempt to interpret such findings as they are not biologically meaningful. Despite our efforts to minimize such results, our analysis yielded a few significant negative heritability enrichment/depletion signals, which are not biologically interpretable.

We believe that the ever-increasing sample sizes of neuroimaging GWASs will boost the statistical power of heritability-based evolutionary analyses, enabling more precise heritability enrichment/depletion estimates. Methodological improvements in the complex trait evolution field, which allow investigation of selection by making use of genotype and phenotype data at the individual level[62], are likely to provide results that are complementary to heritability-based methods. Finally, the continued accumulation of ancient DNA data from extinct hominins, as well as comparative genomics, epigenetics, and transcriptomics data from living relatives of humans, will uncover new genomic regions of interest, fueling the future of human brain evolution research.

In summary, here we shed light on the complex links between common genetic variants associated with various aspects of human sulcal organization, and evolutionary annotations of the human genome such as fetal brain HGEs, Neandertal introgressed fragments, and archaic deserts. Our results showed a robust link between interindividual variability in the morphology of five sulcal structures and fetal brain HGEs, suggesting involvement of enhancer elements gained on the human lineage over the last ∼30 million years in sulcal evolution of our species. Among all fetal brain HGEs that shape our sulcal anatomy, a specific HGE element adjacent to the *ZIC1*/*ZIC4* locus overlaps with a strong GWAS signal associated with variability in the width of the lefthemisphere central sulcus. This overlap provides an important concrete example of how enhancer elements that are gained over the course of human evolution may help shape sulcal structures. Our results demonstrate how integrating neuroimaging genetics with evolutionary annotations of the human genome provides a promising method for future human brain evolution studies by filling the gap between gross anatomical and in vitro neuronal model–based studies.

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Author contributions

Ole Goltermann (Conceptualization, Formal analysis, Investigation, Visualization, Writing—review & editing), Gökberk Alagöz (Conceptualization, Formal analysis, Investigation, Supervision, Visualization, Writing—original draft, Writing—review & editing), Barbara Molz (Conceptualization, Investigation, Supervision, Writing—review & editing), and Simon Fisher (Conceptualization, Funding acquisition, Supervision, Writing—review & editing).

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Conflict of interest statement

None declared.

Data availability

GWAS summary statistics used in this study are publicly available at https://enigma-brain.org/sulci-browser. All scripts used for analyses are publicly available on the Gitlab repository: https:// gitlab.gwdg.de/ole.goltermann/evolutionarysulcusmorph/-/tree/main/Analysis.

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Supplementary materials

The online version contains supplementary material available at https://academic.oup.com/cercor/article/34/3/bhae078/7624986#supplementary-data.

Chapter 4

The shared genetic architecture and evolution of human language and musical rhythm

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Abstract

This study aimed to test theoretical predictions over biological underpinnings of previously documented phenotypic correlations between human language-related and musical rhythm traits. Here, after identifying significant genetic correlations between rhythm, dyslexia and various language-related traits, we adapted multivariate methods to capture genetic signals common to genome-wide association studies of rhythm (N = 606,825) and dyslexia (N = 1,138,870). The results revealed 16 pleiotropic loci ($P < 5 \times 10^{-8}$) jointly associated with rhythm impairment and dyslexia, and intricate shared genetic and neurobiological architectures. The joint genetic signal was enriched for foetal and adult brain cell-specific regulatory regions, highlighting complex cellular composition in their shared underpinnings. Local genetic correlation with a key white matter tract (the left superior longitudinal fasciculus-I) substantiated hypotheses about auditory–motor connectivity as a genetically influenced, evolutionarily relevant neural endophenotype common to rhythm and language processing. Overall, we provide empirical evidence of multiple aspects of shared biology linking language and musical rhythm, contributing novel insight into the evolutionary relationships between human musicality and linguistic communication traits.

4.1 Introduction

The human brain has intricate neural circuitry to process complex communicative signals and behaviours, including speech and music, and the extent of biological overlap between these facets is an important question for the field of neurobiology. Individual differences in rhythm-related skills are correlated with variability in language-related skills, implicating potentially shared underlying neural and genetic architectures[1]. Previous research on the relationship between rhythm and language-related skills used a wide range of task-based tests to measure aspects of rhythm (for example, beat synchronization, rhythm perception and production, and metrical perception) and spoken and written language abilities (for example, word recognition, spelling and phonological awareness). For instance, rhythm perception skills can be measured by quantifying participants' ability to discriminate differences in durations of adjacent tones in melodies, whereas beat synchronization skills can be quantified on the basis of participants' success in tapping along to a metronome[2] or to the beat (pulse) of musical excerpts[3]. Similarly, language-related skills, such as word reading ability, can be measured as the ability to sound out words quickly and accurately in a limited amount of time[4], and spelling skills can be measured by testing participants' abilities to correctly spell out a number of words read aloud by the tester[5]. Importantly, even though language and rhythm measurement tasks involve different signals and stimuli and capture different skills, studies of individual differences often show phenotypic correlations between the different traits[6]. Nayak et al.[1] compiled information from 25 studies that identified significant positive phenotypic correlations between rhythm and language-related skills, synthesizing findings on a total of 397 children and 606 adults. Consistent associations have been found between rhythm perception, beat synchronization and language-related skills including speech perception, word reading and grammatical skills at various phases in the lifespan from pre-school age through to later adulthood. Despite these lines of evidence showing phenotypic associations between non-linguistic rhythmic processing and language skills, empirical evidence at the intersection between the neurobiological, genetic and evolutionary grounds of these traits remain to be discovered.

Various theoretical frameworks[7,8,9], such as the revised vocal learning hypothesis[10], provide overarching perspectives on how rhythm and multiple facets of human communication might relate in a neurodevelopmental and evolutionary context. According to the revised vocal learning hypothesis, human vocal learning ability is a pre-adaptation for predictive and tempo-flexible beat synchronization, and beat processing and vocal learning rely on overlapping neural circuits. This view is in line with neural reuse theories, such as neuronal recycling[11] and massive redeployment hypotheses[12], which suggest overlapping neurobiological circuits for language- and rhythm-related skills. Neural reuse theories claim that cultural innovations, such as reading, invade evolutionarily older brain substrates via the reallocation of an existing neural circuit to a new behaviour. Some argue that cognitive systems, such as language and music, are better understood as different uses of similar information processing mechanisms[13], yet the genetic and evolutionary bases of putative shared neural circuits are largely unknown.

To address prominent theories on the evolution of language development and musical rhythm in humans[10], evidence so far has been taken largely from psychology, neuroscience and cross-species comparisons rather than genetics[14,15]. We believe that identifying potential shared genetic architecture between language-related disorders and musical rhythm abilities, and probing the evolutionary past of the implicated genomic regions, can help reveal the neural and biological characteristics of our species that made rhythm and language an asset to human

development and behaviour. Importantly, individuals with rhythm impairment have been suggested to show higher predisposition to language-related difficulties, such as developmental language disorder and dyslexia (atypical rhythm risk hypothesis, ARRH)[16]. Given that disorders of language and reading can have long-term health impacts, identifying genetic factors that they share with rhythm impairment may enhance future possibilities for diagnoses and treatment. Moreover, basic science concerning the biological substrates of these fundamental human traits will be informed by new approaches to their potentially shared genetic architecture.

Our work built on two recent genome-wide association studies (GWAS) that represent by far the most well-powered genetic investigations of rhythm-/language-relevant traits so far, one for musical rhythm (beat synchronization, hereafter referred to as rhythm; 'can you clap in time with a musical beat?' N_{cases}(yes) = 555,660, $N_{controls}$(no) = 51,165)[17] and the other for dyslexia (developmental reading/spelling difficulties; 'have you been diagnosed with dyslexia?' N_{cases}(yes) = 51,800, $N_{controls}$(no) = 1,087,070)[18], both performed on a 23andMe Inc. research cohort in individuals of European ancestry and both classified as binary traits. We used the dyslexia GWAS as a proxy for the genetic underpinnings of language- and reading-related aspects of human communication, as dyslexia often co-occurs with a number of speech/language disorders[19,20,21,22]. Beat synchronization GWAS was used as a proxy for musical rhythm skills, as beat perception and synchronization are considered to be important features of musical experiences in present-day humans[23,24]. We applied a three-stage analytic pipeline to investigate shared genetics and biology: (1) genome-wide genetic correlations between rhythm and dyslexia (as well as other language-related traits) using linkage disequilibrium score regression (LDSC)[25], (2) multivariate GWAS (mvGWAS) of rhythm impairment and dyslexia using genomic structural equation modelling (SEM)[26] and (3) post-mvGWAS analyses of the shared genomic infrastructure as windows into its evolution and biology (Fig. 1a).

4.2 Results

In the first stage, we estimated genetic correlations between rhythm and dyslexia, as well as quantitative measures of language or reading performance[27], educational traits[28] and brain–language-related endophenotypes[29,30] by using LDSC[25]. We found moderate but significant genetic correlations between rhythm and dyslexia (magnitude of the genetic correlation (r_g) (s.e.m.) = −0.28 (0.02), $P_{FDR} = 2.05 \times 10^{-31}$), five quantitative language or reading measures, three educational traits and two language-relevant neuroimaging endophenotypes (Fig. 1b and Supplementary Table 1). In contrast there were negligible and non-significant genetic correlations with non-verbal intelligence quotient (IQ) (r_g (s.e.m.) = −0.004 (0.047), $P_{FDR} = 0.94$) and overall school performance (r_g (s.e.m.) = −0.066 (0.040), $P_{FDR} = 0.11$) (Fig. 1b and Supplementary Table 1). Thus, rhythm is genetically correlated not only with dyslexia, but also with multiple language-related phenotypes including word and non-word reading, non-word repetition, phoneme awareness, having better language skills than mathematics, and language resting-state functional connectivity ($|r_g|$ median of 0.184 and range of 0.004–0.376), providing empirical genetic evidence for the ARRH. The absence of significant genetic correlations between rhythm and cognitive traits, such as non-verbal IQ and overall school performance, provides evidence that genetic sharing between rhythm and dyslexia is not driven by general cognition. These results represent the first direct empirical support for a shared genetic architecture underlying previously observed phenotypic correlations between rhythm and language-related traits[1], such as dyslexia (Pearson correlation of −0.04, 95% confidence interval (CI) −0.05 to −0.04, t = −25.96, d.f. of 363,285, $P < 2.2 \times 10^{-16}$).

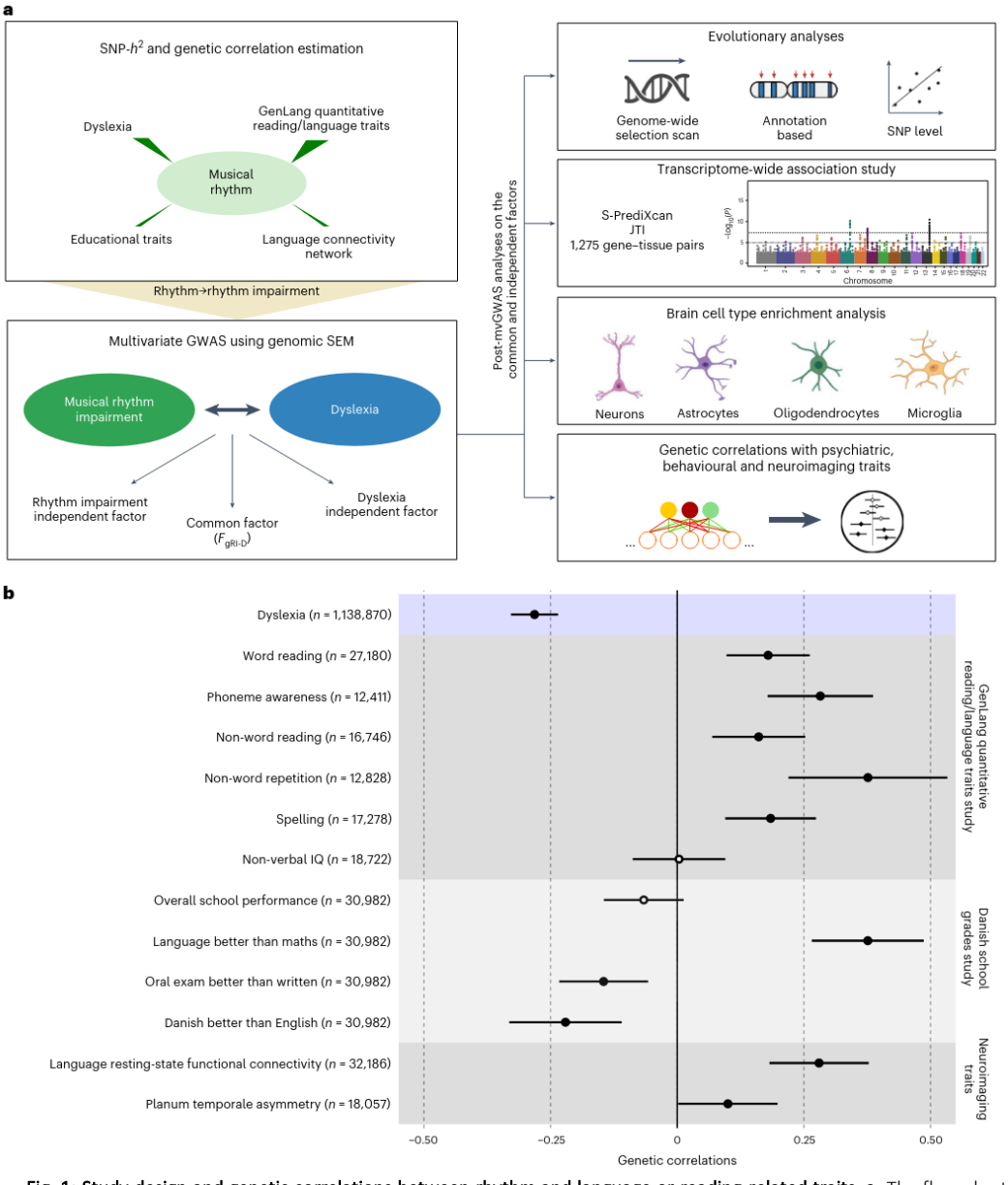

Fig. 1: Study design and genetic correlations between rhythm and language or reading-related traits. a, The flow chart shows the analyses performed in our study. SNP-heritability (SNP-h^2) and genetic correlations were estimated using LDSC. The effect directions in the rhythm GWAS summary statistics were flipped to obtain a proxy to probe rhythm impairment. Genomic SEM was used to identify common and independent genetic factors of rhythm impairment and dyslexia. For post-mvGWAS analyses, we adopted various methods including LDSC partitioned heritability, GCTB SBayesS, LAVA and manual SNP lookups. **b**, The genetic correlation analysis results between musical rhythm and a set of language- and reading-related traits. The x axis indicates the magnitude of the genetic correlation (r_g), with error bars extending one s.e.m. below or above the correlation estimate. The y axis shows 13 language or reading-related, cognitive, educational and neuroimaging traits. GWAS sample sizes (N) are reported next to the trait names. P values were estimated using a two-sided test and were FDR corrected for 13 tests ($P_{FDR} < 0.05$). Significant genetic correlations ($P_{FDR} < 0.05$) are indicated by full circles. Panel a created with BioRender.com.

Given that dyslexia is a neurodevelopmental disorder with effects particularly apparent in the written language domain (evident from reading and/or spelling difficulties)[18] and that other work has shown rhythm impairments associated with dyslexia[19,20,21,22], we expect it to be genetically and phenotypically linked to impairment in rhythm (hereafter referred to as rhythm impairment) rather than rhythm ability (this expectation is supported by the negative sign of the genetic correlation observed in the first stage of our pipeline above). Thus, we reversed the effect directions in the binary rhythm GWAS summary statistics to align genetic effect directions for rhythm and reading impairments. We then performed a mvGWAS on the rhythm impairment and dyslexia GWAS to probe the validity of the ARRH at the genetic level, using a bivariate extension of genomic SEM[26] that we developed (Methods). This allowed us to tease apart the genetic effects shared between rhythm impairment and dyslexia from those that are unique to each. We specified a measurement model with a shared genetic factor ($F_{gRI\text{-}D}$, where RI-D stands for rhythm impairment-dyslexia), which recaptured the genetic correlation between two traits ($\sigma^2_{FgRI\text{-}D}$ (s.e.m.) = 0.28 (0.03)). Similar to Grotzinger et al.[31], we then applied both the common pathway model (CPM), which regresses single nucleotide polymorphisms (SNPs) from FgRI-D (Supplementary Fig. 1), and the independent pathway model (IPM), which regresses SNPs directly onto the genetic components of the two traits (Supplementary Fig. 1). Thus, we were able to obtain a quantitative per-SNP score quantifying the extent to which any given SNP influences rhythm impairment or dyslexia independent from $F_{gRI\text{-}D}$, that is, the bivariate genetic heterogeneity (Q_b).

Our mvGWAS analysis with the CPM resulted in a new set of summary statistics representing the genetic overlap between rhythm impairment and dyslexia, and identified 18 genome-wide significant ($P < 5 \times 10^{-8}$) loci associated with $F_{gRI\text{-}D}$ (Fig. 2a and Supplementary Table 2) after genomic control (GC) correction (Supplementary Fig. 2). We estimated the SNP heritability of $F_{gRI\text{-}D}$ as 13% (s.e.m. of 0.005) by LDSC[25]. The strongest mvGWAS signal came from the SNP rs28576629 ($P = 3.79 \times 10^{-14}$) on chromosome 3 (Fig. 2a), an intronic variant in *PPP2R3A*, a gene encoding a regulatory subunit of protein phosphatase 2 (ref. 32). We validated the genomic SEM CPM results using two additional mvGWAS methods: (1) N-weighted genome-wide association meta-analysis (GWAMA)[33] and (2) cross-phenotype association analysis (CPASSOC)[34]. Both methods captured highly similar genomic architectures to the one captured by the CPM (Supplementary Fig. 3), confirming that the shared genetics of rhythm impairment and dyslexia could be identified consistently regardless of analytic tool. The IPM resulted in two new sets of summary statistics capturing the genetic factors of rhythm impairment and dyslexia that are independent from $F_{gRI\text{-}D}$, so-called independent factors (Supplementary Fig. 4). We used the IPM results to obtain Q_b and mapped the per-SNP Q_b scores onto CPM mvGWAS results to dissociate the homogeneous (hereafter referred to as pleiotropic) signals from the signals driven by a single GWAS (Fig. 2a). We identified 27 significant genome-wide ($P < 5 \times 10^{-8}$) heterogeneous loci in the Q_b results (Fig. 2a and Supplementary Table 3), and two of these loci are co-localized with two CPM signals on chromosome 20 (30,690,943–31,189,993 and 47,514,881–47,821,129), which are mvGWAS signals that are driven by the dyslexia GWAS (Fig. 2a). Thus, our analysis revealed two distinct patterns for CPM mvGWAS hit loci: 16 highly homogeneous (putatively pleiotropic) and two heterogeneous loci indicating different levels of GWAS significance, effect sizes and/or opposite effect directions for these two loci in the rhythm impairment and dyslexia GWASs (Fig. 2b shows the representative loci of each type).

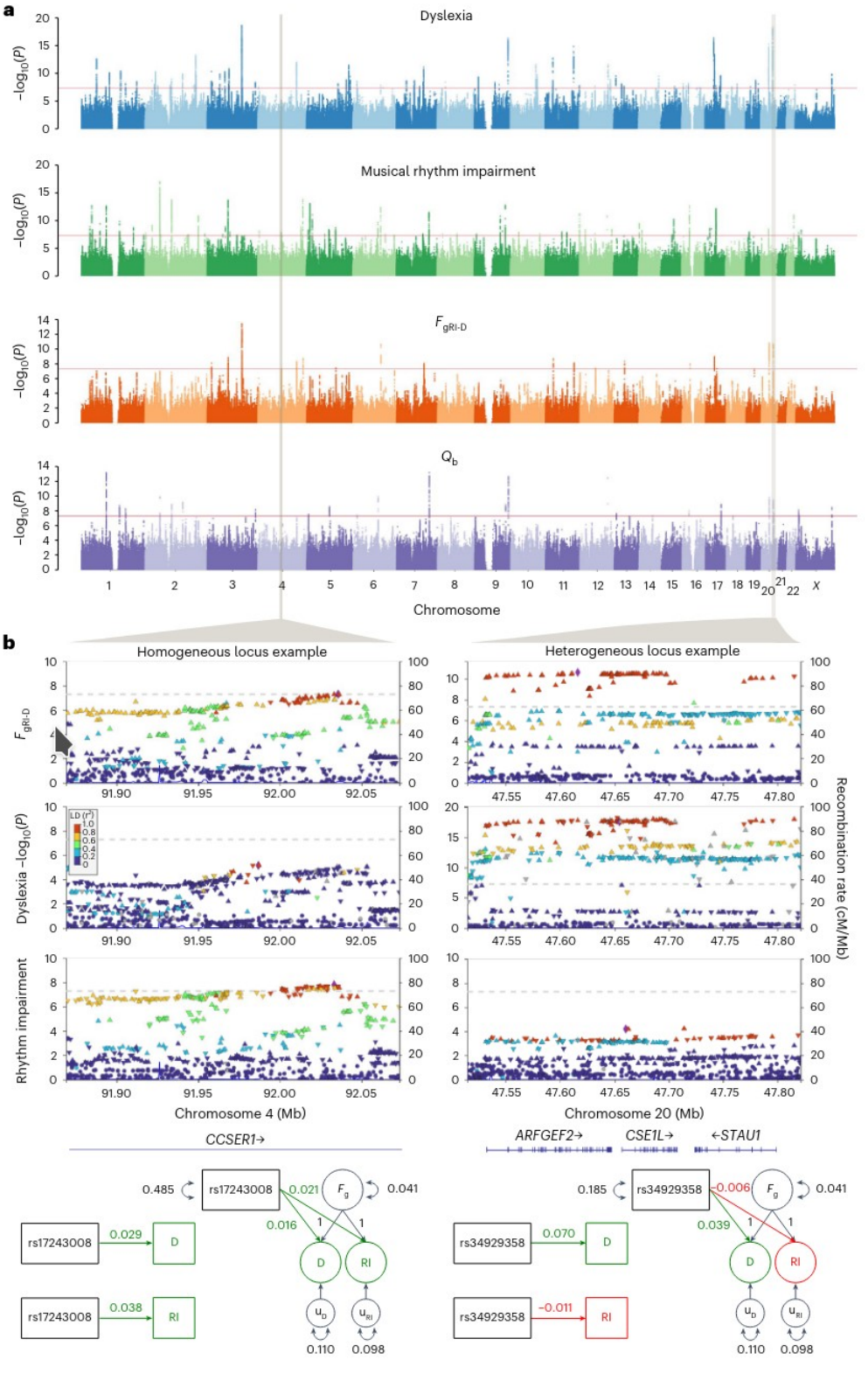
a
Dyslexia
Musical rhythm impairment
F_{gRI-D}
Q_b
$-\log_{10}(P)$
Chromosome
b
Homogeneous locus example
Heterogeneous locus example
F_{gRI-D}
Dyslexia $-\log_{10}(P)$
Rhythm impairment
LD (r^2)
Recombination rate (cM/Mb)
Chromosome 4 (Mb)
Chromosome 20 (Mb)
CCSER1→
ARFGEF2→
CSE1L→
←STAU1
rs17243008
rs34929358
0.485
0.021
0.041
0.016
0.029
0.038
0.185
−0.006
0.039
0.070
−0.011
0.110
0.098

Fig. 2: Manhattan plots for univariate and mvGWASs and heterogeneity, including examples of highly homogeneous and heterogeneous loci in $F_{gRI\text{-}D}$ results. a, Manhattan plots of dyslexia, musical rhythm GWASs, the common factor ($F_{gRI\text{-}D}$) mvGWAS and the heterogeneity between dyslexia and musical rhythm impairment GWASs (Q_b). The y axes show $-\log_{10}$(P) values; the x axes show chromosomal positions and datapoints represent SNPs. Dyslexia and musical rhythm GWASs were previously performed and published and are included here for illustration purposes. The mvGWAS results and heterogeneity estimates were obtained using a CPM in genomic SEM. Loci that pass the genome-wide significance threshold ($P < 5 \times 10^{-8}$) in $F_{gRI\text{-}D}$ and Q_b Manhattan plots are listed in Supplementary Tables 2 and 3. The red lines correspond to the genome-wide significance threshold ($P < 5 \times 10^{-8}$). $N_{rhythm\ impairment}$ = 606,825, $N_{dyslexia}$ = 1,138,870. **b**, LocusZoom plots of example homogeneous and heterogeneous loci, chosen on the basis of Q_b P values. The y axes show $-\log_{10}$(P) values, and the x axes show chromosomal positions of each SNP. Each triangle represents a SNP and the direction of the triangle indicates the sign of the GWAS effect (upwards, positive effects; downwards, negative effects). Colour codes correspond to the linkage disequilibrium with the lead SNP. The SNP loadings on D and RI indicate GWAS effect sizes and directions (left), whereas the SNP loadings in the SEM diagrams show IPM effect sizes and directions (right) of two example SNPs, reflecting the homogeneous versus heterogeneous architecture of the example loci, respectively. F_g, common genetic factor of dyslexia and musical rhythm impairment; D, dyslexia GWAS; RI, musical rhythm impairment GWAS; cM, centimorgan; u variables, residual variance not explained by the common factor.

Next, we performed a transcriptome-wide association study (TWAS) using $F_{gRI\text{-}D}$ summary statistics, and whole-blood and 13 GTEx brain tissue phenotype weights[35,36] with S-PrediXcan[37] (Supplementary Table 4). Our TWAS analysis identified 1,275 significant ($P_{FDR} < 0.05$) gene–tissue pairs and 315 significant ($P_{FDR} < 0.05$) unique genes associated with $F_{gRI\text{-}D}$ after false discovery rate (FDR) correction (Fig. 3a and Supplementary Table 5). Some of the top significant gene–tissue pairs associated with $F_{gRI\text{-}D}$ are *AC072039.2* expression in brain nucleus (Z-score of −7.74, $P_{FDR} = 1.17 \times 10^{-9}$), *PPP2R3A* expression in cerebellum (Z-score of 7.49, $P_{FDR} = 2.43 \times 10^{-9}$) and putamen (Z-score of 7.47, $P_{FDR} = 2.43 \times 10^{-9}$) and *FOXO3* expression in anterior cingulate cortex (Z-score of 6.07, $P_{FDR} = 1.15 \times 10^{-5}$) (Fig. 3a). Functional enrichment analysis of the significant ($P_{FDR} < 0.05$) TWAS genes using PANTHER[38] did not identify any significant enrichments in Gene Ontology (GO) and PANTHER GO-Slim terms[38,39,40,41] after accounting for multiple testing (Supplementary Tables 6–11). Overall, our S-PrediXcan analysis highlighted 315 unique genes linked to $F_{gRI\text{-}D}$, including significant gene–tissue pairs (such as *FOXO3* expression in the anterior cingulate cortex and *PPP2R3A* expression in the putamen) involving brain regions with known relevance for music processing[42,43].

To investigate the neurobiology of genetic variation shared between rhythm impairment and dyslexia at cell type resolution, we performed LDSC partitioned heritability analysis[44] using cell type-specific regulatory region annotations of neurons, microglia, astrocytes and oligodendrocytes45. We found robust significant SNP heritability enrichments in the promoters of neurons (enrichment (SEM) of 8.14 (1.55), $P_{FDR} = 3.38 \times 10^{-5}$), oligodendrocytes (enrichment (SEM) of 7.98 (1.53), $P_{FDR} = 3.38 \times 10^{-5}$), astrocytes (enrichment (SEM) of 7.72 (1.59), $P_{FDR} = 1.1 \times 10^{-4}$) and microglia (enrichment (SEM) of 4.47 (1.63), $P_{FDR} = 0.04$), as well as enhancers of neurons (enrichment (SEM) of 4.43 (0.35), $P_{FDR} = 7.96 \times 10^{-18}$) and astrocytes (enrichment (SEM) of 2.73 (0.58), $P_{FDR} = 4.35 \times 10^{-3}$) (Fig. 3b and Supplementary Table 12). Consistent with the original rhythm and dyslexia GWAS reports[17,18], $F_{gRI\text{-}D}$ relates to brain structure in part by common effects at regulatory regions within multiple cell types, including neuronal and various non-neuronal cells, such as oligodendrocytes. This may suggest that the $F_{gRI\text{-}D}$ might impact myelination and white matter connectivity patterns that could potentially instantiate neural overlap between rhythm and reading-related aspects of language[1,10,46].

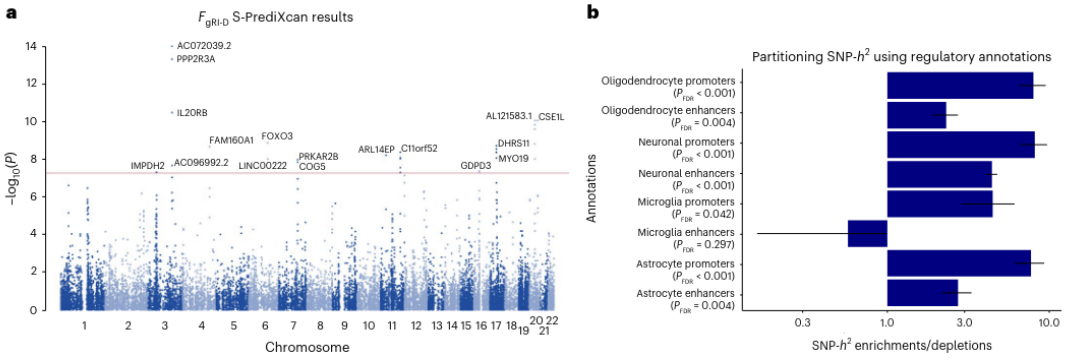

Fig. 3: S-PrediXcan and LDSC partitioned heritability results for regulatory brain cell type annotations. a, Manhattan plot showing TWAS results on 13 brain tissue and whole-blood tissues. Each data point corresponds to a genetissue pair. The y axis shows $-\log_{10}$(P) values, and the x axis shows chromosomal positions of each gene–tissue pair. The most significant gene–tissue association pair is shown for each gene. The red line corresponds to the genome-wide significance threshold ($P < 5 \times 10^{-8}$). **b**, Bar plots showing LDSC SNP-h2 enrichment and depletion estimates for the common genetic factor of musical rhythm impairment and dyslexia ($F_{gRI\text{-}D}$; $N_{rhythm\ impairment}$ = 606,825 and $N_{dyslexia}$ = 1,138,870) on the x axis and eight brain cell type-specific regulatory annotations on the y axis. P values were estimated using a two-sided test and FDR corrected for eight tests. The exact enrichment or depletion P values are reported in Supplementary Table 12. Error bars represent s.e.m., with whiskers extending one standard error below or above the SNP-h^2 enrichment or depletion estimate.

To test the validity of links between rhythm impairment and dyslexia risk and proneness to certain neuropsychiatric disorders proposed by the ARRH, we moved on to investigate relationships of $F_{gRI\text{-}D}$ with psychiatric, neurological and behavioural traits, examining patterns of genetic correlations with common and independent factors in more detail. First, we curated 88 sets of GWAS summary statistics including traits that were significantly genetically correlated either with rhythm or dyslexia in the original GWAS reports[17,18] and three additional education-related traits[28] (Supplementary Table 13). To reduce the statistical burden of multiple testing correction in our consequent analyses, we subset this initial set of 88 traits on the basis of their levels of genetic correlation among themselves. To do so, we estimated pairwise genetic correlations, and identified the most highly correlated traits ($|r_g| > 0.80$; Supplementary Fig. 5). We then performed hierarchical clustering, obtaining one representative trait from each cluster of highly correlated traits (Supplementary Fig. 6). This approach yielded 49 traits that were relatively genetically independent (see Methods for details), for which we estimated the genetic correlations with $F_{gRI\text{-}D}$, and with the summary statistics obtained by the IPM (Supplementary Fig. 7 and Supplementary Table 14). Genetic correlations between $F_{gRI\text{-}D}$ and the assessed traits ranged from −0.56 to 0.46, and mostly lay between the genetic correlation estimates for independent factors (Supplementary Fig. 7), supporting that $F_{gRI\text{-}D}$ indeed captures the common genetic factor of rhythm impairment and dyslexia. We found significant negative correlations between $F_{gRI\text{-}D}$ and non-word repetition (r_g (s.e.m.) = −0.513 (0.099), $P_{FDR} = 7.03 \times 10^{-7}$) and phoneme awareness (rg (s.e.m.) = −0.562 (0.058), $P_{FDR} = 3.78 \times 10^{-21}$), validating the $F_{gRI\text{-}D}$ construct's link to reading- and language-related traits. Positive genetic correlations were observed for attention deficit hyperactivity disorder (rg (s.e.m.) = 0.237 (0.029), $P_{FDR} = 3.69 \times 10^{-15}$), autism spectrum disorder (rg (s.e.m.) = 0.075 (0.035), $P_{FDR} = 4.529 \times 10^{-2}$) and insomnia (rg (s.e.m.) = 0.200 (0.027), $P_{FDR} = 6.04 \times 10^{-13}$), suggesting shared genetic liability with neuropsychiatric traits that have been phenotypically linked to rhythm[47]. In total, $F_{gRI\text{-}D}$ showed significant ($P_{FDR} < 0.05$) genetic correlations with 37 of the 49 selected psychiatric/ neurological/behavioural traits with varying magnitudes and directions, including attention

deficit hyperactivity disorder, Parkinson's disease, health satisfaction and loneliness ($|r_g|$ median of 0.146 and range of 0.06–0.56). Consistent with the ARRH, the directionality of genetic correlations suggest that decreased rhythm impairment/dyslexia risk may be associated with resilience to certain neuropsychiatric disorders. These genetic correlations also reflect a shared genomic architecture underlying rhythm, dyslexia and social traits, showing that social function and co-evolution hypotheses of rhythm and communication skills[48,49,50] are plausible from a genetic perspective. Future work will be needed to disentangle possibly shared genomic substrates of the evolution of social interaction, language and music.

Even though reading is a recent human innovation, it recruits language-related brain circuits[51,52], which have undergone biological evolution on the lineage leading to humans. Similarly, dyslexia manifests overtly as a reading or spelling disorder, yet in many cases this reflects underlying deficits in aspects of oral language (for example, phonological awareness)[19–22]. Given this link between spoken language and reading, and in light of theoretical frameworks positing co-evolution of rhythm and language-related skills in humans[10,48,49,50,53], we leveraged genomic methods to investigate the evolution of the overlap between rhythm and the reading-related aspect of language over a range of timescales (Fig. 4a). We first performed LDSC partitioned heritability analysis using five evolutionary annotations tagging foetal brain human-gained enhancers[54], Neanderthal introgressed alleles[55], archaic deserts[56] and primate-conserved and accelerated regions[57] (Fig. 4a). This revealed significant SNP heritability depletions in Neanderthal introgressed alleles, and significant enrichments in primate-conserved regions for all traits (Fig. 4b and Supplementary Table 15), in line with findings for many other complex human traits[58,59]. We then used the SBayesS function of the GCTB package[60] to probe the effect size-minor allele frequency relationship ($\hat{S}$)—an essential component of the complex trait genetic architecture influenced by natural selection[60]. Similar to most cognitive and behavioural traits[60], we found moderate levels of negative selection acting on $F_{gRI\text{-}D}$ ($\hat{S}$ (s.d.) = −0.51 (0.05)) and the independent factors of dyslexia $\hat{S}$ (s.d.) = −0.47 (0.06)) and rhythm impairment ($\hat{S}$ (s.d.) = −0.49 (0.06)) (Fig. 4d and Supplementary Table 16). Next, we performed MAGMA gene set analysis[61] to investigate links between genes that are co-located with various evolutionary annotations, spanning a timescale from ~8 million to ~35,000 years ago, which were not testable via partitioned heritability analysis owing to low SNP coverage (Methods). Specifically, we tested whether genetic variation associated with $F_{gRI\text{-}D}$ was enriched in genes that overlap with four evolutionary annotations (Supplementary Tables 17–20): (1) ancient selective sweep sites[62], (2) human accelerated regions[63,64,65,66], (3) differentially methylated regions (DMRs) between anatomically modern humans and archaic humans[67] and (4) DMRs between anatomically modern humans and chimpanzees[67]. These gene set-based analyses did not yield any significant enrichment signals (Supplementary Table 21), indicating an absence of evidence for associations between $F_{gRI\text{-}D}$ and these four annotations. We then extended our MAGMA gene set enrichment analysis to look for potential links between $F_{gRI\text{-}D}$ and genomic substrates of songbird vocal learning, in line with theoretical predictions10, and prior associations with the genetic architecture of beat synchronization[68]. To do so, we used nine gene sets that were curated by Gordon et al.[68] and therein converted to human homologues for the purposes of gene enrichment analyses; each set represents differential gene expression patterns associated with vocal learning phenotypes (for example, song versus silence or number of motifs sung) in Area X and other key regions of zebra finch neural circuitry related to song learning. Intriguingly, we found significant enrichments in four Area X-related gene sets (Supplementary Table 22) using the $F_{gRI\text{-}D}$ summary statistics. These findings may

suggest overlapping molecular mechanisms between songbird vocal learning, human rhythm and human language, supporting theories of cross-species convergent evolution of vocal learning and beat perception[10,69].

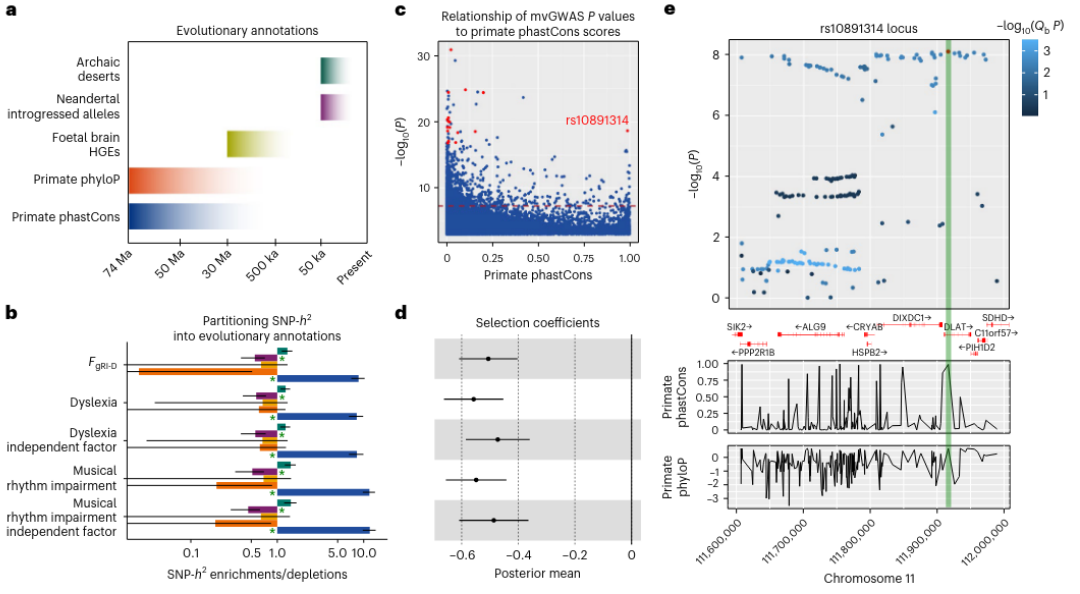

Fig. 4: Evolutionary analyses of dyslexia, rhythm impairment, FgRI-D and independent factors. a, The timescales covered by the evolutionary annotations that we used. HGEs, human-gained enhancers; Ma, million years ago; ka, thousand years ago. **b**, LDSC partitioned SNP-h^2 enrichment and depletion estimates on the x axis and annotation-trait pairs on the y axis ($N_{rhythm\ impairment}$ = 606,825 and $N_{dyslexia}$ = 1,138,870). Colour coding of the bars corresponds to evolutionary annotations in a. P values were estimated using a two-sided test. Green asterisks indicate statistical significance after FDR correction for 25 tests ($P_{FDR} < 0.05$). Error bars represent s.e.m., with whiskers extending one standard error below or above the SNP-h^2 enrichment or depletion estimate. The exact enrichment or depletion P values are reported in Supplementary Table 15. **c**, A scatter plot showing the association between F_{gRI-D} mvGWAS $-\log_{10}(P)$ values on the y axis and primate phastCons scores on the x axis. Lead SNPs in 17 genome-wide significant ($P < 5 \times 10^{-8}$) loci are highlighted in red (one genome-wide significant lead SNP does not have a phastCons score and is not shown here). The dashed red line indicates the mvGWAS genome-wide significance threshold ($P < 5 \times 10^{-8}$). **d**, The GCTB SBayesS selection coefficient estimates as posterior means shown on the x axis, for each trait on the y axis (note that the y axis is shared with b). The error bars represent s.d., with whiskers extending one s.d. below or above each coefficient. The vertical line is fixed at zero. ($N_{rhythm\ impairment}$ = 606,825 and $N_{dyslexia}$ = 1,138,870). **e**, The results of a manual look-up of the SNP rs10891314, showing its co-localization with *DLAT*. Each data point is a SNP, with the y axis showing F_{gRI-D} mvGWAS $-\log_{10}(P)$ values and the x axis showing genomic locations. Colour coding reflects Q_b (heterogeneity) scores. The primate phastCons and primate phyloP graphs show patterns of conservation and accelerated evolution within the locus. rs10891314 is shown in red, and its phastCons and phyloP scores are shown with the green bar.

To follow up the significant partitioned SNP heritability enrichments in primate-conserved regions, we investigated the association between F_{gRI-D} mvGWAS P values and per-SNP primate phastCons scores[57] for 38,164 clumped SNPs ($P < 0.05$, $r^2 < 0.06$) from F_{gRI-D} summary statistics (Fig. 4c), and found that one of the F_{gRI-D} genome-wide significant ($P < 5 \times 10^{-8}$) hits, rs10891314, had an exceptionally high phastCons score, probably because it is a missense variant (Fig. 4c). We zeroed-in on this genome-wide significant hit as an example locus and dissected patterns of Q_b, and conservation or accelerated evolution in primates (Fig. 4e), confirming the sharp increase in conservation rate for the SNP rs10891314. The Human Genome Dating Atlas[70] estimates this polymorphism to be 11,199 generations old (95% CI), corresponding to ~280,000

years ago assuming 25 years per generation, around the time period when the oldest known *Homo sapiens* fossils have been dated[71]. rs10891314 is located in the *DLAT* gene, which is associated with a rare neurodevelopmental disorder, pyruvate dehydrogenase E2 deficiency. This condition is characterized by neurological dysfunction, dystonia and learning disability mainly appearing during childhood[72]. *DLAT* is highly conserved and loss-of-function intolerant (probability of loss-of-function intolerance 6.68) (ref. [73]), which makes this particular missense variant an interesting candidate for increasing susceptibility to rhythm impairment and dyslexia.

After assessing evolutionary signatures on $F_{gRI\text{-}D}$ at the genome-wide and SNP levels, we extended our investigations of rhythm–language co-evolution by integrating with independent data from neuroimaging genetics. To do so, we estimated local genetic correlations between $F_{gRI\text{-}D}$ and fractional anisotropy (FA) measures of five left hemispheric white matter tracts (Supplementary Fig. 8 and Supplementary Table 23), involved in the dorsal or ventral streams of spoken language, and theorized as key components of rhythm–language convergent evolution[10,69]. Using local analysis of (co)variant association (LAVA)[74], we identified a significant local genetic correlation between $F_{gRI\text{-}D}$ and the left hemispheric superior longitudinal fasciculus (SLF) I (r_g = 1, P_{FDR} = 0.02) (Supplementary Table 24) on a ~2 Mb region on chromosome 20 (30,569,660–32,484,506), which encompasses several genes including *EFCAB8*, *BAK1P1* and *SUN5* (Supplementary Fig. 9). SLF-I is the dorsal division of SLF connecting the superior parietal and superior frontal lobes[75] (Supplementary Fig. 8). SLF has been shown to have functional links to musical rhythm[76,77], and SLF-I subdivision is involved in the regulation of motor behaviour[78,79]. This finding is partially consistent with the hypothesized role of the dorsal stream in supporting co-evolution of phonological processing and beat synchronization[4].

4.3 Discussion

We showed robust genetic correlations among musical rhythm, dyslexia and a number of reading- and language-related traits, providing genetic evidence for the ARRH. Traits, such as non-word repetition, phoneme awareness and having better language skills than mathematics at school, showed significant positive correlations with rhythm skills, which makes these particular traits interesting candidates to study in future genetic studies of shared biology of rhythm and language. Importantly, we also found a significant correlation between rhythm and language resting-state functional connectivity, suggesting shared genetic and neuronal architecture for rhythm and reading-related aspects of language.

The bivariate genomic SEM approach that we developed allowed us to identify genetic overlaps between rhythm impairment and dyslexia and to present a map of homogeneous and heterogeneous genetic effects, shedding light on patterns of pleiotropy between the two[1]. Among 18 genome-wide significant loci associated with the common factor of dyslexia and rhythm impairment, the strongest mvGWAS signal comes from a locus tagged by the SNP rs28576629 that is mapped to *PPP2R3A*, a gene implicated in the negative control of cell growth and division, suggesting a putative role for this gene in dyslexia and rhythm impairment prevalence[80]. Given that we validated our common factor results using two additional mvGWAS methods, we believe that the shared genetic factor that we captured represents a solid first glimpse into the shared genetics of dyslexia and rhythm impairment. Results of this kind might potentially contribute, together with information on other risk factors, towards improved diagnostics of individuals' propensity for reading- and rhythm-related problems, to enable special educational support. However, given the highly polygenic and environment-dependent

nature of behavioural traits, the early risk identification power of our results remains to be explored[81].

Our post-mvGWAS analyses enhance understanding of the aetiology of rhythm and language (on which reading depends) by revealing intricate links across rhythm impairment, dyslexia and various aspects of evolutionary past and neurobiological function, including gene expression in brain tissue, brain cell type-specific gene regulation and a local genetic correlation with a tract linked to regulation of higher aspects of motor behaviour[75]. Our TWAS results validated the association between *PPP2R3A* and the common factor of dyslexia and rhythm impairment, and narrowed down the overall relevance of this gene for rhythm and reading skills into its expression profile in cerebellum and putamen. We believe that our TWAS results constitute a potentially important gene–tissue pair list to study the links between genetic variants, gene expression in the brain and subsequent effects on neurodevelopment using experimental assays. Our SNP heritability enrichment results in cell type-specific regulatory regions point to multiple brain cell types for follow-up work, without pinning down a specific neuronal or non-neuronal brain cell type, indicating a complex cellular composition in the brain supporting rhythm and language. Heritability enrichment signals in brain-specific regulatory regions provide additional evidence that the dyslexia and rhythm GWASs largely capture the neurodevelopmental aspects of these traits. The genome-wide genetic correlation analysis between the common factor and a set of behavioural traits yielded two important findings. First, the genetic correlation directions and magnitudes point to a link between rhythm impairment and dyslexia risks, and neuropsychiatric disorders, in line with the ARRH. Second, language-related traits, such as non-word repetition and phoneme awareness, yielded the strongest genetic correlations with the common factor of dyslexia and rhythm impairment, further supporting the statistical validity and language or reading relevance of $F_{gRI\text{-}D}$.

The evolutionary analyses aimed to provide empirical genetic data as groundwork towards understanding potential evolutionary forces acting jointly on human rhythm and language-related skills[53,82]. Our significant SNP heritability depletion findings in Neanderthal introgressed alleles is in line with findings for other complex human traits[58], indicating a reduced contribution of Neanderthal alleles to reading- and rhythm-related skills. Similarly, heritability enrichment findings in primate-conserved regions converge with studies that previously found significant enrichments in these loci for complex disease traits[59]. We also noted a trend for the majority of mvGWAS lead SNPs to have lower primate conservation scores, indicating weaker constraint on these variants in the primate clade, which may have relevance for the evolution of language and rhythm-related traits on the human lineage. This observation lacks statistical confirmation, but would be in line with prior behavioural and neural findings showing a lack of complex musical rhythm detection and synchronization in species, such as macaque monkeys[83]. Interestingly, one mvGWAS hit locus, mapped to the *DLAT* gene, stood out as strongly conserved among primates, which makes this gene a potential candidate for future experimental investigations in this area. Our analyses showed significant enrichments of $F_{gRI\text{-}D}$-associated variants in gene sets curated from transcriptome studies of songbird vocal learning, specifically in a key nucleus of the zebra finch brain, Area X. This link between human genetic variants shaping human reading and rhythm skills, and genes involved in songbird song production (for example, number of motifs sung) in Area X is in line with prior literature[68], further supporting the importance of shared genetic substrates. Finally, the significant local genetic correlation that we identified between SLF-I and $F_{gRI\text{-}D}$-associated variants in a ~2 Mb region on chromosome 20 represents an interesting example of potential pleiotropic associations between language- and musical

rhythm-related traits and white matter microstructure. This finding is particularly interesting as SLF-I is involved in motor behaviour regulation[78,79], suggesting the presence of shared genetic and neuroanatomical elements between motor aspects of language and musical rhythm. It is also plausible that the shared genetic factors underlying language- and musical rhythm-related skills influence a broader range of cognitive processes rather than being confined to the intersection between language and musical rhythm. Here, we note that future local and genome-wide genetic correlation analyses between $F_{gRI\text{-}D}$ and a larger selection of neuroanatomical traits (for example, anterior and posterior segments of the arcuate fasciculus, inferior fronto-occipital fasciculus) and other imaging modalities are necessary to reveal the shared genetics and neuroanatomy of languageand musical rhythm-related traits.

Our study has several limitations. First, the original dyslexia and rhythm GWASs were performed on European ancestry samples owing to data availability reasons. The lack of large-scale GWASs in non-European populations and the strong sampling bias in large cohorts towards European ancestry individuals hinder a global picture of the genetic architecture of these traits, weaken the replicability of GWAS findings in diverse populations84 and limit the interpretability of post-GWAS evolutionary analysis results. This is especially true for behavioural and psychiatric traits that are more prone to be affected by cultural, socioeconomic and environmental factors, which is also reflected in the weaker transferability of polygenic scores across ancestries for such traits[85]. We believe that important steps to solve this will include encouraging and contributing to the generation of large-scale databases with genotype or phenotype data of individuals from diverse genetic ancestries, disentangling the unique gene–environment interactions in other populations, developing GWAS methods and study designs to more carefully take potential confounders into account and improving the reliability of behavioural phenotype measurement techniques. Second, the self-report-based phenotype descriptions in the original GWASs are not ideal measures, but rather represent robust validated proxies that uniquely enable scaling up of data collection to very large cohorts. There is a compromise between the practical convenience of self-report-based data collection from hundreds of thousands of individuals, which is extremely challenging using task-based measurements, and introducing self assessment-related uncertainties to the data. We note that the phenotype in the dyslexia GWAS was not simply self assessment but rather self-report of having received a positive dyslexia diagnosis, and that the genetic architecture was found to be stable across the lifespan, with a genetic correlation of 0.97 between younger (<55 years) and older subgroups (>55 years) of participants[18]. Moreover, the construct validity of the rhythm GWAS phenotype is supported by associations between the self-reported and directly measured rhythm skills[17], and polygenic scores trained on the rhythm summary statistics correlate with scores on a rhythm discrimination test[86,87], further supporting the view that genetic signals associated with self-reported beat synchronization ability are an appropriate reflection of rhythm-related skills. Moreover, the genetic correlations between dyslexia, rhythm impairment, $F_{gRI\text{-}D}$ and other speech and language-related phenotypes suggest that the original GWASs largely capture relevant genetic factors. Third, even though potential confounders, such as age and sex, were included as covariates in the dyslexia and rhythm GWAS regression models, we cannot fully exclude residual effects of such factors and other confounders. The original dyslexia GWAS addressed the impacts of age (as noted above) and sex by performing sensitivity analyses using age- and sex-specific GWAS, which showed little effect of either of these two factors on GWAS results[18]. Fourth, the majority of genetic factors shaping human language and musical rhythm skills are probably fixed in all human populations. Hence, post-GWAS evolutionary analyses,

which leverage present-day variation to probe links between genetic variants and evolutionary annotations, are not ideal to study the origins of traits that probably emerged at earlier periods of hominin evolution. Methodological developments in the complex trait evolution field, and the integration of ancient DNA data from older timepoints of human evolution into polygenic selection analysis methods would greatly help to resolve these challenges in future studies. Finally, relationships between heritability and evolution are quite complex. The individual contributions of common genetic variants to heritability are jointly shaped by selection and demography[88], which limits the evolutionary interpretation of heritability-based methods.

Despite such constraints, our study represents a step towards characterizing the shared genetic architecture between rhythm- and language-related traits, and provides a valuable analytic pipeline tackling the shared genetics and evolution of rhythm and reading-related aspects of language from various angles. We reveal complex links across common DNA variants, genes, genomic loci, white matter structures and human behaviour, making a first set of links across the immensely long causal chain spanning these layers. Developing and applying more sophisticated methods to dissociate environmental confounds from genetics will allow future studies to obtain a better understanding of the genetics and evolution of human language and musicality.

4.4 Methods

GWAS summary statistics

Beat synchronization and dyslexia GWAS summary statistics[17,18] were obtained from 23andMe Inc., a customer genetics company. Both GWASs were performed on European ancestry individuals through online participation. All participants provided informed consent according to 23andMe's human subject protocol, which is reviewed and approved by the external Association for the Accreditation of Human Research Protection Programs, Inc.-accredited institutional review board, Ethical and Independent Review Services, a private institutional review board (http://www.eandireview.com). The 23AndMe sample prevalence of dyslexia is 4.6% (N_{total} = 1,138,870, mean age 51 years), and sample prevalence of beat synchronization is 92% (N_{total} = 606,825, mean age 52 years). Summary statistics files were reformatted and harmonized to include required columns (for example, SNP ID, beta, beta s.e.m. and P value) for each mvGWAS tool following the guidelines in the original publications of each tool. To obtain rhythm impairment summary statistics, effect sizes in the binary beat synchronization GWAS summary statistics were multiplied by −1 so that the effect directions were reversed. This yielded a set of GWAS summary statistics comprising SNP effects contributing to rhythm impairment, which was used for the subsequent mvGWAS analysis with dyslexia. We applied GC correction to both sets of summary statistics for all non-LDSC-based analyses. For LDSC-based analyses (including genomic SEM), uncorrected summary statistics were used as input, as GC correction biases the LDSC SNP heritability estimates downwards. The resulting set of summary statistics from genomic SEM was GC corrected.

SNP heritability and genetic correlation estimations

We used LDSC[25] (v1.0.1) to estimate the SNP heritabilities and genetic correlations. For rhythm impairment and dyslexia, we estimated the total SNP heritability on a liability scale using population and sample prevalence information from the original studies (sample prevalence of 0.045 for dyslexia and 0.085 for rhythm impairment, and a population prevalence of 0.050 for

dyslexia and 0.048 for rhythm impairment). Genetic correlations were estimated using bivariate LDSC between rhythm, dyslexia, GenLang quantitative reading or language-related traits[27], Danish School Grades GWAS[28] and all external summary statistics except for the planum temporale asymmetry and the language resting-state functional connectivity, which were assessed as described below.

To estimate genetic correlations between rhythm and planum temporale asymmetry[30] and between rhythm and language resting-state functional connectivity[29], we used an approach proposed by Naqvi et al.[89] applicable to unsigned multivariate statistics, as the mvGWAS effect sizes or beta values, which are required to run genetic correlation analysis using LDSC, were not available for these traits. We evaluated the amount of shared signal between each pair of GWASs by estimating the Spearman correlation of the average SNP P values within approximately independent linkage disequilibrium (LD) blocks[90]. We first filtered the genome-wide SNPs using the HapMap3 reference panel without the major histocompatibility complex region (https:// github.com/bulik/ldsc). We then split the genome-wide SNPs into 1,703 approximately independent blocks[90]. For each approximately independent LD block, we computed the average SNP $-\log_{10}(P)$ value. We then estimated a rank-based Spearman correlation using the averaged association value (n = 1,703) for each LD block. A standard error of the Spearman correlation was estimated using statistical re-sampling with 10,000 bootstrap cycles with replacement from the 1,703 LD blocks.

mvGWAS

To investigate the shared genetic variance of rhythm impairment and dyslexia, we performed mvGWASs using three tools: genomic SEM[26], N-weighted GWAMA[33] and CPASSOC[34]. These tools use GWAS summary-level data and account for genetic correlation and sample overlap using the cross-trait LD score regression intercept.

Genomic SEM (common and independent pathway models). First, we reformatted our summary statistics for LDSC (munged) and genomic SEM, following standard guidelines (https://github.com/ GenomicSEM/GenomicSEM/wiki). We then used the multivariable extension of LDSC to estimate the 2 × 2 empirical genetic covariance matrix between rhythm impairment and dyslexia and their associated sampling covariance matrix. We specified a measurement model (Supplementary Fig. 1), where a shared genetic factor ($F_{gRI\text{-}D}$) was estimated to capture the observed genetic covariance between rhythm impairment and dyslexia. Given that the number of observed parameters for any 2 × 2 covariance matrix equals 3, we constrained all paths between $F_{gRI\text{-}D}$ to 1. The final CPM was fit to a genetic covariance matrix that incorporates the tested SNP (Supplementary Fig. 1). SNPs were regressed from $F_{gRI\text{-}D}$, and residuals were freely estimated. The 1,000 Genomes Phase 3 reference panel91 was used as the reference panel to calculate SNP variance across traits. Effective population size per-GWAS was calculated as $4 \times N_{cases} \times (1 - N_{cases}/N_{total})$. Both the reference panel and effective population sizes were then fed into the sumstats function and summary statistics were prepared for the meta-analysis. We applied genomic correction to the CPM results on the basis of the genomic inflation index estimated by LDSC (λ_{GC} = 1.62; Supplementary Fig. 2). The final IPM, was fit to the same matrices incorporating the SNP effects, but with the SNP effect being directly regressed from the traits. The final bivariate heterogeneity score, Q_b, was obtained by subtracting by a chi-squared difference test, where the chi-squared of the IPM is subtracted from the chi-squared of the CPM ($Q_b = \chi^2$ CPM $- \chi^2$ IPM) (ref. [31]). A high Q_b value indicates that

the association between the SNP and rhythm impairment or dyslexia is not well accounted for by the factor F_g. We then used the intersect function of bedtools (v. 2.29.2)[92] to identify the overlaps between genome-wide significant ($P < 5 \times 10{-}8$) Q_b (Supplementary Table 3) and CPM loci (Supplementary Table 2), as well as ±1 Mb surroundings of each CPM locus.

CPASSOC. Following the CPASSOC manual[34], we used the median sample size for each summary statistics file, as 23andMe SNPs can have varying sample sizes. We removed SNPs with a Z-score larger than 1.96 or less than −1.96, and extracted a 2 × 2 genetic correlation matrix for dyslexia and rhythm impairment. Then we generated an M × K matrix of summary statistics where each row represented a SNP, and two columns represented dyslexia and rhythm impairment Z-scores. We finally performed the S_{hom} test, and obtained a vector of P values for M SNPs using the pchisq function in R (4.0.3).

GWAMA (N-weighted). To account for sample overlap, we first generated a matrix of cross-trait intercepts using the intercepts of LDSC genetic correlations between dyslexia and rhythm impairment summary statistics. We then performed N-weighted GWAMA by feeding the cross-trait intercept matrix and a vector of SNP heritabilities of each trait using the multivariate_GWAMA function.

TWAS

We conducted a TWAS using S-PrediXcan framework[37] and the joint-tissue imputation (JTI) TWAS derived models from GTEx v8 tissues[35]. PrediXcan predicts gene expression from the genotype profile of each individual by using the JTI model weights, which were trained on GTEx[93], and validated on PsychEncode[94] and GEUVADIS[95]. These SNP expression weights represent the correlations between SNPs and gene expression levels. To overcome the requirement for individual-level genotype data, Barbeira et al.[37] derived a mathematical expression, implemented in S-PrediXcan framework, which effectively yields similar outcomes to PrediXcan using GWAS summary statistics. S-PrediXcan and JTI weights account for LD and collinearity problems owing to high expression correlation across tissues[35]. We filtered the 17q21.31 inversion region (~1.5 Mb long), which has multiple phenotypic associations with brain-related traits[96] to minimize the impact of this high-LD region on our results. We then corrected TWAS P values for 192,905 gene–tissue pairs, and used Z-scores and P_{FDR} of the significant ($P_{FDR} < 0.05$) pairs to assess gene–$F_{gRI\text{-}D}$ associations.

Gene set enrichment and pathway analyses

We used PANTHER to run statistical overrepresentation analysis in three GO and three PANTHER GO-Slim terms (biological process, molecular function and cellular component)[38,39,40,41] with 315 unique genes that we obtained from TWAS. We used 20,102 genes that we tested in TWAS as the background gene set. Results were FDR corrected for all GO and GO-Slim terms (n = 15,028).

LDSC partitioned heritability with cell type-specific annotations

We used eight human genome annotations by Nott et al.[45] tagging promoter and enhancer regions of neurons, oligodendrocytes, microglia, and astrocytes using LDSC partitioned heritability analysis[44] following the guidelines in the LDSC Wiki page (https://github.com/bulik/ldsc/wiki/Partitioned-Heritability). All enrichment analyses were controlled for the baselineLD model v2.2. Enrichment P value results were FDR corrected for eight tests.

Genetic correlations using GWAS summary statistics from neuropsychiatric or behavioural phenotypes

We first compiled 88 traits that were significantly genetically correlated either with rhythm (impairment) or dyslexia in the original respective GWAS papers[17,18]. We filtered these traits to avoid unnecessary multiple testing burden and to focus on genetically independent phenotypes. We identified 46 traits that are more than ±80% genetically correlated with at least one other trait. Next, we created a distance matrix from the correlation estimates and performed hierarchical clustering using Ward's method[97] as the linkage method, which maximizes the within-cluster homogeneity to identify trait clusters. We identified seven clusters using the so-called elbow method, and chose the most informative and representative trait for each cluster on the basis of the highest correlation between traits and the cluster principal component. We added these seven cluster-representative traits to the remaining 42 traits and used LDSC to estimate genetic correlations with $F_{gRI\text{-}D}$ and two independent factors. Genetic correlation P values were FDR corrected for 49 tests.

Partitioned heritability analysis with custom evolutionary annotations

We used LDSC[25] (v1.0.1) to estimate partitioned SNP heritability enrichments or depletions in foetal brain human-gained enhancers[54], Neanderthal introgressed alleles[55], archaic deserts[56], conserved loci in the primate phylogeny (Conserved_Primate_phastCons46way annotation from baselineLD) and genomic loci that have a primate phyloP score[57] less than −2 (presumably suggesting accelerated evolution). All annotations were controlled for baselineLD model v2.2. Foetal brain human-gained enhancers were also controlled for foetal brain active regulatory elements from the Roadmap Epigenomics Consortium database[98].

MAGMA gene set analysis with custom evolutionary and songbird vocal learning gene lists

We compiled four additional evolutionary genomic annotations for MAGMA gene set analysis[61], which cover timescales from ~8 million years ago to ~35,000 years ago: ancient selective sweeps[62], human accelerated regions[63,64,65,66], anatomically modern human-derived DMRs[67] and human versus chimpanzee DMRs[67]. These annotations either tag regulatory or selective sweep sites, and cover less than 1% of the total number of well-imputed SNPs in 1,000 Genomes Phase 3 reference panel, which makes them unsuitable for LDSC partitioned heritability analysis. We listed the genes that fall within ±1 kilobase of each locus tagged by each annotation, and filtered these initial gene lists for protein-coding genes using the National Center for Biotechnology Information's (NCBI) hg19 genome annotation[99]. The resulting protein-coding gene lists were used for MAGMA gene set enrichment analysis for $F_{gRI\text{-}D}$ summary statistics. We first performed gene annotation by integrating SNP locations from the summary statistics, and gene locations from NCBI hg19 genome annotations. We then performed a gene analysis using SNP P values and 1,000 Genomes Phase 3 European panel[91]. We finally applied a gene set analysis using results from gene annotation and gene analysis, and four gene-sets. Enrichment P values were FDR corrected for four tests. In the second part of our MAGMA analysis, we used nine additional songbird brain-expressed gene sets from Gordon et al.[68] and performed a separate gene set enrichment analysis for $F_{gRI\text{-}D}$. Enrichment P values were FDR corrected for nine tests.

Genome-wide negative selection estimation

We performed SBayesS analysis on the rhythm impairment, dyslexia, $F_{gRI\text{-}D}$, and two independent factor GWAS summary statistics using the GCTB software (version 2.02)[60] to quantify the level of negative selection acting on these traits. SBayesS estimates total SNP heritability, polygenicity and the relationship between variants' minor allele frequencies and effect sizes, and generates a genome-wide negative selection metric ($\hat{S}$), which ranges from 0 to −1. S estimates that are closer to −1 are interpreted as a sign of strong negative selection[50], whereas estimates closer to 0 can suggest positive selection[60].

LAVA local genetic correlations with white matter connectivity measures

To identify local regions of the genome that might make shared contributions to rhythm, language and evolutionarily relevant brain circuitry, we tested local genetic correlations between $F_{gRI\text{-}D}$ and white matter connectivity measures. We performed GWASs of selected brain imaging traits using data from the UK Biobank[100]. For these GWASs, UK Biobank data first underwent sample and genetic quality control and brain imaging data processing, followed by genome-wide association analysis.

Sample quality control. This study used the UK Biobank February 2020 release (research application number: 79683). All participants provided informed consent, and the study was approved by the North West Multi-Centre Research Ethics Committee. For individuals with both diffusion-weighted magnetic resonance imaging (MRI) and genotyping data, we excluded participants with unusual heterozygosity (principal components corrected heterozygosity >0.19), high missingness (missing rate >0.05), sex mismatches between genetically inferred sex and self-reported sex as reported by Bycroft et al.100. We further restricted our analyses to individuals with white British ancestry as defined by Bycroft et al.[100] to avoid any possible confounding effects related to ancestry. This resulted in 31,465 individuals (mean age of 55.21 years old, range 40–70 years old, 16,497 females) passing the sample quality control.

Genetic quality control. The imputed genotypes were obtained from the UK Biobank portal. These data underwent a stringent quality control protocol. We excluded SNPs with minor allele frequencies below 1%, Hardy–Weinberg P value below 1×10^{-7} or imputation quality INFO scores below 0.8. Multi-allelic variants that cannot be handled by many programs used in genetic-related analyses were removed. This resulted in 9,422,496 autosomal SNPs that were analysed in the GWAS.

Neuroimaging phenotypes. The diffusion-weighted MRI data were acquired from a 3-T Siemens Skyra scanner using the following parameters: isotropic voxel size (resolution) of 2 × 2 × 2 mm, five non-diffusion-weighted images (b = 0 s mm^{-2}), diffusion-weighting of b = 1,000 and 2,000 s mm^{-2} with 50 directions each, and acquisition time of 7 min. Whole-brain diffusion-weighted MRI scans were acquired in vivo, and fed into diffusion tensor imaging (DTI) fitting toolbox to assess brain microstructure. This analysis created the DTI outputs, including FA quantitative diffusion maps. Next, the DTI FA images were fed into the tract-based spatial statistics analysis[101], resulting in the skeletonized images. Details of the image acquisition, quality control and processing are described elsewhere (refer to https:// biobank.ctsu.ox.ac.uk/crystal/crystal/docs/brain_mri.pdf for the full protocol)[102]. We did not use the imaging-derived phenotypes released by the UK Biobank. Instead, we averaged the skeletonized images of five standard-space tract masks defined by Rojkova et al.[103] by following

a processing protocol similar to the ones applied by the UK Biobank and the ENIGMA teams (http://enigma.ini.usc.edu/protocols/ dti-protocols). Five left hemisphere white matter tracts that we investigated here are the long segment of the arcuate fasciculus; the SLF subdivisions I, II and III; and the uncinate fasciculus.

Genome-wide association scanning. GWASs were performed separately for each of the neuroimaging phenotypes using imputed genotyping data, with PLINK (v1.9)[104]. We made use of categorical and continuous variables controlling for covariates in the GWASs including age, sex, genotype array type and assessment centre. To avoid possible confounding effects related to ancestry, we used the first ten genetic principal components capturing population genetic diversity. These covariates are considered in a pre-residualization step: a multiple linear regression of the endophenotype vector on the covariates is performed, and they are replaced by their corresponding residual. Additionally, a rank-based inverse normalization is performed to ensure that the distributions of endophenotypes are normally distributed.

Local genetic correlations. We identified a list of overlapping loci using 2,495 LD blocks covering the whole human genome provided in the LAVA[73] partitioning algorithm GitHub repository (https://github. com/cadeleeuw/lava-partitioning) and 1,609 genome-wide significant ($P < 5 \times 10^{-8}$) SNPs in our $F_{gRI\text{-}D}$ summary statistics. This resulted in 18 LD blocks. We then used LAVA to estimate local genetic correlations between $F_{gRI\text{-}D}$ and the five aforementioned white matter tracts. LAVA estimates local heritability for each of these 18 LD blocks, and for each considered trait. For the loci that explained a significant proportion (nominally significant SNP heritability estimate, $P < 0.05$) of the total SNP heritability of $F_{gRI\text{-}D}$ and white matter tracts, we proceeded to perform bivariate local genetic correlation. This extra step of filtering on the basis of local SNP heritability estimates is not mandatory but recommended[73]. Finally, we obtained local genetic correlation estimates and associated P values, which we FDR corrected for 14 tests.

Box 1 - types of rhythm impairment phenotypes

Rhythm impairment, which is also described as atypical rhythm, refers to impaired (significantly less accurate) performance on a musical rhythm perception or production task (for example, rhythm and interval discrimination, rhythm or metre processing, beat perception and synchronization and isochronous motor timing or tapping)[16]. It is a broad construct that covers time-based amusia and beat deafness, inaccurate beat synchronization, related impairments in sensitivity to rhythmic patterns and metre of music and inconsistent motor timing[105,106]. The population prevalence of rhythm impairments is estimated to be between 3.0% and 6.5% (ref. [17]). The ARRH claims that rhythm impairment is comorbid with developmental language and speech disorders. While the underlying mechanisms of rhythm impairment are largely unknown, ARRH suggests a shared neurobiological and genetic ground for these traits[16].

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The full GWAS summary statistics from the original 23andMe discovery studies set have been made available through 23andMe to qualified researchers under an agreement with 23andMe that protects the privacy of the 23andMe participants. Datasets will be made available at no

cost for academic use. Please visit https://research.23andme.com/coll aborate/#dataset-access/ for more information and to apply to access the data. Participants provided informed consent and volunteered to participate in the research online, under a protocol approved by the external AAHRPP-accredited insttutional review board, Ethical and Independent Review Services. As of 2022, Ethical and Independent Review Services is part of Salus institutional review board (https:// www.versiticlinicaltrials.org/salusirb). The primary neuroimaging genetics data used in this study are available via the UK Biobank website www.ukbiobank.ac.uk. The GWAS summary statistics of FA measures of five white matter tracts, which were derived from the UK Biobank brain imaging dataset, are publicly available at the MPI Archive (accession link: https://hdl.handle.net/1839/d99a85d0-537f46a2-af19-ee5310311ec8). Genome annotation of the human genome assembly (hg19) was downloaded from the NCBI database (https:// www.ncbi.nlm.nih.gov/datasets/gene/GCF_000001405.25/). Source data are provided with this paper.

Code availability

All scripts used for analyses are publicly available via the GitHub repository at https://github.com/galagoz/pleiotropyevo. This study used openly available software, specifically PLINK (http://zzz.bwh.harvard. edu/plink/) and S-PrediXcan (https://github.com/hakyimlab/MetaXcan). JTI-TWAS prediction models trained on GTEx v8 are available at the PredictDB website (http://predictdb.org and https://github.com/ gamazonlab/MR-JTI/tree/master). The human frontal lobe probabilistic atlas used is available at https://storage.googleapis.com/bcblabweb/ open_data.html.

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Author contributions

G.A., E.E., N.J.C., R.L.G. and S.E.F. designed the research. G.A., E.E., Y.M. and G.B. performed the research. G.A., E.E. and Y.M. analysed the data. G.A. wrote the initial draft of the paper. E.E., Y.M., G.B., P.F., M.G.N., M.L., R.L.G. and S.E.F. provided critical feedback and commented on the paper.

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Competing interests

P.F. is employed by and holds stock or stock options in 23andMe, Inc. The other authors declare no competing interests.

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Supplementary materials

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Chapter 5

Spatial and single-nucleus transcriptomic profile of a chimpanzee frontal pole

This chapter is available as a preprint:

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Abstract

Chimpanzees, our closest living relatives, share a vast amount of our genetic code, with the majority of differences found in non-coding regions of the genome. Functional and gene regulatory differences drive phenotypic divergence, including the distinctive brain anatomy of humans compared to chimpanzees and other apes. However, little is known about species differences in gene expression, and how they relate to the evolution of neuroanatomy and cognition. This is primarily due to the limited availability of great ape brain samples and challenges in comparative spatial transcriptomic studies. Here, we present the first spatial transcriptomic data from a chimpanzee brain based on post mortem tissue from an adult female, who was euthanised due to poor health. We focus on the frontal pole, a brain region that has undergone significant evolutionary changes in size and organisation since the last common ancestor of humans and chimpanzees, and is considered critical for cognitive evolution. We examined the gene expression profiles and cell-type composition of the frontal pole on the left hemisphere, including both neuronal and non-neuronal cell types across cortical layers and white matter. By integrating our spatial transcriptomic data with a publicly available single-nucleus transcriptomic dataset of the chimpanzee dorsolateral prefrontal cortex, we mapped the spatial distribution of 29 chimpanzee brain cell types. This study represents a first step towards characterisation of spatial gene regulatory differences between the brains of non-human great apes and humans.

5.1 Introduction

Gene expression changes are suggested to be key drivers of phenotypic divergence between closely related species[1,2,3,4,5,6], such as humans and chimpanzees. Regulatory changes are often caused by genetic alterations at gene regulatory sequences that impact factors such as chromatin state, transcription factor binding affinity and splicing[7]. These molecular changes can alter the temporal and spatial patterns of gene expression during development and/or in adulthood, and lead to species-specific phenotypes via mechanisms such as acquisition of a new function by a cell type, relocation of an existing cell type and emergence of a completely new cell type[8]. In the context of brain evolution, such molecular and cellular changes can lead to microstructural and anatomic changes in white- and grey-matter, eventually driving cognitive and behavioural changes[9,10,11]. Investigating this long and complex causal chain from regulatory sequence alterations up to neuroanatomic and behavioural changes is quite challenging, but can help to better understand the evolutionary and biological foundations of human neuroanatomy and behaviour.

The advent of RNA-sequencing technologies over the last decade allowed the examination of gene expression at bulk, single-cell/nucleus and spatial levels[12,13,14]. Such technological advances fuelled cross-species comparative transcriptomic studies, and led to important discoveries such as a primate-specific striatal interneuron population[15] and genes expressed in primary motor cortex with human-specific co-expression patterns[16]. Despite such important insights, differences in transcriptional and developmental programmes shaping human and non-human great ape neuroanatomy are largely unknown. Drawing on prominent views on human brain evolution and recent findings from molecular evolution studies, Pollen et al.[8] suggested that shedding new light on the spatiotemporal gene expression programmes involved in human and non-human great ape neurodevelopment is crucial to better understand how the human-specific aspects of our brains have evolved.

The recent availability of spatial transcriptomics methods enabled the investigation of spatial gene expression patterns, as well as the localisations of and interactions between different cell types[17,18,19]. While spatial transcriptomics data from various cortical and sub-cortical human brain regions have accumulated over the last few years[20,21,22,23], such insights from non-human great ape brains are completely missing due to the scarcity of good-quality great ape brain samples.

Here, we present the first spatial transcriptomics data from a non-human great ape brain, alongside a comprehensive pipeline – from necropsy to data generation and analysis – for high-quality, fresh-frozen chimpanzee brain spatial transcriptomics. The brain tissue was opportunistically obtained from an adult female chimpanzee at the Saarbrücken Zoo (Germany), which had to be euthanized due to a poor medical prognosis (unrelated to our study). We focused our efforts particularly on the left hemisphere frontal pole, which is a region known to be substantially different in size and organisation between humans and chimpanzees[24,25], and is suggested to be involved in various aspects of cognition such as self-awareness, decision-making and metacognition[26,27]. By combining our fresh-frozen brain sample preparation method with 10x Genomics Visium, a well-established sequencing-based spatial transcriptomics method[28,29], and a set of analytic tools, we were able to generate good quality chimpanzee brain spatial transcriptomics data. Finally, we integrated our Visium data with a publicly available single-nucleus transcriptomics dataset of chimpanzee dorsolateral prefrontal cortex using a Bayesian model implemented in cell2location[30], and annotated cell

type abundances on chimpanzee frontal pole. Our study establishes a comprehensive framework for investigating spatial gene regulatory profiles of great ape brains, and paves the way for future great ape brain comparative spatial transcriptomics studies.

5.2 Results

Histological and transcriptomic quality control of chimpanzee brain tissue sections and comparison with publicly available human data.

We first performed a set of qualitative and quantitative controls to ensure the suitability of our sample for the Visium protocol. We measured the integrity and concentration of RNA isolated from two tissue samples obtained from two tissue blocks in the left frontal pole, and observed that both RNA integrity and concentration measures were sufficient to proceed with spatial transcriptomics data generation (Table S1). We then performed cryosectioning on two adjacent left hemisphere frontal pole tissue blocks, and obtained two pairs of 10-µm thick consecutive tissue sections, one pair from each block (Fig. 1A, Fig. S1). We placed these tissue sections on a Visium spatial gene expression slide and performed haematoxylin and eosin (H&E) staining to characterise the anatomical morphology, revealing roughly marked white matter-grey matter borders, blood vessels and gyrification patterns, as expected (Fig. 1B).

We then performed spatial transcriptomic profiling (median sequencing depth=283×106, sequencing depth range=221×106-316×106), and measured gene expression of 23,012 genes in 13,399 spatially barcoded Visium spots across four sections (Table S2). Importantly, our analysis yielded a mean of 5,002 unique molecular identifiers (UMI) and 1,879 genes per Visium spot, which is close to that of human brain-derived spatial transcriptomics data available in literature (Fig. 1B, Table S3)[20,21,22,23]. We observed that the UMI count distribution patterns differ between white and grey matter, a phenomenon also reported in a prior human brain spatial transcriptomics study[22] which is potentially due to the lower RNA content in axons compared to neuronal cell bodies.

To further investigate the overall morphology of the chimpanzee frontal pole, we quantified the nucleus density in our tissue sections and compared our findings to a publicly available human dorsolateral prefrontal cortex dataset[21]. Our image analysis, which leveraged the high-resolution H&E staining images, identified 2.7 nuclei per Visium spot on average across all spots from four tissue sections (Table S3). We also found that 9.5% of all spots harboured a single nucleus, whereas 10.3% did not contain any nuclei (Fig. S2). These measures are similar to the human brain-derived nucleus count estimates in Maynard et al. (average nuclei per spot=3.3, percentage of spots with one nucleus=15.0, percentage of spots with zero nucleus=9.7)[21].

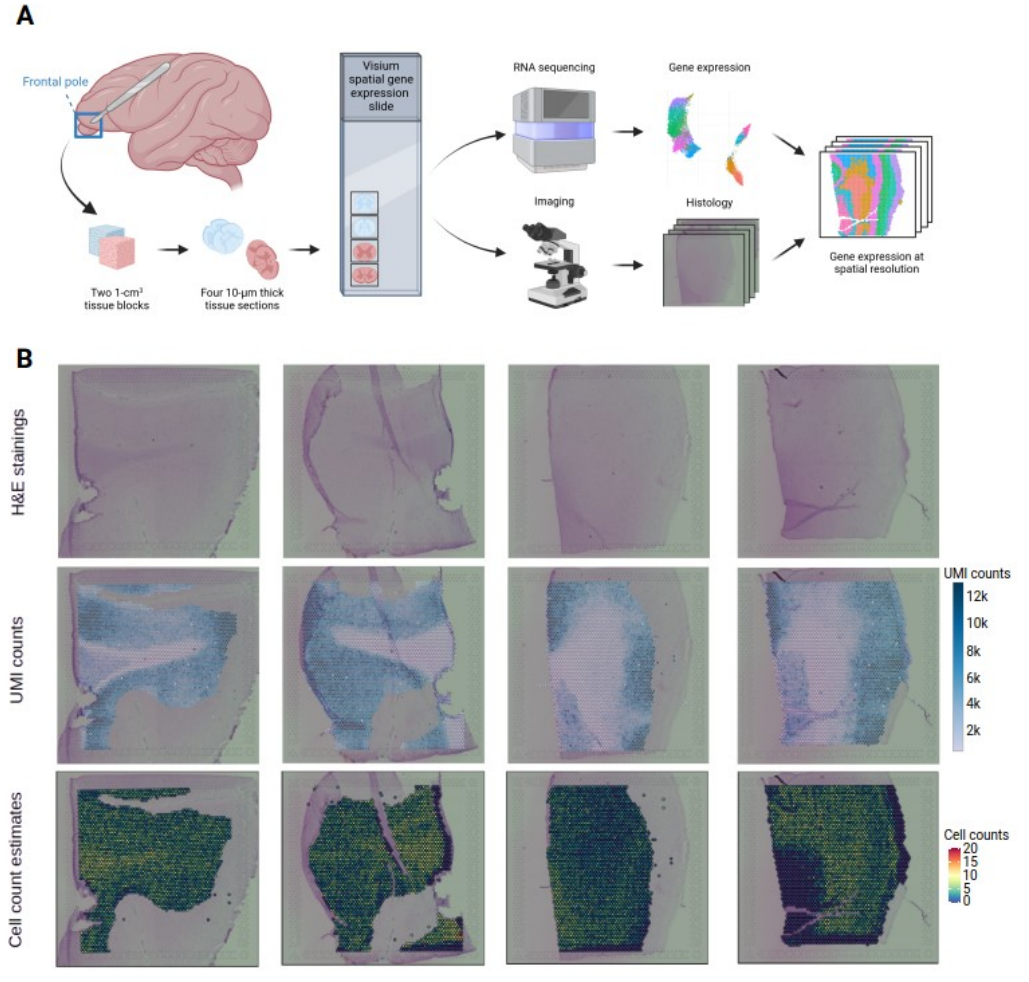

Figure 1: Spatial transcriptomics workflow and an overview of the chimpanzee frontal pole tissue sections. (A) Schematic shows an overview of our spatial transcriptomics data generation and analysis pipeline. **(B)** H&E staining of all sections that are investigated in this study, depicting the overall tissue morphology (haematoxylin stains nuclei with purple, eosin stains extracellular matrix and cytoplasm with pink). Unique molecular index counts and cell count estimates are depicted as spot-plots.

Before conducting spatial transcriptomics analyses, we performed immunohistochemical protein staining to gain an initial overview of the cytoarchitecture in our chimpanzee frontal pole samples. We stained eight tissue sections, which were obtained from the same two tissue blocks used for spatial transcriptomics. To reveal the white matter-grey matter boundary and to highlight deep- and outer-layer neurons, we stained for canonical protein markers that are known to be abundant at certain layers of the human cortex[21,31], including a pan-neuronal marker (NeuN), two deep layer neuronal markers (CTIP2 and TBR1), an upper layer marker (BRN2), and a white matter marker (MBP). Layer-specific localisations of canonical markers in human brain studies were rather ambiguous in our staining images (Fig. S3). However, the white matter-grey matter distinction that we observed in H&E images and UMI count distributions overlapped with the spatial patterns revealed especially by anti-NeuN, -TBR1 and -BRN2 staining.

Spatial variation in gene expression reflects grey-white matter distinction and the laminar structure in grey matter.

After performing quality control to ensure data quality was comparable to that of publicly available human brain-derived spatial transcriptomics data, we integrated and normalised the spatial transcriptome data from our tissue sections by using Harmony[32] to correct for batch effects (Fig. S4). We applied further filtering on genes and Visium spots (as described in Methods), and removed spots located under folded tissue sections, which yielded 12,614 spots in total. We then identified 12 gene expression clusters of Visium spots from across all tissue sections by using a Markov Chain-Monte Carlo clustering algorithm provided in BayesSpace[33] (Fig. 2A, Fig. S5). To investigate the spatial distribution of these transcriptomically-defined clusters, we mapped the cluster identity of each Visium spot back to its corresponding histology image. We observed that the 12 gene expression clusters mainly reflect i) transcriptomic differences between white and grey matter, and ii) cellular and gene expression variability across layer-like laminar structures within the grey matter of the chimpanzee frontal pole (Fig. 2B). This transcriptomically-defined laminar spatial organisation, including the specific order of gene expression clusters across the axis spanning from the white matter towards the outer layers of grey matter, was consistent among all four sections. Thus, these clusters appear to reliably capture the distinct molecular characteristics of the various cortical layers.

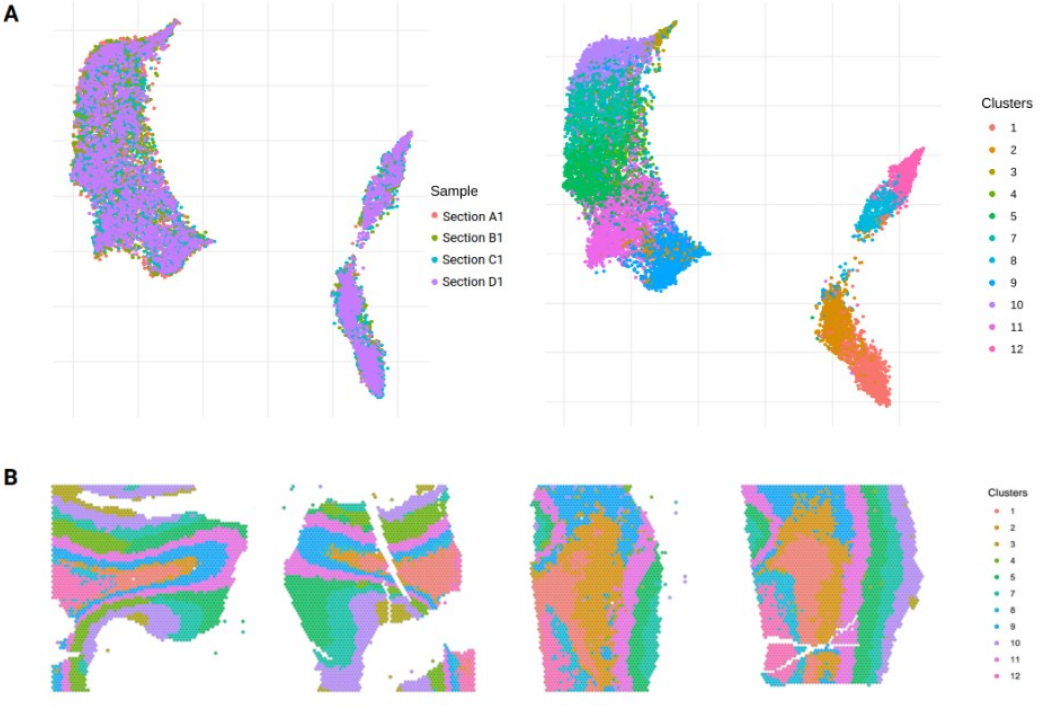

Figure 2: UMAP and spatial representations of gene expression clusters. (A) Unsupervised clustering of Visium spots based on their gene expression profiles after integration of data from four sections. Colours indicate which tissue section each spot belongs to (left) and 12 gene expression clusters identified by the BayesSpace (right). **(B)** Spatial map

of gene expression clusters on histology images. Colours correspond to the same 12 gene expression clusters with the UMAP on top right.

Integration with a publicly available single-nucleus RNA-sequencing dataset and cell type deconvolution analyses reveal cell subclass organisation in the chimpanzee frontal pole.

To investigate the spatial organisation of various brain cell types in the chimpanzee frontal pole, we deconvoluted our spot-level data to cellular level. To do so, we integrated the Visium spatial transcriptomics data with a publicly available single-nucleus RNA-sequencing (snRNA-seq) dataset[34] derived from dorsolateral prefrontal cortex tissue samples of four adult chimpanzees (158,099 nuclei). This reference dataset contains annotations of 29 neuronal, glial, and non-neuronal cell subclasses (Table S4) based on gene expression profiles, providing a high-resolution cellular map of the chimpanzee dorsolateral prefrontal cortex, which is a large cortical area located adjacent to the frontal pole[35].

We first quantified RNA and cell abundances to estimate RNA detection sensitivity per-spot for all sections using cell2location[30] (Fig 3A, Fig. S6-7). Spatial variation in RNA detection sensitivity estimates overlapped with the spatial distribution pattern of UMI counts, which indicates that low RNA counts in white matter can hinder cell2location's RNA detection sensitivity. We then generated a cell subclass-specific gene expression signature reference by training a Bayesian model on the single-nucleus gene expression data from Ma et al.[34] using cell2location[30]. We integrated these reference gene expression signatures with our spatial transcriptomics data, and trained an RNA detection sensitivity-aware model to estimate per-spot cell subclass abundances for 29 cell subclasses (Fig. S8). Overlaying the abundance of four neuronal subclasses with layer identity (layer 2-3 intratelencephalic, layer 3-5 intratelencephalic type 1, layer 3-5 intratelencephalic type 3, and layer 6 corticothalamic neurons), as well as oligodentrocytes and astrocytes, further highlighted the laminar structure of the chimpanzee grey matter, and the distinction between grey and white matter. The localisations of four selected neuronal subclasses converged with the spatial organisation of 12 gene expression clusters identified by unsupervised clustering (Fig. 3B), which suggests that these clusters also have layer identities potentially with unique cellular compositions. The spatial order of the four neuronal subclasses with cortical layer identities, as identified by Ma et al.[34], match their expected anatomical order along the axis from the outer zone of grey matter to white matter,

again supporting the convergence of reference data-based cell subclass mapping and unsupervised clustering analysis results.

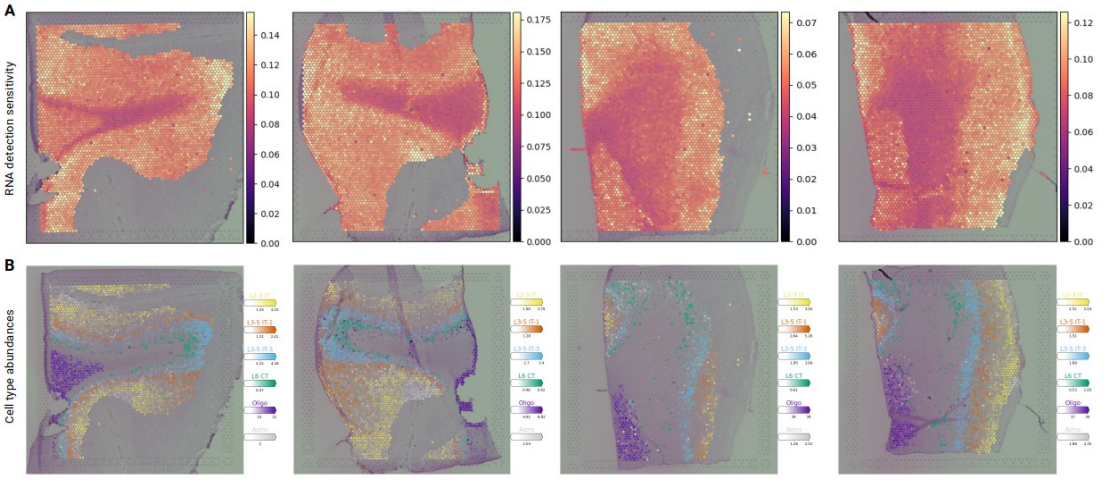

Figure 3: RNA detection sensitivity and cell subclass abundance estimates by cell2location. (A) Per-spot RNA detection sensitivity measures for each section. **(B)** Cell subclass abundances for six selected subclasses [L2-3 IT (yellow): Layer 2-3 intratelencephalic; L3-5 IT-1 (orange): Layer 3-5 intratelencephalic Type 1; L3-5 IT-3 (blue): Layer 3-5 intratelencephalic Type 3; L6 CT (green): Layer 6 corticothalamic; Oligo: Oligodendrocytes (purple); Astro: Astrocytes (grey)].

5.3 Discussion

Here, we have established and validated in pilot experiments a workflow to study spatial gene expression in great ape brains, covering steps from necropsy, tissue extraction and freezing, through to spatial transcriptomics data generation and analysis. We successfully generated high-quality spatial transcriptomics data from a chimpanzee brain using the Visium platform, with comparable RNA quality and quantity measures to that of publicly available human brain-derived data. We have applied a set of integrative omics tools to our dataset, and successfully profiled the spatial gene expression patterns and cell type composition in the chimpanzee frontal pole.

We compared a number of standard spatial transcriptomic data quality and quantity measures between our chimpanzee brain-derived data and three publicly available human brain-derived spatial transcriptomics datasets[20,21,22,23]. These comparisons provided a key checkpoint before proceeding with data analysis, as the experimental and analytic protocols for spatial sequencing technologies were not tested on non-human great ape brains prior to this study. We observed that the average number of unique RNA molecules, genes and cells per Visium spot in chimpanzee frontal pole and human cortex tissue sections are similar, suggesting that spatial transcriptomics data from these two species can be analysed in a comparative design. Our findings support previous observations[22] showing that the number of unique RNA molecules per-spot in white matter is lower compared to grey matter, which might be related to the lower RNA content in the axons compared to cell bodies. Yet, we note that the sample size of our study does not have sufficient statistical power to reach generalisable biological conclusions on the molecular characteristics of the chimpanzee frontal pole. Nonetheless, obtaining similar data quality and quantity measures with prior human brain spatial transcriptomics studies

enabled us to proceed with spatial gene expression data analysis. Previously, such comparative spatial transcriptomics studies were performed between human and mouse samples, revealing distinct cell types and species-specific organisation of gene expression[36]. Our study paves the way for similar comparative studies across humans and non-human great apes, which may provide important insights into species-specific aspects of our brains.

Our gene expression data-driven clustering analysis identified spots with distinct transcriptomic profiles. The main axes of gene expression variation were driven by the transcriptomic and cell type composition differences between white and grey matter, and across cortical layers. Despite the limited sample size of our study, this observation is in line with various human brain spatial transcriptomics studies, indicating the overall spatial patterns of gene expression can be recapitulated even in small datasets. Similarly, our observation that the gene expression clusters are organised in a laminar pattern is in line with the well-known six layered structure of the mammalian cortex[37]. To support our findings from unsupervised clustering, we performed single-nucleus data integration and cell type abundance estimation analysis, which replicated this laminar distribution and showed that the gene expression clusters overlap with cell subclasses with cortical layer identity in the expected layer order[38]. Our results from the unsupervised clustering and the reference data-based cell type mapping analyses converged, validated the histologically identified laminar structure of the chimpanzee cortex[39,40], and provided a first glance into the fine-grained cell type organisation in the chimpanzee cortex.

The main limitation of the present study is that tissue sampling was restricted to the left hemisphere frontal pole of a single female chimpanzee brain. Thus, even though we were able to observe the laminar organisation of gene expression in the adult chimpanzee frontal pole, we refrain from drawing cortex-general, species-general or comparative conclusions. In addition, the absence of a chimpanzee reference transcriptome, and the incomplete annotation of the chimpanzee genome may introduce a bias to quantify the expression of genes that are well-annotated. Ongoing efforts to generate high quality and high coverage primate genome assemblies[41,42] hold great promise for the availability of better annotated great ape genomes in the near future that will help to avoid this bias in future studies. Secondly, the relatively long post mortem interval prior to brain extraction (27 hours) and freezing may lead to RNA degradation. To circumvent this potential limitation, we cooled down the body immediately after euthanasia, and managed to retrieve non-degraded RNA from the brain samples. Finally, we were not able to recover sufficient UMI and gene counts from some Visium spots, potentially due to technical reasons including uneven freezing pace of different parts of the tissue sections and condensation formed due to fluctuations or suboptimal temperature fluctuations within the cryostat when transferring cryosections onto Visium slides. We recommend using a cryostat with a stable chamber, holder and blade temperature to overcome similar issues.

In summary, our study represents a promising first step towards the spatial transcriptomic profiling of non-human great ape brains. We believe that the rapid enhancement of the spatial biology technologies[43] and efforts like ours to apply these methods to non-human great apes pave the way for the generation of comprehensive great ape brain cell atlases. Combined with the ongoing efforts of human brain cell atlases[23,44], future comparative spatial research on human and non-human great ape brains can shed light on the spatiotemporal characteristics of gene expression, which drive human-specific aspects of neurobiology and cognition.

5.4 Materials and Methods

Necropsy and sample preparation

For this study, we collected a 53-year-old female captive chimpanzee (Pan troglodytes troglodytes) brain in July 2021 at the Saarbrücken Zoo in Germany, following her euthanisation due to poor medical prognosis caused by a right abdominal cavity tumour. The body was cooled at 3-5 °C, and the brain was extracted 27 hours post mortem. We first cut apart the cerebellum and the cerebrum. The left and right hemispheres were then separated by cutting the corpus callosum. Left and right hemispheres were sliced coronally into ~1-cm thick slices, which were freshly frozen in isopentane (-80 °C). Then, half of the rostral-most slice of the left hemispheric cerebrum (i.e. the frontal pole) was cut into five ~1 cm3 tissue blocks on dry ice. After visually inspecting these tissue blocks' suitability (e.g. having large and flat surface) for cryosectioning and Visium spatial transcriptomics protocols, we selected two tissue blocks for RNA quality and quantity control measurements, and another two blocks for performing spatial transcriptomics (see Fig. S1 for details) and immunohistochemistry.

Sample processing and Visium data generation

We cryosectioned (CM1900, Leica) the selected frozen tissue blocks at a chamber and blade temperature of -20 °C, and a holder platform temperature of -12 °C. We took two 10-µm thick consecutive sections from each block, obtaining four tissue sections in total. Sections were placed on a pre-chilled (down to -20 °C) Visium Spatial Gene Expression Slide (Visium Spatial Gene Expression Slide & Reagent Kit, 10x Genomics, kit no. 1000184, Visium slide no. V13J17-280), and immediately attached sections to the slide by warming the back of the slide. Following the Methanol Fixation, H&E Staining and Imaging for Visium Spatial Protocols (catalogue no. CG000160 Rev C), we immersed and incubated our Visium slide in pre-chilled methanol (-20 °C) for 30 minutes to fixate the tissue sections, after which we carried out H&E staining and tissue imaging. We took brightfield images of H&E stained sections using an AxioScan Z1 slide scanner (Zeiss) with a Hitachi HV-F292SCL camera, a Plan-Apochromat 20x/0.8 M27 objective and Zeiss Zen v.2.6 (Blue Edition) software.

We then proceeded with permeabilisation of the tissue sections (for 18 minutes) and reverse transcription, followed by second strand synthesis and denaturation following the Visium Spatial Gene Expression User Guide (catalogue no. CG000239 Rev F, 10x Genomics). We performed qPCR to determine the number of cDNA amplification cycles required to reach ~25% of the peak fluorescence value, and then ran 16 cycles of cDNA amplification. Finally, we performed library preparation using the BioRad T100 Thermal Cycler for PCR reactions following the Spatial Gene Expression Library Construction section of the Visium Spatial Gene Expression User Guide (catalogue no. CG000239 Rev F, 10x Genomics). The libraries were sequenced on an Illumina NovaSeq 6000 System with 150 bp paired-end reads using the standard sequencing protocol for Visium fresh-frozen libraries (Read 1: 28 cycles, i7 index: 10 cycles, i5 index: 10 cycles, Read 2: 91 cycles). The four libraries were sequenced to a median depth of 283×106 reads (range: 221×106-316×106).

Histology image analysis and nucleus segmentation

Following the methodology provided by Maynard et al.[21] (also see https://www.mathworks.com/help/images/color-based-segmentation-using-k-means-clustering.html), we applied K-means colour-based nucleus segmentation to the high-resolution

H&E staining images. We first converted each image from RGB to CIELAB colour-space by using the rgb2lab function in Matlab (vR2019a). CIELAB colour-space quantifies luminosity measures (from black to white), as well as two chromacity spectrum measures (along red-green and blue-yellow axes). We extracted two chromacity layers from each image, which allowed us to visually inspect and identify the number of different colours in each image. We then used the imsegkmeans function to generate binary masks for each manually observed colour in the histology images, which allowed us to obtain a binary mask per section tagging all nuclei which were dyed in purplish blue by haematoxylin. Following the nuclei number estimation per Visium spot protocol implemented by Maynard et al., we integrated these binary masks with individual spot coordinates that we obtained from data processing by SpaceRanger. By overlaying the binary masks with Visium spot coordinates, we estimated the number of nuclei that fell in each spot and generated a summary table including per-spot estimates.

Visium spatial transcriptomics data processing

We integrated raw sequencing data (FASTQ files) and the low-resolution H&E images to perform read alignment against the chimpanzee reference genome Clint_PTRv2 (a.k.a. pan_tro6; accession number: GCF_002880755.1). We rescaled the H&E images to 2000x2000 pixels by using ImageJ (v1.53). We then performed a standard QC of the sequencing data using default parameters in SpaceRanger (v1.2.2) (see Table S1 for data quality control measures). The resulting count matrices (feature-barcode matrices), which contain UMI counts of each gene-spot pair, were then used for data analysis.

Following the analysis pipeline used by Maynard et al.[45], we imported mapped and preprocessed Visium data and the matching low-resolution H&E image for each sample into R using SummarizedExperiment[46]. We obtained a large SingleCellExperiment object which includes data from 13,399 Visium spots across four sections, with a mean of 5,002 UMIs and 1,879 genes per spot. First, we performed spot-level quality controls of UMI count, gene count and percentage of mitochondrial reads by using perCellQCMetrics and quickPerCellQC functions from the Scran package (v1.18.7). We did not filter out spots based on these measures, as the UMI and gene count variance across spots largely reflected biological differences between grey and white matter. We then removed the spots located under folded parts of tissue sections, resulting in high-quality data from 12,614 spots across four sections. Second, following the data filtering protocol from Wong and Sha et al.[22], we performed gene filtering by removing the genes that are expressed in less than 0.01% of all spots, and all mitochondrial genes. A total of 23,012 genes passed these filtering steps.

Visium spatial transcriptomics data analysis

In order to prepare raw data for unsupervised gene expression clustering, we first computed the log-normalised gene expression counts per spot and estimated the first 50 principal components based on gene expression variation among the top 2,000 highly variable genes using the spatialPreprocess function from the BayesSpace R package[33]. We then performed a principal component analysis to estimate the top 50 axes of gene expression variation across Visium spots from four sections using the runUMAP function of the scater R package[47]. We first used these top 50 principal components to perform Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction and explored data variation axes prior to data harmonisation. We then integrated principal component analysis results with the main SingleCellExperiment object, and corrected for technical variation across spatial transcriptomics

libraries (i.e. batch effects) using the runHarmony function from the Harmony R package[32], which took the log-transformed gene expression count data combined with the top 50 principal components. By combining this enhanced data object with user-specified batch or sample information, Harmony corrected for technical variation in our data while preserving transcriptomic variation. We generated a new UMAP after data harmonisation to ensure that batch effects did not impact main axes of variation in our data.

Next, we selected the number of gene expression clusters to use in our clustering analysis by using the qTune function of BayesSpace. We estimated average pseudo-log-likelihoods for a set of cluster numbers (q=2 to 20), plotted log-likelihoods as a function of q and chose the q value around the elbow of this plot (q=12) as the number of gene expression clusters. We then performed clustering analysis for all spots across four sections using the spatialCluster function of BayesSpace (q=12, total number of iterations=20,000, burn-in iterations=1,000, seed number=149). We projected these data-driven gene expression clusters of Visium spots onto H&E images and observed the spatial distribution of 12 gene expression clusters across four sections using the clusterPlot function of BayesSpace.

Single-nucleus RNA-sequencing data-supervised cell type deconvolution analysis

We used a Bayesian model named cell2location[30] to integrate our Visium spatial transcriptomics data with a publicly available chimpanzee dorsolateral frontal cortex-derived single nucleus-RNA sequencing dataset[34]. The dataset from Ma et al. contains transcriptomic data from 29 neuronal, glial and non-neuronal cell subclasses (see http://resources.sestanlab.org/PFC/). Following the cell2location tutorial for Visium spatial transcriptomics data analysis (see https://cell2location.readthedocs.io/en/latest/notebooks/cell2location_tutorial.html), we imported mapped and pre-processed Visium data, and the matching low-resolution H&E images for each sample, into Python (v3.9) by using the read_visium function from the scanpy package[48]. We filtered out mitochondrial genes from our Visium data.

Next, we downloaded processed single-nucleus RNA-sequencing data from the Ma et al. study (see http://resources.sestanlab.org/PFC/), imported the raw count matrix to Python, and removed non-chimpanzee samples from the data. We applied a permissive gene selection in this dataset by excluding genes with less than 1.1 mean expression among all spots and genes that are not expressed at least in 0.03% of all spots, which yielded 13,279 genes. Then, we estimated reference cell subclass signatures using cell2location, which follows a two-step procedure: i) negative binomial regression to combine and harmonise single-nucleus RNA-sequencing data across batches/samples, and ii) computation of average gene expression levels per gene for each cluster by training a model (training epochs=250). We performed standard quality control steps to check the accuracy of this model by checking the evidence lower bound (ELBO) loss history and reconstruction accuracy, and then proceeded with integrating the reference data set with our Visium data.

We performed cell type deconvolution analysis for each Visium section independently by using the default cell2location model to estimate cell type abundances in each Visium spot. We informed this model with the average per-spot cell number estimate that we obtained from the aforementioned high-resolution image analysis. Once the model was trained (training epochs=30,000), we performed a quality control to check the ELBO loss history and reconstruction accuracy measures (Fig. S6), and spatially visualised cell abundances for 29 cell

subclasses by using the 5% quantile of the posterior distribution, which represents cell abundances in which the model has high confidence.

Immunohistochemistry

Cryosections (10-µm thick) adjacent to those used for Visium were fixated in 4% PFA at room temperature for 10 minutes, followed by 1X PBS washes (3 x 5 minutes). Then, we applied antigen retrieval by incubating sections in pre-warmed citrate buffer (pH 6.0) (Merck, C9999) at 90 °C for 10 minutes in a water-bath, which were then cooled down to room temperature for 30 minutes. Sections were washed again with 1X PBS (3 x 5 minutes). After washing, we drew hydrophobic barriers between sections using an immunostaining pap pen (Kisker Biotech, MKP-1) to prevent cross-binding of different antibody combinations. Sections were then incubated in blocking and permeabilisation buffer containing 5% donkey serum (Sigma, S30-100ML) and 0.25% Triton (Sigma Aldrich, X-100) in PBS at room temperature for one hour. Primary antibodies: NeuN (Millipore, MAB377, 1:2000), BRN2 (Cell Sig., 12137S, 1:500), CTIP2 (Abcam, ab18465, 1:500), MBP (Sigma, NE1019-100UL, 1:1000) and TBR1 (Sigma, ab2261, 1:500) were diluted in blocking solution and incubated with tissue sections at 4 °C for 48 hours. Sections were then washed with 1X PBS (3 x 5 minutes), and incubated with the appropriate fluorescently labelled donkey- or goat-raised secondary antibodies (Invitrogen, 1:200) at room temperature for one hour. Sections were washed again with 1X PBS (3 x 5 minutes). We incubated sections with Hoechst solution in 1X PBS (Invitrogen, H3570, 1:1000) to stain cell nuclei at room temperature for five minutes. We then incubated sections in 0.01% Sudan black (Sigma Aldrich) solution at room temperature for five minutes. Sections were washed with 70% ethanol (1 x 30 seconds) and with 1X PBS (1 x 5 minutes), then mounted using Fluorescent Mounting Medium (Dako, S3023) without DAPI, and covered with 40 mm coverslips.

Data availability

The raw and processed chimpanzee left frontal pole Visium spatial transcriptomics data were deposited in the Max Planck Institute Archive and are avaialble on request (https://hdl.handle.net/1839/00f642f3-ac12-44e3-a3d3-f384884dc3e7).

Code availability

All scripts used for the analysis are available on the project GitHub repository (https://github.com/galagoz/chimpbrain-st).

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Author contributions

G.A., M.W., and S.E.F. designed research; J.K. provided the *post mortem* tissue; T.G. and C.J. performed necropsy and tissue freezing; C.J. and M.M. performed tissue block cutting and sample shipment; M.W. and G.A. generated the data; G.A. analysed the data; G.A. wrote the initial draft of the paper; M.W., R.M.W., C.C., P.G., and S.E.F. supervised research; all co-authors provided critical feedback and commented on the manuscript.

Competing interest

The authors declare no conflict of interest.

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Supplementary materials

The online version contains supplementary material available at https://www.biorxiv.org/content/10.1101/2025.02.16.638510v1.supplementary-material.

Chapter 6

General discussion

6. General discussion

6.1 Summary of the thesis

In this thesis, we adopted a highly interdisciplinary approach, combining computational and experimental methods to understand the genetic and molecular underpinnings of human brain evolution. We generated and analysed a wide range of data types, including neuroimaging and behavioural genomics, comparative genomics, ancient DNA, and transcriptomics. Additionally, we generated the first spatial transcriptomics data set from a chimpanzee brain. By integrating the results of four key investigations into human language and brain evolution (Figure 1), this thesis aims to provide a unified perspective on the genetic and molecular factors shaping human brain evolution.

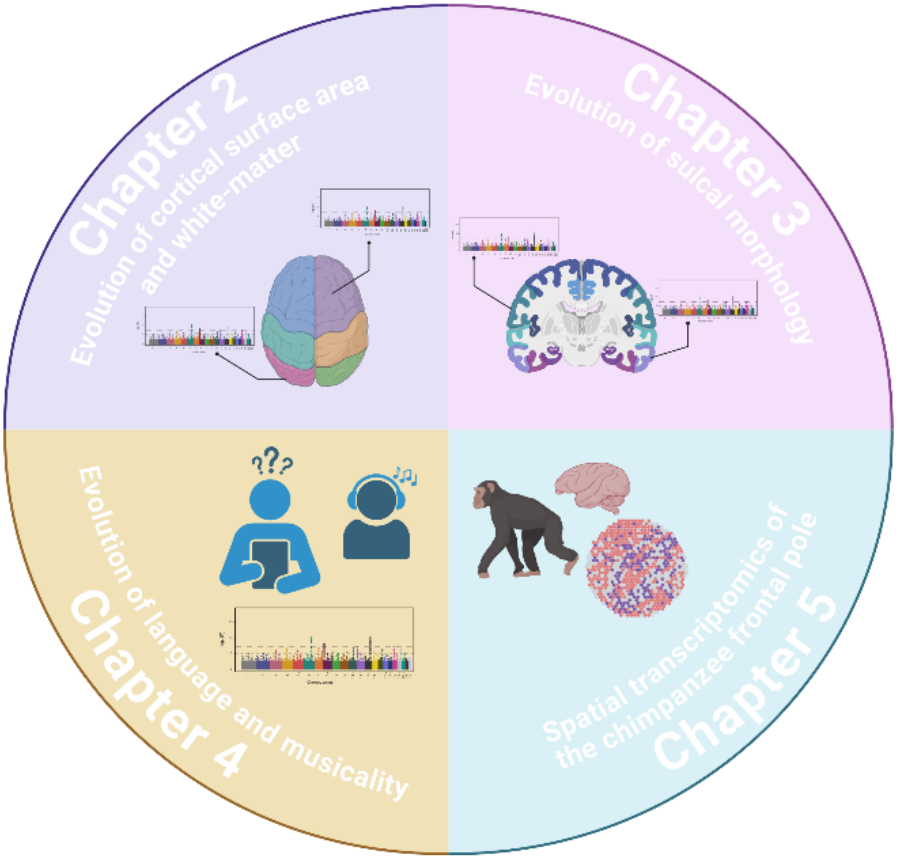

Figure 1: A graphical representation of the four research chapters focussing on diverse aspects of human language and brain evolution.

In ***Chapter 2***, we investigated the present-day inter-individual variability and evolutionary past of human cortical surface area and white-matter connectivity. Building on the analytic pipeline established by Tilot et al.[1], we traced the genetic signatures of recent selection and deeper evolutionary events, such as *Homo neanderthalensis-Homo sapiens* admixture. We first

conducted a targeted replication of the evolutionary findings reported by Tilot et al.[1], and observed that the recent selection signal for cortical surface area could not be replicated in our independent study sample. However, the SNP-heritability enrichment in human-gained enhancers for specific cortical regions was replicable for pars orbitalis, a region involved in language processing. Thus, our findings partially align with those of Tilot et al., suggesting a link between human-gained enhancer elements active in foetal brain tissue and the anatomy of language-related cortical regions. We then expanded our investigation to explore the genetic architecture and evolutionary past of 33 regional and global cortical surface area (N=30,332) and white-matter connectivity (N=29,924) measures from left and right brain hemispheres using a large-scale structural MRI and genotype data set from the UK Biobank[2]. Given the functional and anatomical laterality of complex behaviours, such as the predominantly left lateralisation of language-related processes[3], focussing on left and right hemispheres independently enabled us to investigate the genetic bases of neurobiology of language at hemispheric and regional resolution. To minimise ancestry- and population stratification-related confounding, we focussed on a relatively ancestrally homogeneous subset of individuals. Our results revealed significant SNP-heritability enrichments in foetal brain human-gained enhancers for cortical surface area of several regions, including the left hemisphere pars triangularis [Enrichment(SEM)=9.65(3.04), P_{FDR}=0.015] and pars opercularis [Enrichment(SEM)=9.61(3.47), P_{FDR}=0.044]. Additionally, we identified SNP-heritability depletion in Neandertal introgressed fragments for a connectivity measure of the left hemisphere uncinate fasciculus [Enrichment(SEM)=0.01(0.28), P_{FDR}=0.022], a white-matter tract involved in mapping sound to meaning. Finally, we conducted an in-depth dissection of an example genomic locus, demonstrating how a human-gained enhancer element, a set of genetic variants associated with the left hemisphere pars triangularis surface area, and a gene implicated in neurogenesis, *ZIC4*, are interacting.

Building on the aforementioned post-genome wide association study (GWAS) evolutionary analysis pipeline that we adopted and refined, in ***Chapter 3***, we examined a set of publicly available GWAS summary statistics on sulcal morphology derived from an earlier release of the UK Biobank neuroimaging genomics data set (N=26,530). Specifically, we analysed GWAS summary statistics of 450 hemispheric sulcal parameters and four sulcal shape descriptors (i.e., mean depth, surface area, width and length) to investigate the links between common genetic variants shaping human sulcal organisation and the evolutionary annotations that we validated and used in ***Chapter 2***. Leveraging findings from recent comparative primate neuroimaging studies, we established two analysis streams: i) a targeted analysis focussing on 14 pre-selected sulcal parameters with known connections to primate- and human-specific aspects of brain evolution, and ii) an exploratory analysis targeting 139 sulcal parameters with available GWAS summary data. Our analyses revealed significant SNP-heritability enrichments in foetal brain human-gained enhancers for several sulcal structures, including the central sulcus in both hemispheres. This finding suggests a potential link between the genetic underpinnings of regional sulcal anatomy and human-gained enhancers. Notably, a common genetic variant near the *ZIC4* gene showed genome-wide significant association with the width of the left hemisphere central sulcus, and directly overlapped with a foetal brain human-gained enhancer element. These findings, combined with results from ***Chapter 2***, suggest a convergent pattern linking cortical anatomy-associated common genetic variants, foetal brain human-gained enhancers and neurodevelopmentally important genes *ZIC4* and *ZIC1*.

In ***Chapter 4***, we shifted our focus to another aspect of human evolution, examining the shared genetics and evolutionary origins of language/reading- and musical rhythm-related traits. A key objective of this chapter was to follow up on previous findings of phenotypic correlations between these traits, and investigate whether these links also manifest at the genetic level. We first identified significant genetic correlations between musical rhythm skills and various language- and reading-related abilities, including word reading, non-word repetition and dyslexia. Secondly, we started building a data-driven ground for resolving long-standing hypothetical debates over the co-evolution of human language and musical skills. Using genomic structural equation modelling and two previously published large-scale GWASs of dyslexia[4] and musical rhythm[5], we performed a multivariate GWAS of dyslexia and musical rhythm impairment, uncovering the pleiotropic genetic landscape of these traits. This analysis identified 16 pleiotropic loci contributing to their shared genetic architecture. We further explored the shared genetic aetiology of dyslexia and musical rhythm impairment, revealing intriguing genetic links to various neuroanatomic structures, behavioural traits, and psychiatric disorders. Notably, we identified a genetic link with the connectivity of a segment of the superior longitudinal fasciculus (i.e., SLF-I), which may suggest potential involvement of this white-matter tract in the co-regulation of language and musicality-related behaviours. Thirdly, we investigated the evolutionary history of these traits, using a battery of analytic tools and the shared genetic factor of dyslexia and musical rhythm impairment as proxies for human language and musicality. Our analyses confirmed previously observed evolutionary characteristics of complex human traits, such as significant SNP-heritability enrichment in primate-conserved regions[6] and depletion in Neandertal introgressed fragments, in line with the literature[7]. We detected moderate genome-wide negative selection acting on dyslexia, musical rhythm impairment, and their shared genetic factor, consistent with trends observed for other complex traits[8]. Finally, we focussed on a genomic locus of potential importance for the shared genetics and co-evolution of language and musicality. This locus harbours a genome-wide significant genetic variant associated with both dyslexia and musical rhythm impairment, and is highly conserved within the primate clade. Our analyses revealed that this variant is located on *DLAT*, a gene implicated in a rare neurodevelopmental disorder characterised by neurological dysfunction and childhood learning disability.

In ***Chapter 5***, we approached human brain evolution from a different perspective, taking on the challenge of generating the first set of spatial transcriptomics data from a post-mortem chimpanzee brain. The primary goal of this chapter was to test the feasibility of applying current spatial transcriptomics data generation and analysis techniques to great ape brain tissues, therefore paving the way for future comparative investigations of human and non-human primate (NHP) brains. When used in a comparative design, spatial transcriptomics enables the testing of previously unaddressed hypotheses such as the disproportionate molecular and cellular divergence of the association cortex[9] as opposed to other brain regions that are relatively conserved among humans and NHPs. We collaborated with leading scientists in primate brain and behaviour research to obtain high-quality brain tissue samples from the left frontal pole of a chimpanzee brain. The brain sample was obtained post-mortem from a female captive chimpanzee named Takki, who was euthanised due to poor prognosis. The tissue samples were fresh-frozen in isopentane to preserve the tissue integrity of the outer layers of the cortex. Using the Visium (10x Genomics) spatial transcriptomics data generation protocol, we successfully generated high-quality gene expression data from four tissue sections spanning grey and white matter. We then proceeded with data integration and harmonisation from

across four tissue sections, followed by unsupervised clustering to identify distinct gene expression clusters. Additionally, we integrated our results with a publicly available single-nucleus RNA sequencing dataset from the chimpanzee dorsolateral prefrontal cortex. This integration allowed us to map the spatial distribution of 29 neuronal, glial, and non-neuronal cell types in the chimpanzee brain, providing a first glimpse into the spatial transcriptomic and cellular organisation of the chimpanzee frontal pole. Our work provides a foundation for future comparative spatial transcriptomic studies of human and NHP brains, setting the stage for more complex comparative investigations of the molecular and cellular underpinnings of primate brain evolution.

In summary, this thesis provides an interdisciplinary framework for studying human language and brain evolution, leveraging neuroimaging and behavioural genomics, and great ape brain transcriptomics. The neuroimaging genomics investigations (***Chapters 2*** & ***3***) highlight the utility and power of large-scale neuroimaging genomics datasets in identifying the genetic bases of human neuroanatomy and uncovering the evolutionary characteristics of these genetic underpinnings. Our behavioural genomics work presents a novel bivariate extension of genomic structural equation modelling, enabling the mapping of pleiotropic landscapes of any two complex traits and exploring the evolutionary origins of putatively related or co-evolved phenotypes. Finally, we generate the first spatial transcriptomics data from a chimpanzee brain, work that establishes a robust end-to-end data generation and analysis pipeline, laying groundwork for future comparative transcriptomic investigations of primate brains.

6.2 Placing the findings in scientific context

The natural genetic and phenotypic variation observed in present-day human populations offers an invaluable resource for mapping genotype-phenotype associations. Our findings on human cortical surface area, sulcal morphology, white-matter connectivity, and language and musicality reveal novel genomic loci underlying the polygenic nature of these traits, and illuminate some of the links between human evolution and genetic underpinnings of complex human traits. By leveraging diverse data resources and analytic pipelines, this work provides evidence for the perspective of a complex relationship between genes, cells, neuroanatomy, and the evolution of human language and brain[10].

6.2.1 Contributions to the brain evolution field

Our neuroimaging genomics work (***Chapters 2*** & ***3***) uncovered associations between common genetic variants, cortical and white-matter structures, and a set of evolutionary annotations in the human genome. A key contribution of ***Chapter 2*** to the complex trait genetics field was the finding showing lack of replication for previously reported signals of recent polygenic selection on cortical surface area[1] in an independent and ancestrally homogeneous study sample of comparable size. This result aligns with earlier studies demonstrating the sensitivity of Singleton Density Score (SDS) analysis to population stratification[11]. Additionally, the absence of genetic evidence for recent selection on human cortical anatomy over the last ~2,000-3,000 years is consistent with deeper time frames for the evolution of human cortical anatomy. This view is further supported by our partially successful replication of the partitioned SNP-heritability findings by Tilot et al.[1], linking common genetic variants associated with the surface area of the pars orbitalis with foetal brain-active enhancer elements gained on the human lineage after the Old World monkey and ape branches split[12]. Our findings suggesting a deeper evolutionary past

for changes in the surface area of regions of the cortex align with views suggesting a longer time requirement for the evolution of complex human traits than those previously hypothesized.

Beyond our replication efforts, LDSC-based SNP-heritability partitioning analyses were used to test for links between the genetic underpinnings of hemispheric cortical structures and a set of evolutionary annotations of the human genome. To achieve this, we first re-assessed the suitability of the evolutionary annotations previously used by Tilot et al.[1], and attempted to curate new ones from the comparative genomics and ancient DNA literature. An important criterion for assessing the analytic suitability of an evolutionary annotation for LDSC-based partitioned heritability analysis is its respective SNP-coverage[14]. As a rule of thumb, the ratio of SNPs located within the genomic loci that are part of an annotation to the background set of SNPs in the reference panel is required to be above 1% to minimise false positive heritability enrichment/depletion results. To this end, we curated and calculated the SNP-coverage of various evolutionary annotations from recent comparative genomics and ancient DNA literature[14,15,16]. Unfortunately, we did not manage to identify any novel annotations with sufficient SNP-coverage for partitioned heritability analyses. However, we enhanced the SNP coverage of three of the previously used annotations by Tilot et al.[1], namely foetal brain human-gained enhancers, Neandertal introgressed fragments, and archaic deserts. We enhanced the SNP-coverage of these annotations by either merging biologically related ones (e.g., human-gained enhancers active at various stages of foetal brain development) or by adding SNPs in full linkage disequilibrium (r^2=1) with the originally annotated SNPs to the annotation. These refinements allowed for more statistically stable estimates of SNP-heritability enrichment/depletion estimates compared to earlier versions of these annotations, adding to the post-GWAS evolutionary analysis pipeline established by Tilot et al.[1].

Another important contribution was to investigate the evolution of cortical structures in left and right hemispheres independently, for the first time. Given the functional and anatomic left hemisphere lateralisation of language-related brain regions, such as that observed for *Broca's* and *Wernicke's* areas, this approach enabled us to probe the evolution of language-related cortical areas at hemispheric resolution. By combining our enhanced evolutionary annotations with left and right hemisphere-specific neuroimaging measure-derived GWAS summary data, we identified the aforementioned SNP-heritability enrichment signals in foetal brain human-gained enhancers for the surface area of the left hemisphere pars triangularis and pars opercularis. These findings also align with previous findings indicating anatomic divergence in the inferior frontal gyrus between macaques and humans[17], suggesting a role for noncoding genetic variants and cross-species enhancer activity changes in cortical evolution. In addition, we identified some regions with significant SNP-heritability enrichment in human-gained enhancers for both hemispheres, such as the cuneus, lingual gyrus and supramarginal cortex, regions that have been implicated in functions such as self-projection and vision. These findings are consistent with previous work showing substantial differences in the organisation of primary visual cortex across NHPs and humans[18].

In addition to SDS and SNP-heritability-based analyses, we dissected the functional and evolutionary characteristics of trait-associated loci, as in the example of the *ZIC1/ZIC4* region. Though it is a qualitative way of linking evolutionary annotations of the human genome with the genetic variants shaping complex traits, this approach can help identify putative regions and genes of interest for experimental validation studies. Our comparative genomic investigations revealed a putative hominin-specific polymorphism at a genetic variant that showed genome-

wide significant association with the surface area of left hemisphere pars triangularis. This finding exemplifies cross-species divergence of noncoding variants, potentially contributing to cortical evolution by altering *ZIC1*/*ZIC4* expression in foetal human brain tissue. On the other hand, our significant SNP-heritability depletion finding in Neandertal introgressed fragments for the left hemisphere uncinate fasciculus represents an intriguing link between *H. neanderthalensis-H. sapiens* admixture, present-day human cortical anatomy and language. Given that the left uncinate fasciculus is part of the ventral language stream and plays a role in mapping sound to meaning[19], the significant heritability depletion in Neandertal introgressed fragments may indicate a greater role for *Homo sapiens*-specific alleles in the evolution of anatomical infrastructure of language. Additionally, this finding is in line with the broader observation of depleted heritability within Neandertal introgressed fragments for a wide set of complex human traits[7]. Future work illuminating the links between heritability and evolution will help refine SNP-heritability-based analytic pipelines, and strengthen the conclusions drawn from such studies.

In ***Chapter 3***, using our established pipeline, we investigated genome-wide significant genetic variants associated with hemispheric regional sulcal structures in a present-day European population, based on a publicly available GWAS summary data set. Our significant SNP-heritability enrichment finding in foetal brain HGEs for both left and right hemisphere central sulci was particularly intriguing, given the previous findings showing no morphological lateralisation of this sulcus among primates[20]. Of note, our functional and evolutionary annotation of the genomic locus tagged by rs884370, which is a variant genome-wide significantly associated with the width of the left hemisphere central sulcus, converged with results from ***Chapter 2***. This suggests a potentially important role for this locus in human cortical evolution, which harbours multiple human-gained enhancer elements upstream of the *ZIC4*/*ZIC1* genes. Given the known role of *ZIC4* and *ZIC1* in neurogenesis[21], these findings exemplify the intricate interplay between common genetic variants, neuroanatomical traits (i.e., cortical surface area and sulcal width) and human-gained enhancers. Although the effect sizes of common genetic variants are typically insufficient to individually produce substantial phenotypic changes, this locus provides hints into how common variants may contribute to polygenic evolution via alteration of gene regulatory mechanisms.

Overall, our analytic pipeline for investigating the evolution of neuroanatomic structures using GWAS summary data represents a reliable framework for narrowing down the broad research questions regarding human brain evolution, by pointing to a set of genetic variants and evolutionary annotations that may be of particular interest. In light of the polygenic and omnigenic models of complex trait genetics and evolution[22,23], future GWASs with larger sample sizes and improved genetic association methods will likely provide deeper and more precise insights into the links between common variants and the human cortical anatomy, fueling more robust evolutionary and functional post-GWAS analyses. In the meantime, our work has demonstrated links between foetal brain human-gained enhancers and diverse cortical structures including cortical surface area, white-matter connectivity, and sulcal morphology, suggesting that human-macaque (or more broadly ape-Old World monkey) differences in epigenetic marks and gene expression programs in foetal brains might have played a critical role in human brain evolution. Moreover, our findings have converged on an intriguing link between common genetic variants associated with cortical anatomy, human-gained enhancer elements, and *ZIC1*/*ZIC4* genes. Future experimental validation studies aiming to dissect the relationship of human-gained enhancers in close proximity of these two genes using neuronal cellular and

organoid models might illuminate the putative importance of this locus in human brain evolution. Finally, some of the notable advantages of this analytic pipeline over available complex trait evolution methods[11,24,25] are as follows: i) it is based on GWAS summary-level data, facilitating human genetic data privacy- and anonymity-related issues, as well as easing data storage and processing, ii) it allows investigation of the links between a complex trait of interest and three evolutionary annotations at once, each informative about a different aspect and timeline of human evolution, and iii) the overlap analysis between evolutionary annotations and trait-associated genome-wide significant loci enables a gene- and variant-level perspective on complex trait evolution, as opposed to methods generating a single genome-wide measure of selection. However, our approach also has a number of limitations, which will be discussed further in subsequent sections of this chapter (see section 6.3.1 in particular).

6.2.2 Contributions to the language and musicality evolution field

Our genomic analysis using dyslexia and musical rhythm GWAS summary data from two recent large-scale studies[4,5] represents one of the largest multivariate GWAS investigations to date, incorporating genotype and self-reported behavioural data from over one million individuals (***Chapter 4***). By employing a diverse set of genetic and statistical tools, we investigated the intricate relationships among dyslexia and language/reading-related skills, musical rhythm, neuroanatomic traits and human evolution. This work provided the first genetic evidence for significant correlations between musical rhythm skills and dyslexia, alongside several language/reading-, education- and cognition-related traits. These findings converge with previous observations of phenotypic correlations between language- and musicality-related traits[26], and the Atypical Rhythm Risk Hypothesis, which suggests that individuals with impaired musical rhythm skills are more likely to experience language/reading difficulties[27]. For instance, we revealed significant correlations between musical rhythm skills and language/reading-related traits, including non-word repetition and phoneme awareness. These measures are known to capture aspects of expressive and receptive language abilities, further supporting hypotheses on the shared genetic and evolutionary mechanisms underlying language and musicality.

Following up on the aforementioned findings of genetic correlations among dyslexia, musical rhythm skills, and a number of other complex traits, we developed a bivariate extension of genomic structural equation modelling to identify genomic loci jointly associated with dyslexia and musical rhythm impairment. This analysis revealed 16 loci with genome-wide significant associations with both traits, uncovering a shared genetic substrate, which we named $F_{gRI\text{-}D}$. We could then go on to dive into the functional, neurobiological and evolutionary characteristics of the genetic factors shared by dyslexia and musical rhythm impairment. A particularly intriguing finding from our neurobiological analyses was a local genetic correlation signal between $F_{gRI\text{-}D}$ and connectivity of the SLF-I white-matter tract, suggesting a connection between the processes contributing to development of this tract and the shared genetic factors co-influencing reading and musical rhythm impairments.

Our evolutionary investigations of $F_{gRI\text{-}D}$ GWAS signals revealed that one of the 16 pleiotropic loci shared between dyslexia and musical rhythm impairment exhibits higher conservation among primates than any other identified loci. The lead SNP of this locus is located in the *DLAT* gene, which is implicated in a rare neurodevelopmental disorder. This finding suggests a potential role for this variant in the proper development of neuroanatomic structures underlying language/reading- and musicality-related abilities. Additionally, we uncovered an

intriguing link between $F_{gRI\text{-}D}$ and genes that have been associated with songbird vocal learning in prior work. Specifically, we identified significant enrichment of $F_{gRI\text{-}D}$-related genetic variants in a set of genes co-expressed in Area X, a brain region critical for song learning in zebra finches[28,29]. These results provide a cross-species perspective, highlighting possible evolutionary parallels between mechanisms of human language and musicality, and vocal learning in songbirds. Overall, our evolutionary findings validate the complex, polygenic evolutionary history of human language/reading- and musicality-related traits. Furthermore, they represent the first genetic evidence to inform and support empirical discussions on the potential co-evolution of language and musicality.

6.2.3 Contributions to the molecular primate brain evolution field

In ***Chapter 5*** of this thesis, we shifted our methodological approach, transitioning from genetic association studies to spatial transcriptomics. We established a comprehensive, end-to-end pipeline from post-mortem brain tissue extraction to the spatial mapping and quantification of cell types in the chimpanzee frontal pole. Considering the likely connections between cell type evolution in the brain and the cognitive specialisations of our species, shedding light on the molecular and cellular characteristics of our closest living relatives, chimpanzees, is crucial. Our work offers insights into the cellular and molecular landscapes of the chimpanzee brain, paving the way for a deeper and more comprehensive understanding of human brain evolution.

With this in mind, we performed spatial transcriptomics on a chimpanzee left hemisphere frontal pole sample in collaboration with the *Evolution of Hominoid Brain Connectomics* (EBC) Consortium, who have assembled one of the largest primate brain banks in the world. The main contributions of this chapter to the human brain evolution field are as following: i) generating and analysing the first set of spatial transcriptomics data from a great ape brain sample, ii) applying single nucleus-spatial transcriptomics integrative analytic approaches to chimpanzee brain-derived datasets for the first time, iii) spatially annotating 29 transcriptomically identified neuronal, glial and non-neuronal cell subtypes, and uncovering the transcriptomic organisation of the chimpanzee frontal pole. Moreover, using standard histological methods and image analysis, we quantified the cell density in chimpanzee brain sections and compared these findings with existing human brain-derived data. This comparison revealed a relatively lower cell density in chimpanzees than in humans. Immunostainings with canonical deep and upper layer neuronal markers, such as TBR1 and BRN2/POU3F2, indicated varying levels of layer specificity. However, more extensive research is needed to draw clearer conclusions about the validity and applicability of human brain markers in chimpanzee brains.

Overall, this chapter makes a substantial contribution to the field of primate brain evolution by presenting a comprehensive pipeline that spans from brain tissue acquisition to the generation and analysis of spatial transcriptomic data. In conjunction with ongoing efforts to create cellular atlases of adult human and NHP brains[30,31], our findings and workflow establish a foundation for future comparative studies in primate brain spatial transcriptomics.

6.3 Limitations and potential sources of bias

6.3.1 Biases in GWAS and post-GWAS evolutionary studies

GWAS methods leveraging large population cohorts have a number of limitations. A primary issue is the overrepresentation of individuals of European ancestry, which can drive ancestry-specific genetic findings and hinder drawing species-wide conclusions. Additionally, allele

frequency and linkage disequilibrium differences across genetic ancestries can lead to population- or ancestry-specific SNP-trait associations and SNP-heritability estimates, reducing the transferability of polygenic risk scores across ancestries[32]. To address this, diverse databases such as Biobank Japan[33] (established in 2003 as a clinical database) and Mexican Biobank[34] (established in 2023 as a nation-wide genotype-phenotype database), as well as SNP reference panels and genotyping arrays for diverse genetic ancestries represent promising steps towards a more comprehensive understanding of human genetic variation at the species-level. Another challenge is that population stratification, which is systematic genetic and environmental structure within human populations, can cause false-positive SNP-trait associations[35,36]. There are a number of potential drivers of population stratification such as cryptic relatedness (i.e., unknown close genetic relatedness among the individuals within the study sample)[37], assortative mating (i.e., non-random choice of partners, specifically based on having a similar phenotype)[38], and dynastic effects (i.e., intergenerational transmission of social and economic resources, creating privileged individuals)[39]. Among these factors, the impacts of socioeconomic status-driven population stratification can be empirically observed in large-scale datasets such as the UK Biobank[40,41], as it leads to secondary biases such as the participation bias in volunteer-based datasets. Although current population GWAS designs integrate a number of genetic principal components to GWAS regression models to mitigate this issue[42], residual confounding often persists[43], impacting GWAS and post-GWAS evolutionary analyses. Third, a limitation specific to ***Chapters 2*** and ***3***, which are based on the UK Biobank neuroimaging genomics dataset, is that the UK Biobank is not representative of the UK population[40,41]. As this is a dataset comprising data from volunteers, the dataset is skewed towards older and female individuals with higher socioeconomic status. This bias particularly limits drawing evolutionary and species-wide conclusions from research findings based on the UK Biobank study sample. Fourth, a limitation specific to ***Chapter 4*** is the reliance on GWAS summary data from self-reported behavioural traits collected by an ancestry testing company (23andMe, Inc.). While the ease of data collection via self-report increases sample sizes and statistical power to detect SNP-trait associations, the self-reported and -assessed nature of phenotypes introduces ambiguity into data measurement and reliability, limiting their precision for genetic and evolutionary studies. Fifth, GWASs focus on common variants and short DNA insertions/deletions, missing contributions from rare and structural DNA variants, which are critical to resolve the "missing heritability" issue[44]. Emerging fields such as GWAS-by-whole genome and GWAS-by-exome sequencing are making substantial contributions to addressing this limitation[45]. Finally, behavioural and social traits are particularly sensitive to environmental confounds[36], as reflected in greater differences between population- and family-GWAS-based SNP-heritability estimates[36], and lower predictive accuracy of polygenic scores for such traits[46]. Overall, for robust insights into complex trait genetics and reliable post-GWAS evolutionary analyses, these limitations must be addressed. Advances in understanding gene-gene[47] and gene-environment interactions[48], within sibship effects[49], dynastic effects[50], and causal inference methods[51] are promising for the future of the field. These developments have the potential to reduce the prevalence of "just-so stories"[52] in the GWAS field, and strengthen the biological and evolutionary interpretations derived from genome-wide association studies.

Similarly, there are a number of limitations to the complex trait evolution methods used in this thesis. First, given the known sensitivity of Singleton Density Score (SDS) analysis to population stratification[53,54], we aimed to minimise the influence of residual population stratification by performing GWASs and SDS analysis on a relatively ancestrally homogeneous subset of

individuals in the UK Biobank (White British participants). After taking these precautions, our targeted replication study in ***Chapter 2***, which aimed to validate previous findings of recent selection regarding cortical surface area measures reported by Tilot et al.[1], was not successful. Secondly, we frequently used LDSC-based SNP-heritability partitioning analysis to investigate the links between a set of evolutionary annotations of the human genome, and the genetic underpinnings of human neuroanatomy. While this approach provided intriguing insights, it requires larger sample sizes and higher statistical power to produce SNP-heritability enrichment/depletion estimates with greater statistical confidence (i.e., reduced standard errors). The lack of statistical power of population GWASs may hinder the detection of many plausible SNP-heritability enrichment/depletion signals. Additionally, the scope of the evolutionary annotations curated and used in this thesis does not cover all genomic loci that are relevant for human language and brain evolution. However, the continual growth of neuroimaging genomics datasets suggests that well-powered studies conducted in relatively genetically homogeneous population cohorts will be possible in the near future. This will likely enable more stable SNP-heritability enrichment/depletion estimates. Similarly, the fast pace of advances in ancient DNA research, and the increasing quality and quantity of available NHP reference genomes fuel comparative genomics and hominin evolution studies, holding great promise for the availability of more precise and comprehensive evolutionary genomic annotations over the next decade. Third, investigation of the links between genomic fragments introgressed from archaic humans (e.g., Neandertals, Denisovans) into *Homo sapiens* genomes and phenotypic variability in present day humans is particularly challenging, because it is known that the introgressed alleles are often subject to purifying selection and are found in low frequencies across present day populations[55], making them invisible to methods such as GWAS. Finally, it is important to note that genetic variants with large effects on human neuroanatomy likely have reached fixation across human populations due to strong selective constraints. Such variants, being common to all members of our species, are not polymorphic and therefore cannot be detected by genetic association studies. This limitation highlights the need for complementary approaches, such as genomic and functional investigations comparing humans and NHPs, to identify genetic changes that are shared across all humans and may have played crucial roles during our speciation.

6.3.2 Limitations of primate brain spatial transcriptomics investigations

Studying gene expression in primate brains presents numerous methodological and sample acquisition-related challenges and limitations. The most significant limitation is the extreme rarity of high-quality captive and wild NHP brain samples, a consequence of the global decline in primate populations[56]. Acquiring NHP brain samples at earlier developmental stages is particularly difficult due to ethical and conservation reasons, which impedes efforts to identify critical cross-species differences in neurodevelopmental programmes. This issue results in small sample sizes for comparative primate brain transcriptomics studies, often limited to data from a few species, individuals and brain regions. Consequently, these studies face statistical power limitations, making it challenging to derive species-general biological conclusions. However, despite their own limitations[57], neural cellular and organoid models are valuable alternatives, enabling the investigation of early stages of human and NHP neurodevelopment and providing access to the gene expression profiles of cell types unique to early neurodevelopmental stages. Secondly, many NHP species lack good-quality reference genomes, genome annotations and reference transcriptomes. This issue complicates transcriptomic data analysis, and obstructs such analyses for some understudied primate species. The recent availability of genome

assemblies for hundreds of primate species and improvements in the quality of NHP reference genomes due to advances in long-read DNA sequencing technologies are promising developments[58]. Third, the lack of standardised protocols for brain sample extraction and tissue freezing introduces technical variability among studies. Combined with the limited sample sizes in primate transcriptomics research, this variability makes it challenging to distinguish between inter-individual, inter-study and cross-species differences. A recent study has proposed a comprehensive pipeline aimed at improving tissue quality and standardising the workflow to address these challenges[59]. Finally, there is a strong bias towards studying the molecular properties of the cortex, given its suggested role in many human cognitive specialisations and higher-order behaviours. However, recent research has highlighted the interplay between cortical and subcortical regions, as well as cerebellum, in orchestrating complex cognitive functions such as language[60]. Ongoing initiatives, such as efforts by the Allen Institute to create transcriptomic and cellular maps of human and NHP brains, hold great promise for identifying the genetic and molecular bases of human brain specialisations.

6.4 Future prospectives and suggestions for the field

Recent advances in the GWAS field, including improved genotyping arrays, larger SNP panels, and the ever-increasing sample sizes via large-scale collaborative efforts and biobanks, have greatly enhanced the precision of GWAS effect size estimates and the statistical power to detect SNPs with larger effects[61]. Assuming the field will continue moving forward at an increasing pace over the next decades, on both the technological and data analytic side, the complex trait genetics field will likely produce human genotype-phenotype datasets with better quality and in higher quantity. This may enable the generation of "saturated maps" of common variants shaping many complex phenotypes, similar to what has been achieved for human height[62]. If combined with improvements in GWAS design, the current gap between common and rare genetic variant studies may start to close, as a much broader set of genetic variants with diverse allele frequencies and effect sizes can be detected and studied. Combined with these advances in genetic association studies, the complex trait evolution field also holds promise to shed light on the complex web of interactions between genetic variants, genes, molecular mechanisms, cells and complex phenotypes. The availability of novel sophisticated statistical tools leveraging GWAS summary data[63,64] will enable investigations beyond SNP-heritability-based explorations.

However, I believe there are several critical points that should be considered and addressed in the complex trait genetics field. Given that the demographic, social and biological sources of population stratification can vary substantially across populations and complex traits (e.g., morphometric, disease and behavioural traits), I propose that population- and trait-specific GWAS designs are needed. These "tailored study designs" would better account for potential confounders, and minimise the absorption of environmental effects into GWAS effect estimates due to their specificity to unique sources of confounding. Based on the findings presented in this thesis, I suggest the following measures to address these limitations and to improve the fields of complex trait genetics and evolution: i) incorporating socioeconomic status as a standard covariate in population GWASs, alongside commonly used covariates such as age and sex, ii) increasing the sample sizes of genotype and phenotype data derived from families, and using family GWAS as the primary approach to study the genetics of complex traits, iii) enhancing GWAS methodologies to better disentangle direct and indirect genetic effects, more effectively account for environmental confounders, and develop methods to investigate causal relationships between genetic variants and phenotypes, iv) standardising and improving the

objectivity of phenotype measurements while scaling up sample sizes, and v) promoting ethical and scientific discussions within the GWAS community regarding the scientific and social value of genetic investigations of social and cultural phenomena/concepts (e.g. intelligence, race, criminality, political tendencies), with an emphasis on discussions over the potential benefits and harms of this type of research for the society. The fifth suggestion may seem out of the context of this thesis, yet I believe it is particularly relevant to the ***Chapter 4*** of this thesis, as that chapter focusses on the genomics and evolution of behavioural traits. The risk of misuse and misinterpretation of GWAS findings by politically motivated groups or individuals[65] poses significant risks with potential political, social and historical implications[66]. This risk is amplified by research designs that are poorly conceived, and are not well planned before execution. Despite the good intentions of most researchers in the complex trait genetics field, continuous caution is necessary to avoid causing harm to the public or enabling the exploitation of research findings. Given the historical burden and legacy of sociobiology[67] and phrenology[68], the fields of behavioural and neuroimaging genomics are especially vulnerable to such threats.

As for the next steps in comparative transcriptomics studies in primate brains, increasing primary brain tissue sample sizes via the continuous expansion of primate brain banks is promising. However, the fast-declining populations of wild primates creates serious concerns regarding the conservation of wildlife and biodiversity, and consequently hinder NHP brain research relying on primary brain samples. Recent technological innovations have also enabled the generation of spatial omics data from formalin-fixed brain tissues, in addition to standard methods focussing on fresh-frozen brain samples[69]. These novel technologies enable researchers to investigate larger collections of archived brain samples, and contribute to resolving the NHP brain sample availability problem. Future comparative omics studies spanning foetal, developmental, and adult stages of primate brains will likely illuminate the molecular divergence and conservation underlying the spatial organisation of cell types and transcriptomic profiles. The ongoing brain atlasing efforts for the developing and adult human and NHP brains are great examples of the next generation of large-scale brain omics investigations, deepening our understanding of the cell types that constitute the brain[30,31,70], by revealing the molecular and cellular maps of the brain at a resolution that has never before been possible. Furthermore, advances in neural organoid methods, including various types such as cerebral, cerebellar and striatal neural organoids, and the recent availability of neural assembloids resembling multiple regions of the human brain, offer promising model systems. However, these model systems come with their inherent limitations too[57]. Finally, the recent and ongoing efforts to improve primate genome assembly qualities using novel long-read sequencing technologies, and their enhanced functional annotations, might facilitate comparative transcriptomic analyses across primates. Hence, the improvement of primary brain tissue acquisition methods from across developmental stages and adulthood, and the struggle against the decrease of NHP populations remain critical tasks for advancing molecular studies of human and NHP brain evolution.

6.5 Towards an integrative study of human brain evolution

This thesis encompasses independent studies in neuroimaging and behavioural genomics, as well as chimpanzee brain spatial transcriptomics, with a particular emphasis on human language and brain evolution. However, it does not explicitly aim to integrate these disciplines. Here, I would like to explore two potential avenues for combining these diverse approaches to investigate human brain evolution more comprehensively.

One well-established integrative approach bridges two data modalities used in this thesis: neuroimaging and transcriptomics[71]. Imaging transcriptomics employs correlative analyses to identify gene expression patterns that align with structural variation in neuroanatomic parameters, such as cortical surface area and cortical thickness[72,73]. The availability of comprehensive brain-wide transcriptomic atlases (e.g., the Allen Brain Atlas), and large-scale neuroimaging datasets (e.g., UK Biobank) has fueled the advancement of this integrative method. While it is commonly applied to human brain-derived data, a notable recent study extended this innovative approach to macaque brains, yielding intriguing insights into macaque neurobiology[74]. In this study, researchers generated a gene expression atlas of the cynomolgus macaque brain ($N_{brain\ regions}$=110, $N_{macaques}$=9) and combined it with structural MRI data from over 160 macaques to examine associations between gene expression and cortical thickness. This integrative analysis identified hundreds of genes associated with cortical thickness, many of which were enriched in neurons and oligodendrocytes[74]. Furthermore, the study revealed a spatially varying gene expression gradient along the dorsolateral-ventromedial axis of the macaque frontal pole, which is suggested to parallel the anatomically identified sensorimotor-to-transmodal gradient in the human cortex[74,75]. Future studies expanding on this approach hold promise for unravelling the complexities of human and NHP neurobiology, enabling multi-modal comparative studies. Given the substantial difficulties in comparative brain transcriptomics studies that are not informed by comparative neuroanatomy, such as challenges in precisely identifying homolog brain regions across species, multi-modal methods combining various lines of brain-derived molecular data represent powerful ways of advancing investigations of primate brain evolution[75,76,77].

Another integrative approach would be bridging genetic association methods with primate genomics data, and applying various genetic association methods to NHP populations. Recent advancements in primate genomics using short-read DNA sequencing have provided deep profiles of genetic variation in approximately 47% of extant primate species, substantially enhancing the quality of reference genome assemblies[78]. These advances have enabled investigations of primate phylogeny, mutation rates and the effects of pathogenic mutations in primates[78,79]. As the size of primate genetic variation datasets continue to grow, sufficiently powered genetic association studies in wild and captive primate populations may soon become feasible. Such studies would allow for the assessment of genotype-phenotype associations and quantitative trait loci profiles within species-specific genomic backgrounds, providing valuable insights into the phenotypic and functional consequences of common and rare variants across species. Two notable public datasets containing macaque genetic and phenotypic data have recently become available[80,81], marking promising initial steps toward genetic association studies in NHPs. The *macaque Genotype And Phenotype* resource (mGAP)[80] includes whole-genome sequencing data and a variety of phenotypic records from 293 rhesus macaques (N_{SNPs}=17,087,212), offering individual-level genotype data for allele carrier look-ups. On the other hand, Wang et al.[81] presents targeted-sequencing and phenotype data from 1,845 rhesus macaques (N_{SNPs}>47,000). By applying a machine learning algorithm and combining macaque and human allele frequencies, the researchers developed a new missense variant pathogenicity score that outperforms existing methods[81]. In summary, the improved cataloguing of NHP genetic variants, along with subsequent well-powered genotype-phenotype mapping in NHP populations, has the potential to i) cross-validate genetic association findings from human studies across species, ii) uncover species- and genomic background-specific functional consequences of genetic variants, iii) broaden our understanding of the molecular processes

underlying disease biology by providing insights from primate models closely related to humans, and iv) illuminate the genetic and molecular divergence mechanisms that drive speciation.

6.6 Conclusion

In conclusion, this thesis identified novel associations between common genetic variants and various aspects of human neuroanatomy at global, hemispheric and regional levels, focussing on cortical surface area, white-matter connectivity, and sulcal morphology. Our evolutionary analyses leveraging neuroimaging phenotype-derived GWAS summary data, uncovered intriguing SNP-heritability distribution patterns for certain cortical structures, diverging from the neutral assumption of full polygenicity. In particular, we found a repetitive pattern of links between a set of regional cortical structures, foetal brain human-gained enhancers, and neurodevelopmentally important *ZIC1* and *ZIC4* genes. Additionally, we took a first step to identify the shared genetic substrate of dyslexia and musical rhythm impairment, revealing 16 genome-wide significant pleiotropic loci. We also identified genome-wide and local genetic correlations among language- and musical rhythm-related traits, white-matter structures, and a set of behavioural traits. These findings support previous reports of phenotypic correlations between language- and musicality-related traits[26]. We then explored molecular brain evolution using spatial transcriptomics, a powerful and newly emerging method[82]. This approach enabled the generation of the first set of spatial gene expression data from a chimpanzee brain, shedding light on the transcriptomic and cellular organisation of the chimpanzee frontal pole. While this pilot study provided intriguing insights, it primarily serves as a foundational effort for future comparative spatial transcriptomics investigations in human and NHP brains.

On a final note, understanding how the human brain has evolved is an exceptionally challenging task, but the value of uncovering a potential answer to this question makes the effort worthwhile. The success of this scientific goal relies on formulating precise, literature-informed and goal-oriented research questions, investing resources in methodologies that are most likely to address the field's pressing research questions, and fostering well-coordinated collaborations across scientific disciplines. I believe such an integrative approach holds great promise to unravel the complexities of human brain evolution, and its underlying molecular and genetic mechanisms.

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Appendices

English summary

The evolution of complex language and human-specific aspects of brain anatomy occurred within the last ~7-9 million years, since the last common ancestral population of humans and chimpanzees. The importance of genetic contributions to human language acquisition, coupled with the tripling of human brain size and substantial reorganisation of neural architecture during this evolutionary period, suggests potential co-evolution of these traits.

As detailed in ***Chapter 1***, this thesis explores the genetic and evolutionary bases of human brain and language using neuroimaging genomics, behavioural genetics, and primate brain transcriptomics. While the six-layered mammalian cortical architecture is broadly conserved, human brain specialisations underlying complex language and higher-order cognitive skills likely stem from cross-species differences in neurodevelopmental gene expression programmes and cell type organisation. Identifying genetic changes driving novel phenotypes, which can consequently contribute to speciation, is crucial for cross-species evolutionary genetic investigations. Natural genetic and phenotypic variation in present-day human populations offers a valuable resource, enabling genotype-phenotype mappings via sophisticated genetic association methods. In the context of human language and brain evolution, genome-wide association studies (GWAS) leveraging large-scale neuroimaging and behavioural datasets stand out as a helpful method. Post-GWAS evolutionary analyses can illuminate the intricate links across genetic variants, genes, molecular pathways, cells, neurobiology, behaviour, and the evolutionary pressures acting on various levels of this chain. On the other hand, spatial transcriptomic investigations on primate brains allow the identification of gene expression and cell type organisation in the brain, providing valuable resources for future comparative transcriptomic work between humans and non-human primates (NHP).

In ***Chapter 2***, we first investigated the genomic architecture of cortical surface area and white-matter connectivity using inter-individual variability in genotypes and phenotypes. Building on a previously established post-GWAS evolutionary analysis framework, we examined the genomic signatures of recent and deeper evolution, using diverse methods and evolutionary genomic annotations focussing on different periods during human prehistory. Our targeted replication study showed that a previous finding, indicating recent selection human cortical anatomy over the last ~2,000-3,000 years, is not replicable when population stratification is relatively accounted for, which is a known confounder for investigation of recent selection. On the other hand, we replicated a previous finding showing a genetic link between pars orbitalis, a key area for language processing, and human-gained enhancer elements, which are gene regulatory elements that are active in foetal human brain tissue, whereas inactive in macaques foetuses. We then performed GWASs for numerous hemispheric and regional cortical surface area and white-matter tract phenotypes using a large-scale neuroimaging genomics dataset from the UK Biobank. Our approach was particularly relevant to study the genetics of neuroanatomic structures underlying language, given the left hemisphere lateralisation of language-related functions. Using this approach, we identified significant SNP-heritability enrichment in human-gained enhancers for the left hemisphere pars triangularis and pars opercularis, indicating a significant contribution of these enhancer elements to the evolution and development of language-related cortical regions. Additionally, we identified a significant SNP-heritability depletion in Neandertal introgressed fragments for the left hemisphere uncinate fasciculus, a white-matter tract involved in mapping sounds to meaning. This finding

suggests a significantly reduced contribution of Neandertal introgressed variants to the heritability of the left uncinate fasciculus, inviting further research on the evolution of this white-matter structure. Finally, we zeroed in on an exemplary genomic locus that harbours a genetic variant associated with variation in the left hemisphere pars triangularis, a number of human-gained enhancer elements, and the neurogenesis-related genes *ZIC1* and *ZIC4*. Despite the low effect sizes of individual common variants, by dissecting the functional and evolutionary characteristics of this locus, we shed light on potential mechanisms through which non-coding variants might influence complex trait evolution.

In ***Chapter 3***, we extended our post-GWAS evolutionary work on neuroimaging phenotypes to sulcal morphology, using publicly available GWAS summary data. We investigated the links between common genetic variants, hemispheric and regional sulcal morphology measures, and evolutionary annotations of the human genome. Our analyses revealed significant SNP-heritability enrichment in human-gained enhancers for the widths of left and right hemisphere central sulcus, a structure that separates primary motor and primary somatosensory cortices. Notably, we found that a left hemisphere central sulcus width-associated genetic variant was localised in a human-gained enhancer element nearby the *ZIC1*/*ZIC4* genes. These results, taken together with our findings from ***Chapter 2***, suggest a consistent link across common genetic variants associated with a set of cortical structures, human-gained enhancers, and neurodevelopmentally relevant genes such as *ZIC1* and *ZIC4*.

In ***Chapter 4***, we focussed our efforts on investigating the genetic architecture and evolution of human language/reading- and musicality-related traits. We first tested whether previously observed phenotypic correlations between language/reading- and musical rhythm-related traits are observable also at the genetic level. Our genetic correlation analyses revealed significant correlations between musical rhythm and diverse language/reading traits, such as word reading, non-word repetition, and dyslexia. Next, we adopted a bivariate extension of genomic structural equation modelling to perform a multivariate GWAS of dyslexia and musical rhythm impairment, which identified 16 genomic loci jointly associated with the two traits. Our post-multivariate GWAS analyses revealed genetic links between the shared genetic factor underlying the two traits, and a set of neuroanatomical, psychiatric, and behavioural traits. An intriguing finding was the local genetic correlation between the shared genetic factor and the connectivity of superior longitudinal fasciculus-I, a white-matter tract involved in the motor aspects of language. Our evolutionary investigations on the shared genetic factor identified significant SNP-heritability enrichment in primate-conserved loci, and depletion in Neandertal introgressed fragments, a pattern that is shared across many complex human traits. Finally, we zeroed-in on an example locus that contains a common variant associated with the shared genetic factor of dyslexia and musical rhythm impairment, which is highly conserved among primates. This variant is located on the *DLAT* gene, which is reported to be linked to a neurodevelopmental disorder affecting learning and cognition.

In ***Chapter 5***, we took a different approach to study human brain evolution by pioneering the application of spatial transcriptomics (Visium, 10x Genomics) to chimpanzee brain tissue. This chapter assessed the feasibility of applying this cutting-edge technique to fresh-frozen chimpanzee brain tissue, paving the way for future studies comparing spatial gene expression and cell type organisation across humans and NHPs. To test this, we collaborated with experts in primatology and neuroscience, and obtained high-quality left hemisphere frontal pole brain

tissue from a female chimpanzee, *Takki*, euthanised for health reasons unrelated to our project. Following the tissue extraction and freezing performed by our collaborators, we generated Visium spatial transcriptomics data from four sections obtained from the frontal pole tissue samples. These data were then integrated with a publicly available single-nucleus RNA-sequencing data set derived from chimpanzee dorsolateral prefrontal cortex. Our integrative analysis allowed us to map the abundance and spatial distribution of 29 transcriptomically identified neuronal, glial, and non-neuronal cell types in the chimpanzee frontal pole. This chapter offers a first glimpse at the spatial organisation of cell types and gene expression within the chimpanzee frontal pole. In parallel, by demonstrating the feasibility of Visium spatial transcriptomics in primate brain tissue, it establishes a solid methodological ground for future comparative spatial transcriptomics studies.

Overall, this thesis present various lines of data shedding light on diverse aspects of human brain and language evolution, as summarised in ***Chapter 6***. While our findings are useful for narrowing down the broad and speculative debates over human brain evolution, we do not claim to present any decisive conclusions on human brain evolution. Instead, we hope that our findings will help refine the existing research questions in the field, and open new avenues for future multi-modal comparative investigations across NHP brains.

Nederlandse samenvatting

De evolutie van complexe taal en mens-specifieke aspecten van hersenanatomie vond plaats in de laatste ~7-9 miljoen jaar, sinds de laatste gemeenschappelijke voorouderlijke populatie van mensen en chimpansees. Het belang van genetische bijdragen aan de menselijke taalverwerving, gekoppeld aan de verdrievoudiging van de menselijke hersenomvang en substantiële reorganisatie van de neurale architectuur gedurende deze evolutionaire periode, suggereert een mogelijke co-evolutie van deze eigenschappen.

Zoals beschreven in ***Hoofdstuk 1***, onderzoekt dit proefschrift de genetische en evolutionaire basis van het menselijk brein en taal met behulp van neuro-imaging genomics, gedragsgenetica en transcriptomics van primatenhersenen. Terwijl de zes-lagige corticale architectuur van zoogdieren in grote lijnen behouden is, komen de menselijke hersenspecialisaties die ten grondslag liggen aan complexe taal en cognitieve vaardigheden van hogere orde waarschijnlijk voort uit verschillen tussen soorten in neurologische genexpressieprogramma's en celtype organisatie. Het identificeren van genetische veranderingen die nieuwe fenotypes aansturen, die vervolgens kunnen bijdragen aan soortvorming, is cruciaal voor evolutionair genetisch onderzoek naar verschillende soorten. Natuurlijke genetische en fenotypische variatie in hedendaagse menselijke populaties biedt een waardevolle bron, die genotype-fenotype mappings via geavanceerde genetische associatiemethoden mogelijk maakt. In de context van menselijke taal- en hersenevolutie zijn genoomwijde associatiestudies (GWAS) die gebruik maken van grootschalige neuro-imaging en gedragsdatasets een nuttige methode. Evolutieanalyses na GWAS kunnen de ingewikkelde verbanden tussen genetische varianten, genen, moleculaire routes, cellen, neurobiologie, gedrag en de evolutionaire druk die op verschillende niveaus van deze keten inwerkt, belichten. Aan de andere kant maakt ruimtelijk transcriptoomonderzoek van primatenhersenen het mogelijk om genexpressie en celtypeorganisatie in de hersenen te identificeren, wat waardevolle bronnen oplevert voor toekomstig vergelijkend transcriptoomonderzoek tussen mensen en niet-menselijke primaten (NHP).

In ***Hoofdstuk 2*** onderzochten we eerst de genomische architectuur van corticale oppervlakte en witte-materie connectiviteit met behulp van inter-individuele variabiliteit in genotypes en fenotypes. Voortbouwend op een eerder vastgesteld post-GWAS evolutionair analyse raamwerk, onderzochten we de genomische signaturen van recente en diepere evolutie, gebruikmakend van diverse methoden en evolutionaire genomische annotaties gericht op verschillende perioden tijdens de prehistorie van de mens. Onze gerichte replicatiestudie toonde aan dat een eerdere bevinding, die wijst op recente selectie van de menselijke corticale anatomie gedurende de laatste ~2,000-3,000 jaar, niet repliceerbaar is wanneer populatiestratificatie relatief in rekening wordt gebracht, wat een bekende verstoorder is voor onderzoek naar recente selectie. Aan de andere kant hebben we een eerdere bevinding herhaald die een genetisch verband aantoonde tussen pars orbitalis, een belangrijk gebied voor taalverwerking, en humaan-verworven enhancerelementen, wat genregulerende elementen zijn die actief zijn in foetaal menselijk hersenweefsel, terwijl ze inactief zijn in makakenfoetussen. Vervolgens voerden we GWASs uit voor talrijke hemisferische en regionale corticale oppervlakte en witte-materie tractypes met behulp van een grootschalige neuroimaging genomics dataset van de UK Biobank. Onze aanpak was met name relevant voor het bestuderen van de genetica van neuroanatomische structuren die ten grondslag liggen aan

taal, gezien de lateralisatie van taalgerelateerde functies in de linker hersenhelft. Met behulp van deze aanpak identificeerden we een significante SNP-erfbaarheid verrijking in menselijke enhancers voor de linker hemisfeer pars triangularis en pars opercularis, wat duidt op een significante bijdrage van deze enhancer elementen aan de evolutie en ontwikkeling van taal-gerelateerde corticale gebieden. Daarnaast identificeerden we een significante SNP-erfbaarheid depletie in Neandertaler introgressed fragmenten voor de linker hemisfeer uncinate fasciculus, een witte-materie tractus betrokken bij het in kaart brengen van geluiden naar betekenis. Deze bevinding suggereert een significant verminderde bijdrage van Neandertaler introgressievarianten aan de overerfbaarheid van de linker uncinate fasciculus en nodigt uit tot verder onderzoek naar de evolutie van deze witte-materie structuur. Tenslotte hebben we ons gericht op een voorbeeld genomische locus die een genetische variant herbergt die geassocieerd is met variatie in de linker hemisfeer pars triangularis, een aantal door de mens gewonnen enhancer elementen en de neurogenese gerelateerde genen *ZIC1* en *ZIC4*. Ondanks de lage effectgroottes van individuele gemeenschappelijke varianten, hebben we door het ontleden van de functionele en evolutionaire karakteristieken van deze locus, licht geworpen op potentiële mechanismen waardoor niet-coderende varianten de evolutie van complexe eigenschappen zouden kunnen beïnvloeden.

In ***Hoofdstuk 3*** hebben we ons post-GWAS evolutionaire werk aan neuroimaging fenotypes uitgebreid naar sulcale morfologie, met behulp van publiek beschikbare GWAS samenvattende gegevens. We onderzochten de verbanden tussen veel voorkomende genetische varianten, hemisferische en regionale morfologische maten van de sulcus en evolutionaire annotaties van het menselijk genoom. Onze analyses toonden een significante SNP-erfelijkheid verrijking aan in bij de mens gevonden enhancers voor de breedte van de centrale sulcus in de linker- en rechterhersenhelft, een structuur die de primaire motorische en primaire somatosensorische cortex scheidt. In het bijzonder vonden we dat een genetische variant die geassocieerd is met de breedte van de centrale sulcus in de linker hersenhelft gelokaliseerd was in een humaan-verworven enhancerelement in de buurt van de *ZIC1*/*ZIC4*-genen. Deze resultaten, samen met onze bevindingen uit ***Hoofdstuk 2***, suggereren een consistent verband tussen gemeenschappelijke genetische varianten die geassocieerd zijn met een set van corticale structuren, humaan-verworven enhancers en neurologisch relevante genen zoals *ZIC1* en *ZIC4*.

In ***Hoofdstuk 4*** hebben we onze inspanningen gericht op het onderzoeken van de genetische architectuur en evolutie van menselijke taal/lees- en muzikaliteit-gerelateerde eigenschappen. We hebben eerst getest of eerder waargenomen fenotypische correlaties tussen taal/lees- en muziekritme-gerelateerde eigenschappen ook op genetisch niveau waarneembaar zijn. Onze genetische correlatieanalyses lieten significante correlaties zien tussen muzikaal ritme en diverse taal/leeskenmerken, zoals woordlezen, niet-woordherhaling en dyslexie. Vervolgens gebruikten we een bivariate uitbreiding van genomic structural equation modelling om een multivariate GWAS van dyslexie en muzikale ritmestoornissen uit te voeren, die 16 genomic loci identificeerde die gezamenlijk geassocieerd waren met de twee eigenschappen. Onze post-multivariate GWAS analyses onthulden genetische verbanden tussen de gedeelde genetische factor die ten grondslag ligt aan de twee kenmerken, en een set van neuroanatomische, psychiatrische en gedragsmatige kenmerken. Een intrigerende bevinding was de lokale genetische correlatie tussen de gedeelde genetische factor en de connectiviteit van de superieure longitudinale fasciculus-I, een witte-materie tractus die betrokken is bij de motorische aspecten van taal. Ons evolutionair onderzoek naar de gedeelde genetische factor

identificeerde een significante verrijking van SNP-erfbaarheid in primaat-geconserveerde loci en depletie in Neandertaler introgressieve fragmenten, een patroon dat gedeeld wordt in veel complexe menselijke eigenschappen. Tenslotte hebben we ons gericht op een voorbeeld locus die een gemeenschappelijke variant bevat die geassocieerd is met de gemeenschappelijke genetische factor van dyslexie en muzikale ritme stoornissen, die sterk geconserveerd is onder primaten. Deze variant bevindt zich in het *DLAT*-gen, waarvan bekend is dat het gekoppeld is aan een neurologische ontwikkelingsstoornis die invloed heeft op leren en cognitie.

In ***Hoofdstuk 5*** hebben we een andere benadering gekozen om de evolutie van het menselijk brein te bestuderen door als eerste ruimtelijke transcriptomics (Visium, 10x Genomics) toe te passen op hersenweefsel van chimpansees. Dit hoofdstuk beoordeelde de haalbaarheid van het toepassen van deze geavanceerde techniek op vers ingevroren hersenweefsel van chimpansees, waarmee de weg werd vrijgemaakt voor toekomstige studies waarin ruimtelijke genexpressie en celtype organisatie tussen mensen en NHP's werd vergeleken. Om dit te testen, werkten we samen met experts in primatologie en neurowetenschappen en verkregen we hersenweefsel van hoge kwaliteit van de linker hemisfeer van de frontale pool van een vrouwelijke chimpansee, *Takki*, die geëuthanaseerd werd om gezondheidsredenen die geen verband hielden met ons project. Na de weefselextractie en het invriezen door onze medewerkers, genereerden we Visium spatial transcriptomics gegevens van vier secties verkregen uit de frontale pool weefselmonsters. Deze data werden vervolgens geïntegreerd met een publiek beschikbare single-nucleus RNA-sequencing dataset afkomstig van chimpansee dorsolaterale prefrontale cortex. Onze integratieve analyse stelde ons in staat om de overvloed en ruimtelijke distributie van 29 transcriptomisch geïdentificeerde neuronale, gliale en niet-neuronale celtypen in kaart te brengen in de frontale pool van de chimpansee. Dit hoofdstuk biedt een eerste blik op de ruimtelijke organisatie van celtypen en genexpressie in de frontale pool van de chimpansee. Door de haalbaarheid van Visium spatial transcriptomics in primatenhersenweefsel aan te tonen, wordt een solide methodologische basis gelegd voor toekomstige vergelijkende spatial transcriptomics studies.

Over het geheel genomen presenteert dit proefschrift verschillende lijnen van gegevens die licht werpen op verschillende aspecten van de evolutie van het menselijk brein en taal, zoals samengevat in ***Hoofdstuk 6***. Onze bevindingen zijn nuttig voor het beperken van het aantal aspecten van de evolutie van het menselijk brein en taal. Hoewel onze bevindingen nuttig zijn voor het beperken van de brede en speculatieve debatten over de evolutie van het menselijk brein, pretenderen we niet om beslissende conclusies te presenteren over de evolutie van het menselijk brein. In plaats daarvan hopen we dat onze bevindingen zullen helpen bij het verfijnen van de bestaande onderzoeksvragen in het veld, en nieuwe wegen zullen openen voor toekomstig multi-modaal vergelijkend onderzoek naar hersenen van mensen met een NHP.

Biography

Gökberk Alagöz was born in İstanbul, Türkiye, on March 17, 1994. After completing his Bachelor of Science degree in Molecular Biology and Genetics at Bilkent University, Ankara, in 2017, he moved to Munich, Germany, for his Master of Science studies. He earned his Master's degree in Evolution, Ecology, and Systematics at Ludwig Maximilians University in Munich (LMU) in 2019. He performed two research trainings in Prof. Wolfgang Enard's laboratory at LMU before conducting his Master's thesis project in Dr. Aylwyn Scally's research group at the Genetics Department of Cambridge University, where he studied the evolutionary past of a developmental gene duplication event in primate genomes. After receiving his Master's degree in 2019, he moved to Nijmegen, Netherlands, in April 2020 and began his doctoral studies under the supervision of Prof. Simon E. Fisher in the Language and Genetics Department of the Max Planck Institute for Psycholinguistics. During his doctoral studies, he investigated the genomics and evolution of human cortical anatomy, language, and musicality by leveraging genome-wide association-based methods, before starting to explore the molecular and transcriptomic characteristics of primate brains in the final year of his doctoral work.

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Research Data Management

This thesis research has been carried out under the institute research data management policy of the Max Planck Institute for Psycholinguistics, Nijmegen, the Netherlands. A summary of this policy can be found at https://www.mpi.nl/page/research-data-management.

All data used in this study are either publicly available on MPI for Psycholinguistics Archive (https://archive.mpi.nl/mpi/) or available on request from 23andMe, Inc. and the UK Biobank. Links to data repositories are provided in the "Data availability" sections of each research chapter. All analyses presented in this thesis were performed using freely accessible software.

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