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Application of Systems Biology Approaches to Unveil the Pathogenic Mechanisms of Neuropsychiatric Disorders

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neuroscience

by

Yanning Zuo

2021

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ABSTRACT OF THE DISSERTATION

Application of Systems Biology Approaches
to Unveil the Pathogenic Mechanisms
of Neuropsychiatric Disorders

by

Yanning Zuo
Doctor of Philosophy in Neuroscience
University of California, Los Angeles, 2021
Professor Weizhe Hong, Co-Chair
Professor Xia Yang, Co-chair

Neuropsychiatric disorders are brain disorders involving complex genetics, molecular, cellular, and circuitry mechanisms. Recent advances in multimodal omics data and systems biology tools have enabled modeling and dissection of the underlying pathogenic mechanisms *in silico*. However, such approaches have not been widely adopted in the neuropsychiatric field. My dissertation focuses on the applications of various system biology approaches, especially network-based multi-omics integration methods, to unveil the pathogenic mechanisms of neuropsychiatric disorders. I firstly reviewed and surveyed the current state of network modeling usage in studying neuropsychiatric disorders. I then applied various system biology tools, including Mergeomics and WGCNA, to identify the molecular, network, cellular, and connectome basis of sex-specific transcriptional changes caused by chronic adolescent exposure to cannabis. I also expanded the network applications from tissue level to single-cell

resolution, and constructed cell-level networks informed by common genetic variants of autism spectrum disorder to predict potential key regulators of the affected networks for drug repurposing. In summary, my studies revealed novel pathogenic mechanisms across multiple levels of biological complexity, provided databases of networks and key regulators for query, and offered potential therapeutic targets for multiple neuropsychiatric disorders.

The dissertation of Yanning Zuo is approved.

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2021

DEDICATION

This dissertation is dedicated to Jieming, Wanjie, Mingliang, Xiaochuan, Sveta, and Dave.

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Chapter 1. Introduction

Neuropsychiatric disorders are complex brain disorders with a high level of genetic heterogeneity and affects 10.7% of worldwide population based on estimation[1]. Decades of research have linked neuropsychiatric disorders to numerous brain regions, circuits, cell types, and molecules. However, the pathogenic mechanisms of neuropsychiatric disorders still remain largely elusive. Studies on molecular pathophysiology of neuropsychiatric disorders are urgently needed to aid the discovery of novel therapeutics.

To address this knowledge gap, my dissertation focuses on unveil the pathogenic mechanisms of neuropsychiatric disorders by taking the advantage of the abundant human genetics and multimodal omics data as well as recent advances in system biology approaches and tools. For example, using network modeling tools to perform multi-tissue multi-omics integrative analysis of ~100 mouse strains in the hybrid mouse diversity panel with a spectrum of non-alcoholic fatty liver disease, Krishnan et al identified candidate regulatory genes and pathways involved in the development of non-alcoholic fatty liver disease, which was further validated by in vitro and in vivo experiments[2]. Such approaches have not yet been widely adopted in the neuropsychiatric disorder research field.

Specifically, I focus on adopting various complementary network-based multi-omics integration methods to identify disturbances in gene networks, specific cell types and subtypes, circuitries, and brain regions in neuropsychiatric disorders. In **Chapter 2**, I review current network modeling methods and survey existing applications of network modeling methods in neuropsychiatric research. This review provides a comprehensive summary of key findings based on network applications in six most commonly studied neuropsychiatric disorders and compares shared and distinct pathways across these neuropsychiatric disorders. In addition, I discuss the limitations

of current applications and propose future directions including engaging the increasing amount of single-cell level datasets.

In **Chapter 3**, I focus on applying system biology tools, including Mergeomics and WGCNA, to identify the molecular, network, cellular and connectome basis of sex-specific transcriptional changes caused by chronic adolescent exposure to Δ-9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis, using bulk-tissue mouse transcriptomic data across multiple brain regions. I found that gene coexpression modules involved in endocannabinoid signaling and inflammation were correlated with memory deficits in the female dorsal medial striatum and ventral tegmental area, respectively. Converging pathways related to dopamine signaling and addiction were altered in the female amygdala and male nucleus accumbens. Moreover, the connectivity map of THC-correlated modules uncovered intra- and inter-region molecular circuitries influenced by THC. Lastly, modules altered by THC were enriched in genes relevant for human cognition and neuropsychiatric disorders. These findings provide novel insights underlying persistent behavioral deficits induced by adolescent exposure to THC in a sex-specific manner.

The increasing abundance of single cell multimodal omics data in the past few years has enabled further dissection of the gene networks at finer granularities. In **Chapter 4**, I expand the network modeling applications to a single-cell resolution, and have constructed Bayesian perturbation networks of autism spectrum disorder informed by common genetic variants. My analyses suggested that gene networks in the caudal ganglionic eminence (CGE) linage inhibitory neurons in the frontal cortex play an essential role in the pathogenesis of ASD across developmental stages. Further, the ASD networks informed by common variants were also highly enriched for genes revealed by ASD rare variants, supporting the omnigenic hypothesis of ASD pathogenesis. Our analysis connects ASD genetics with downstream mechanisms and provides network models to guide target identification for ASD.

In summary, my studies have revealed novel pathogenic mechanisms in chronic adolescent cannabis use and ASD, respectively, across multiple levels of biological complexity, provided databases of networks and key regulators for query, and offered potential therapeutic targets. These studies demonstrate the strength of integrating multimodal omics data with human genetics findings to facilitate the identification of perturbed gene networks and their potential regulators, which warrant future experimental validation and mechanistic studies.

Chapter 2. Unveiling the Pathogenesis of Psychiatric Disorders Using Network Models

2.1 Introduction

Psychiatric disorders, a group of prevalent brain disorders involving complex disturbances in sociocognitive functioning, are a leading cause of disability[3]. Surpassing cancer and cardiovascular disease, psychiatric disorders are estimated to affect 792 mil-lion people worldwide, representing 10.7% of the total population[1]. Nearly one in five adults experiences a psychiatric disorder in the U.S., with major depressive disorder being the leading cause of disability[4]. Given the lifelong morbidity and dearth of rationally designed treatments, it is imperative to understand the pathophysiology of psychiatric disorders. While numerous advances have expanded the scope of genetic analysis, how genetic risk confers pathophysiology remains largely elusive.

Key unanswered questions include: How do polygenetic contributions interact to affect molecular signaling and endophenotypes? Do different combinations of common and rare variants produce distinct manifestations of psychiatric symptoms? How do we meaningfully expand the understanding of these genetic interactions, and how do we leverage such knowledge to promote precision medicine (**Figure 2.1a**)?

One promising approach is to broaden analysis to noncoding regulatory elements and consider their effects within the complete architecture of a functional genome[3,5]. Current analytical methods mostly focus on interpreting common and rare variants located in gene coding regions, but 93% of the disorder-associated loci identified in genome-wide association studies (GWAS) are located in non-coding regions of the genome[6]. However, analyzing global interrelationships between noncoding regulatory elements and rare and common variants is complicated by cell-type heterogeneity, data availability, and incomplete annotation.

In this review, we discuss recent advances in network approaches that may address this complexity. Broadly, for example, network approaches that define the superstructures of interactions and probabilistic models that localize key nodes in this structure could compress the genetic search space to the most important elements. We will firstly introduce the prevailing view on the polygenetic architecture of psychiatric disorders and review genetic studies that have linked rare and common genomic loci to different conditions. We will then discuss several challenges in dissecting the full architecture of psychiatric disorders with this view. We will present advances in multiomics approaches and network methodologies that could address these challenges. We will introduce emerging network tools that are underutilized but promising. Last, we will summarize potential future directions of developing network approaches.

2.2 The scope, characteristics, and genetic architecture of psychiatric disorders

We surveyed six common psychiatric disorders with various levels of heritability (twin heritability ranging from 0.37 to 0.85) and distinct domains of manifestation and etiology: alcohol use disorder, autism spectrum disorder (ASD), bipolar affective disorder, major depressive disorder, post-traumatic stress disorder (PTSD), and schizophrenia. Details regarding disorder characteristics, heritability, and impacts is outside the scope of this review but can be found in previous reviews, such as Sullivan and Geschwind, 2019[3].

Over the past decade, genetic studies have linked both rare and common genomic loci to different disorders and traits[7-13]. One efficient way to characterize the genetic architecture of complex diseases is to search for protein-encoding rare mutations in singletons or multiplex families with extreme phenotypes, which include an early onset, more severe symptoms, or fast progression of diseases[14]. Human genetics studies of extreme psychiatric phenotypes and rare syndromes involving psychiatric symptoms have revealed numerous rare variants in psychiatric disorders. These rare variants include copy number variations (CNVs) and protein-altering point

mutations; particularly for schizophrenia and autism spectrum disorder, 159 and 136 rare variants have been identified, respectively[8, 15-18]. These rare mutations occur at extremely low frequencies in the population, but each with a large effect size. One rare mutation on its own may be sufficient to cause a specific disorder, as in the Rett syndrome[19]. Rare variants often cause loss-of-function of known genes, and it is relatively easier to identify the affected pathways. For instance, synaptic function and transcriptional regulation pathways have been implicated by autism spectrum disorder rare genetic variants. However, rare variants can only explain a small proportion of individuals[3].

By contrast, recent GWAS studies have uncovered common genetic loci with relatively high frequencies in the population but each with small effect size, making common variants challenging to identify unless studied in large populations comprised of as many as 100,000 subjects[19, 20]. Despite the challenges, 353 common loci have been identified for the surveyed psychiatric disorders, amongst which 270 loci are associated with schizophrenia[15]. Such findings illustrate a polygenic model in which many gene loci with small effect sizes and hub genes with moderate to large effect sizes contribute to a disorder[22] (**Figure 2.1b**).

There are several challenges in investigating the polygenic architecture and mechanisms of psychiatric disorders. First, large population samples are needed to overcome statistical hurdles to identify common variants with small effect sizes. Second, interpreting the biological roles of common variants is challenging because the 93% of common variant loci located in non-coding areas of the genome can regulate gene ex-pression in a subtle or indirect way[6]. Third, the set of regulatory actions of a given gene is diverse and varies across cell types and developmental stages. Last, cell-state specific sequencing and functional annotations of such non-coding areas are unavailable or inconsistent.

Multiomics and network approaches can address these levels of complexity by exploiting the structure of physiological contextualization and connectivity to generate otherwise inaccessible insights. Multiomics approaches integrate genetics, functional genomics, transcriptomics, proteomics, and epigenetics. Integrating multiple levels of analysis in this manner can provide unique windows into key driving elements and hypothesized biological functions of genes that would otherwise be opaque to single-level analysis. One way to integrate multiomics data is through network approaches and we will discuss the biological and pragmatic motivations for transitioning from a polygenic model to an omnigenic network model of psychiatric genetics.

2.3 From a polygenic model to an omnigenic network hypothesis of psychiatric disorders

A polygenic model views disorder risk from a multitude of common and rare variants as combinatorial contributions. More recent inferences from the polygenic model and advances in technology and biology have promoted the recognition of an omnigenic model, which views genetic architecture from a network perspective[23, 24]. Networks are graphical models depicting interactions between nodes. From social networks to the World Wide Web, network models emphasize the structure of interconnections between nodes, which may have apparent commonalities across domains. In biological networks, nodes are biological entities such as genes, proteins, non-coding RNAs, and metabolites. Nodes are organized hierarchically and may be described in terms of their topology or interconnections, such as in scale-free architecture[25]. In a scale-free network, most genes have only a few connections with other genes, while a small proportion of genes have a high level of connectivity and are located at the center of the network. These small numbers of genes with high connectivity are referred to as 'core genes' or 'hub genes'. The remaining genes with low connectivity are referred to as 'peripheral genes'[26]. The relationships between entities are illustrated by edges connecting nodes, with the strength of interactions encoded as weights on edges. This view of genetic and

signaling architecture permits not only relationally based biological insights but also hypotheses regarding how certain structures are more vulnerable or robust to disorder.

In the omnigenic model, core genes and peripheral genes contribute differentially to the heritability of complex traits[23]. The small number of core genes usually plays a large regulatory role in the network, thus having large effect sizes. On the other hand, the majority of genes are peripheral genes, which account for most of heritability as a whole, yet each displaying a small effect size. Naturally, one can correspond to rare variants with core genes and common variants with peripheral genes (**Figure 2.1c**). While the polygenic model also allows for rare variants with large effect sizes, the omnigenic model provides additional insights into the underlying gene regulatory relationships responsible for pathogenesis. The assumption that rare variants are core genes in the network, which have larger effect sizes, account for a small percent of heritability and are more phenotype-specific, is supported by existing studies[24, 27, 28]. Thus, we believe that the omnigenic model is superior in reflecting the underlying pathogenic mechanisms of complex psychiatric disorders.

The omnigenic network model calls for systems biology tools to make inferences about pathogenic mechanisms; however, variants alone are insufficient to construct disorder-related networks – additional molecular data that help establish or infer functional relationships are needed. Multiple levels of data from gene expression to protein interactions can be integrated to facilitate the construction of disorder-related networks (**Fig. 2.2**). We describe these approaches in more details below.

2.4 Connecting disorder-related genetic architecture to network models

Integrating and embedding multi-tissue, multiomics data into network architectures offers unprecedented relational insights while anchored to physiologic contexts. Common omics data

for network construction include genetics, transcriptomics, proteomics, and epigenomics[29]. Transcriptomic datasets derive from microarray, RNA-seq, and single-cell RNA-seq experiments and are the most used data type in network construction. mRNA and non-coding RNA expression levels can inform gene coexpression, regulation, and causality relationships. Epigenomics data such as histone modification, DNA methylation, non-coding RNA regulation, and open chromatin sites derived from methods including CHIP-seq, ATAC-seq, Hi-C, and methyl-seq, highlight specific gene regulation profiles[30]. Proteomic data can reflect protein-protein physical interaction relationships based on assays such as yeast double hybridization or co-regulation relationships through high throughput methods such as protein chips. Across omics domains, genetic and epigenetic variations contribute to gene expression regulation, which in turn affect protein levels and downstream protein-protein interactions and functions. All these within-datatype and between-datatype relations can be used in network construction.

As mentioned, the vast majority of heritability involves common variants that are often in non-coding areas, which cannot be directly mapped in a gene network. To connect these loci with molecular networks in disease-relevant tissues, functional genomics serves as a bridge between genetics and other omics. Intermediate phenotype quantitative trait loci (iQTL) are mostly used for this purpose, which are genetic loci associated with specific quantitative traits such as gene expression or protein levels, which are intermediate traits between genetics and clinical phenotypes[31]. In terms of quantitative trait associated, iQTLs include expression QTLs (eQTLs), splicing QTLs (sQTLs), histone modification QTLs (hQTLs), methylation QTLs (mQTLs), and protein QTLs (pQTLs)[32]. The most common type of iQTLs studied is eQTLs that define genetic loci that are associated with gene expression. eQTLs can be divided into cis-eQTLs and trans-eQTLs. Cis-eQTLs are adjacent genetic loci that cis-regulate the covariate gene, while trans-eQTLs are distant genetic loci that regulate genes remotely[33]. In a network, cis-eQTLs can help set the corresponding covariate genes as parent nodes. In contrast, trans-

eQTLs can infer the covariate genes as child nodes. As such, eQTL information can also be directly incorporated in network construction. For instance, using eQTLs as an input to Bayesian networks boosts the causal inference and network performance[34]. In this way, one can locate disorder-related common variants in a network by examining the connection of their eQTL covariate genes and further identify hub genes related to common variants in the network.

As efforts devoting to large-scale omics profiling proceed, there has been an accumulation of databases of different data modalities that can be used for psychiatric research (**Table 2.1**). GWAS catalog, LD-hub, and PGC collect the summary statistics of genetic associations of diseases or phenotypes from many GWAS, including numerous psychiatric disorders[35]. The Genotype-Tissue Expression (GTEx) project profiled the genotype, transcriptome, eQTLs, and sQTLs across 54 tissues in a total of 948 donors, including 2642 samples from 13 brain regions[36]. The Encyclopedia of DNA Elements (ENCODE) profiles various transcriptional regulators and epigenomic factors across more than 150 tissues from 4920 samples, including 706 brain samples[37]. Another project focusing on transcriptional regulator profiling is the Functional Annotation of the Mouse/Mammalian Genome (FANTOM), which has released atlases of transcriptional regulatory networks, promoters, enhancers, lncRNAs, and miRNAs[38]. Apart from GTEx, an abundance of bulk tissue RNA-seq and single-cell RNA-seq datasets can be found on Gene Expression Omnibus (GEO)[39]. Lastly, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) curates and profiles protein interactions, with the latest version v11 including 24.6 million proteins from 5090 organisms[40]. All these data resources enable robust network construction integrating multi-tissue multiomics datasets.

2.5 A survey of current and potential network methods and applications in psychiatric research

Networks commonly used in systems biology include gene regulatory networks, protein-protein interaction networks (PPI), literature-curated networks, and hybrid networks (**Table 2.2**). These network models depict the molecular relationships at both cellular and intracellular level, each from a unique perspective. Gene regulatory networks focus on elucidating gene-gene interaction and regulatory relationships, organizing genes based on coexpression clusters or inferred causality and regulatory pairs. PPIs emphasize the physical interaction between proteins, combining protein interaction in-formation from both experiments and computational predictions. Literature-curated networks capture potential gene or protein interaction by mining gene or protein co-occurrence from published research papers. Hybrid networks combine and integrate information from two or more different networks and present a comprehensive summary for a specific tissue[41].

Below we discuss each network method and its applications in psychiatric disorders. We also highlight approaches that are not yet widely adopted in psychiatric research.

Gene regulatory networks

Three main kinds of gene regulatory networks are commonly adopted: gene coexpression networks, causal relationship networks (Bayesian networks), regulator-target pair networks.

1) Gene coexpression networks

Gene coexpression networks are correlation-based networks in which highly co-regulated genes are clustered into modules, illustrating functional clustering of genes and pinpoint core genes based on connectivity. Commonly used methods to generate gene coexpression networks include WGCNA[42] and MEGENA [43]. The key differences between the two types of coexpression networks include module size (large modules in WGCNA vs. more compact modules in MEGENA) and whether a gene can be in multiple modules (not allowed in WGCNA but allowed

in MEGENA). WGCNA has been widely implemented in numerous studies and is one of the most adopted network methods in systems biology and psychiatric research. In contrast, MEGENA has not be broadly applied. In recent comparative applications between the WGCNA and MEGENA for non-psychiatric diseases, the complementary nature of the two methods is strongly supported[2, 44]. It will be interesting to test MEGENA in psychiatric disorders in future studies.

Using WGCNA or MEGENA, one can identify modules associated with certain conditions based on transcriptomic profiles in both cases and controls using the module-trait correlation analysis (**Figure 2.3a**). By annotating disorder-associated modules' biological function, cell types and pathways responsible for pathogenesis can be elucidated. For example, Kapoor et al. examined bulk-tissue gene expression in the prefrontal cortex of subjects with alcohol use disorder as well as controls[45]. They applied WGCNA to the transcriptomic profiles and identified two modules that were significantly correlated with alcoholism. Further pathway analysis suggested that in subjects with alcoholism, there is a down-regulation of calcium signaling and nicotine response pathways in one module and an up-regulation of immune signaling pathways in another module.

Another common application of coexpression networks is first to construct coexpression networks based on transcriptomic profiles from control subjects and then ex-amine the module enrichment level of genes affected in a specific disorder (**Figure 2.3a**), as exemplified in the following study. Parikshak et al. constructed WGCNA gene coexpression networks based on bulk tissue brain RNA-seq data from subjects representing the cortex of early developmental stages spanning post-conception week 8 to one year after birth in the BrainSpan database[46,47]. Autism spectrum disorder rare variant genes are enriched in hub genes of modules functioning in early transcriptional regulation and synaptic development. Spatially, autism spectrum disorder rare variant genes are enriched in superficial cortical layers and glutamatergic projection neurons of the cortex. These findings have been cross-validated experimentally by other studies

using postmortem human brain samples from subjects with autism spectrum disorder, using both bulk-tissue and single nucleus transcriptomics[48,49]. A parallel paper by Willsey et al. also utilized the BrainSpan database and gene co-expression networks to identify pathways and cell-types related to autism spectrum disorder[50]. Rather than using WGCNA, the authors constructed coexpression networks of high confidence autism spectrum disorder 'seed genes' using the Pearson correlation coefficient to choose the top 20 best-correlated genes with a Pearson coefficient higher than 0.7 for each seed gene. Using this method, the authors elucidate an enrichment of autism spectrum disorder rare mutation genes in deep-layer glutamatergic projection neurons of the mid-fetal cortex, consistent with Parikshak et al.

Gene coexpression networks are powerful tools in determining co-regulatory relationships of genes involved in distinct functions and how these modules connect to psychiatric disorders. However, coexpression networks are not directional, and thus unable to provide causal relationships between genes, an important aspect to retrieve upstream regulators. This limitation can be addressed by causal regulatory networks such as Bayesian networks.

2) Bayesian networks

Bayesian networks (BNs) are directed acyclic graphs summarizing causal regulatory relationships between genes. BNs can be generated with transcriptomic data alone, but the incorporation of prior information capturing regulatory information can offer higher prediction accuracy for regulatory relationships[34,51]. cis-eQTLs, trans-eQTLs, and transcriptional factor-target pairs can be used as prior information for causal inference[33]. For instance, genes with cis-regulatory function and transcription factors are as-signed as 'parent nodes' in BN, while genes under trans-regulation or target genes of transcription factors are 'child nodes'. Arrows pointing from the parent nodes to child nodes indicate the inferred direction of causality.

Integrating genetics, transcriptomic, functional genomic inputs, and more, BN can capture causal regulatory relationships in a given tissue and can be used as a 'roadmap' in pinpointing key regulatory genes[51] (**Figure 2.3b**). There have been a few applications of BN in psychiatric research. Scarpa et al. leveraged a combination of WGCNA gene co-expression network, transcription factor-target network, PPI, and BN to identify con-vergence and divergence of biological processes between sleep loss and depression[52]. The authors first measured the affective and sleep patterns of 288 hybrid mice and their genotypes and transcriptional profile in the cortex, hippocampus, hypothalamus, and thalamus. WGCNA coexpression networks were constructed to identify trait-related modules in individual tissues, and BNs were constructed based on the transcriptomic profiles in conjunction with eQTLs derived from the genotype information and transcriptomics. Next, the authors examined the differentially expressed genes (DEGs) from a meta-analysis cohort of human major depressive disorder and from mouse sleep loss datasets. The DEGs of human major depressive disorder and mouse sleep loss model converged on a frontal cortex-derived module enriched in clock genes and immediate early genes (IEGs). Moreover, genes in this module displayed opposite directions of change in major depressive disorder subjects and sleep deficient mice, in line with the fact that many major depressive disorder patients manifest sleep issues and antidepressants affect sleep. The authors then identified the key driver gene of this subnetwork by overlapping the non-directional coexpression modules on the directional BN to identify intramodular regulatory hub genes. An IEG Arc was found as a key driver gene of the clock/IEG network, which may link depression and sleep loss.

Protein-protein interaction and tissue-specific gene expression patterns have also been used to construct BNs, as in GIANT BNs[53]. GIANT BNs contain 31 central nervous system-related tissue-specific functional interaction networks, each constructed based on transcriptomic profiles, protein interaction information, and regulatory in-formation curated from diverse

experiments. Among those, the brain-specific BN was constructed with thousands of curated experiments and was used to predict autism spectrum disorder risk genes and characterize their biological functions by Krishnan et al.[54]. The authors applied this BN as an input to a machine-learning procedure, which was informed by text-mining co-occurrence of genes of high-confidence autism spectrum disorder associations. This approach revealed synaptic transmission, MAPK signaling, histone modification, and immune response to be essential affected functions in autism spectrum disorder, which was cross-validated with previous literature and experiments. Besides, this method was applied to prioritize driver genes in autism spectrum disorder related CNVs, and the authors highlighted PPP4C and MAZ as potential top driver genes in the most common autism spectrum disorder related CNV 16p11.2.

Although not yet widely adopted in psychiatric research, BN has been applied to study many other diseases such as Alzheimer's disease[55], Type II diabetes[56], and non-alcoholic fatty liver disease[2]. BN is very powerful in identifying key driver genes in a biological process based on causality. For example, BN construction in RimbaNET uses Monte Carlo Markov Chain simulations to reconstruct 1000 networks starting with random seeds, and the final BN is a consensus network containing the most shared edges across all the reconstructions[52]. Although this method promotes causal inference, its disadvantages include high computational cost[30, 41, 57], possibility of failing to find the optimal network structure[41], and lack of feedback loops which misses an essential type of gene expression regulation[58].

3) Regulator-target pair networks

Dysregulation of transcriptional factors and non-coding RNAs have been indicated in psychiatric disorders[48,59-61]. A regulator-target pair subnetwork consisting of a gene expression regulator (such as a transcription factor or a non-coding RNA) and its downstream effect genes is termed ‘regulon.’ One can directly explore experiment-derived regulator-target pair networks from

databases such as FANTOM and ENCODE[37,62]. An alternative method is to use the binding sites or transcriptomic information to infer targets of transcription factors or non-coding RNAs.

One piece of software that infers transcriptional factor regulons based on transcriptomic information is Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNe). Repunte-Canonigo et al. applied ARACNe to a rat model of alcoholism and identified Nr3c1, the gene encoding the glucocorticoid receptor, as a master regulator across many brain regions in alcohol-dependent rats[63,64]. The authors then performed an in vivo validation experiment by administering a glucocorticoid antagonist to the nucleus accumbens and ventral tegmental area of alcohol-dependent rats and control animals. A significant decrease in alcohol consumption was observed in alcohol-dependent rats with the glucocorticoid antagonist to either of the two brain regions, while there was no effect in alcohol non-dependent rats. Another method developed based on ARACNe, Reconstruction of Transcriptional regulatory Networks (RTN)[65], was applied by Pfaffenseller et al. to identify differentially expressed regulons in the prefrontal cortex of subjects with bipolar affective disorder[66]. Five regulons (EGR3, TSC22D4, ILF2, YBX1, and MADD as regulators) were identified, with EGR3 showing the most robust significance in two independent human bipolar affective disorder datasets.

Ingenuity Pathway Analysis (IPA), a commercial tool constructed based on a comprehensive curation of different networks from experimental datasets, text-mining literature, and other databases, also contains transcriptional factor and miRNA-target pair networks[67]. Using this tool, Bam et al. predicted many down-regulated miRNAs in PTSD to target IFNG and IL-12, which exhibit increased expression levels in PTSD patients[68]. They also predicted that up-regulation of hsa-miR-193a-5p could decrease the high expression level of IL-12, which may help reduce the excessive inflammation response in PTSD patients.

Another software tool, TargetScan, predicts mRNA targets of miRNA based on conserved sequences in mRNAs[69]. Wu et al. leveraged a combination of coexpression network and miRNA-target regulatory network to identify miRNA dysregulation in autism spectrum disorder[61]. The authors firstly identified differentially expressed miRNAs from autism spectrum disorder case-control brain samples and constructed WGCNA coexpression modules. They then used the TargetScan algorithm to identify mRNA targets of top differentially expressed miRNAs and hub miRNAs in autism spectrum disorder related coexpression modules. The authors illustrated that autism spectrum disorder related risk genes are enriched in miRNA targets and miRNA modules. One miRNA, hsa-miR-21-3p, targets neuronal/synaptic genes down-regulated in autism spectrum disorder, which may play an essential role in pathogenesis.

PPI networks

In a PPI network, the nodes are proteins, and the edges depict the physical interaction relationship between proteins based on experimental datasets or computational simulation. The edges are undirected, and the weights of edges indicate the reliability of the interaction. StringDB is the most commonly used PPI database, with the latest version v11 covering around 25 million proteins from 5099 organisms[40]. StringDB imports and integrates PPI information from other databases, including PINA, MINT, IntAct, DIP, BioGRID, HPRD, and MIPS/MPact. It also contains PPI inferred from text-mining, statistically significant co-occurring genes from the literature, and computationally predicted PPI based on criteria such as coexpression. Other integrated databases, including IPA and GeneMania, also contain PPI resources.

PPIs have been extensively used in psychiatric research to identify hub genes. Commonly used methods of identifying subnetworks and hubs include DAPPLE, DMS, MCODE, and PINA[70-73]. For example, Blizinsky et al. constructed a PPI network related to rare CNVs in schizophrenia using PINA2[74]. MAPK3/ERK1 was identified as the most topologically important hub gene for

the 16p11.2 network. The authors then performed in vitro validation by applying an ERK signaling inhibitor to cultured primary neurons with 16p11.2 microduplication. This treatment successfully reversed the abnormality in these neurons' dendritic arborization, indicating the critical role of MAPK3/ERK1 in maintaining normal neuronal morphology.

PPIs are also used broadly in combination with other networks, such as the coexpression network, to identify disorder-related networks. Gulsuner et al. profiled schizophrenia-related de novo mutations and leveraged a combination of PPI network and coexpression network to examine the functional relevance of these de novo mutations and identify their enrichment in pathways and tissues[75]. By mapping de novo mutation genes onto GeneMania physical interaction data set, the authors constructed an interconnected subnetwork enriched for schizophrenia de novo mutation genes, suggesting that the mutation genes are biologically interacting. The authors then constructed coexpression networks based on BrainSpan data by calculating Pearson correlation coefficients across de novo mutation gene pairs. The most highly connected coexpression network of de novo mutation genes was derived from the fetal cortex. To further examine the interaction topology and gene characteristics, the authors merged the PPI network and coexpression network derived from schizophrenia risk genes. This merged network contains genes in pathways related to neurogenesis and synaptic integrity, with most of the genes expressed high in early fetal development, low in childhood, and high again in early adulthood, which is in line with the onset of schizophrenia in early adulthood.

Since proteins interacting with each other may be co-regulated by the same up-stream regulatory signal, they may also exist in the same coexpression network. Thus, genes that are both coregulated and showing protein interaction may be of higher relevance to a specific condition and should be prioritized for further study as driver genes (**Figure 2.3b**). Parikshak et al. identified two WGCNA coexpression modules enriched for autism spectrum disorder rare de novo variants[46] and further showed that these modules enriched for rare variants are also

significantly enriched for protein interaction. The authors then intersected the WGCNA coexpression network hub genes with PPI network hub genes. Many of the overlapping hub genes are known to harbor autism spectrum disorder associated mutations and interact with other autism spectrum disorder related genes, such as TBR1, NFIA, and KDM6B.

The comprehensive PPI databases provide abundant resources of reliable PPI networks. However, one main limitation of the PPI network is that it does not reflect causality or regulatory relationships. In addition, the PPI network is not tissue-specific and may bring in interactions that are not relevant to the disease tissues.

Literature-based networks

Literature-based networks are curated based on integrating general knowledge such as pathway/function annotation databases and text-mining existing experimental data and literature to capture genes contributing to specific biological functions. Although gene ontology terms can be constructed into a network in terms of the relatedness of biological pathways, genes in this type of network are not interconnected and do not reflect the topological properties of gene-gene relationship[76]. Thus, pathway/function annotation databases such as GO, KEGG, Reactome, and BioCarta are collections of sets of functionally related genes rather than networks. However, pathway annotations can be used in conjunction with other molecular interaction information in network analysis.

Leveraging a knowledge-based and data-driven gene-phenotype likelihood network, Gilman et al. developed NETwork-Based Analysis of Genetic Associations (NETBAG)[77]. The gene-phenotype likelihood network was first constructed by connecting all pairs of human genes. Then weights calculated based on likelihood ratio were assigned to the edges based on naive Bayesian integration of pathway annotations, protein-protein interaction information, shared

gene or protein sequence or structure, and coevolutionary patterns. In this network, genes that are more likely to participate in the same phenotype have a high likelihood ratio weight. To predict genes affected by rare de novo CNVs in autism spectrum disorder using this network, the authors overlaid genes from autism spectrum disorder related CNV regions and found the subnetwork with the highest enrichment p-value. They discovered that genes in these subnetworks mostly participate in synapse development, axon targeting, and neuron motility, which are cross-validated by later studies[46,48-50,54,78]. Gilman et al. also developed NETBAG+ to incorporate more genetic variation, including GWAS loci and de novo single nucleotide variants (SNVs). Further analysis suggested that cortical interneurons, pyramidal neurons, and medium spiny neurons are the most impacted cell types in ASD[78]. The authors also applied NETBAG+ to schizophrenia and identified related subnetworks and their functions[79]. Schizophrenia-related networks function mainly in axon guidance, neuronal cell mobility, synaptic function, and chromosomal remodeling, which largely coincides with ASD but with different mutations.

Similar to NETBAG, Ward et al. constructed phenotypic-linkage networks (PLN) to identify nervous system gene sets related to GWAS loci of mood instability[80]. The authors constructed a nervous-PLN using phenotypes specifically in mouse phenotype ontology (MPO) category Nervous System and integrated GO terms and pathway an-notations with PPI, coexpression relationship, to derive semantic similarity score[81]. Using this nervous-PLN, the authors found genes within loci related to mood instability to function in synapse transmission. Specifically, two candidate genes associated in the network are HTR4 and MCHR1, encoding serotonin and melatonin receptors, respectively, and have been indicated in depression and schizophrenia.

Hybrid networks

Apart from the individual usage of the networks mentioned above, constructing a hybrid network consisting of two or more kinds of networks is also a common practice. As different networks cover different aspects of gene interaction, a hybrid network can leverage the strengths and overcome the disadvantages and limitations of each network type. For example, combining PPIs with BNs integrates both causal gene regulatory in-formation and protein physical interaction, which covers distinct aspects of gene interactions and promotes the identification of hub genes that play regulatory roles at gene and/or protein levels.

Gazestani et al. constructed hybrid networks incorporating knowledge-based, functional, and experiment-derived coexpression networks to identify transcriptional perturbation patterns in leukocytes from ASD cases and control children[82]. The authors first generated a static network which combines information from (a) high-confidence physical and regulatory interactions from the Pathway Commons database, Reactome, and BioGRID; (b) coexpression network based on the transcriptome of the aforementioned case-controlled leukocyte samples; (c) functionally related gene interactions from GeneMania, which includes PPI, coexpression, colocalization, pathways and protein domain similarity information. Gene pairs in each diagnosis group (case or control) were retained to generate diagnosis-specific networks. By comparing the con-trol-specific network and ASD specific network, the authors discovered that the ASD network was enriched for ASD rare mutation genes, as well as their regulatory targets and regulators. RAS–ERK, PI3K–AKT, and WNT–β-catenin signaling pathways were enriched in autism spectrum disorder specific networks, and ASD rare mutations perturbed the network through these pathways.

In addition to constructing literature-curated networks or hybrid networks from scratch, there are numerous existing hybrid network resources available. Huang et al. benchmarked 21 popular human gene or protein networks, including StringDB, Gene-Mania, GIANT, based on a disease gene set recovery test and found that networks with larger size have better performance in

retrieving known disease genes[83]. They then assembled an integrative network which requires edges to be present in at least two networks, called Parsimonious Composite Network (PCNet), which is smaller in size but has the best performance. Although psychiatric disorders were not explicitly tested, this study provides a new network resource and a guideline to choose hybrid network re-sources.

Cross disorder network applications

Due to the genetic correlation and comorbidity across psychiatric disorders, studying pathogenesis mechanisms across disorders may yield therapeutic targets for several disorders or specific endophenotypes. A recent study by Gandal et al. leveraged WGCNA gene coexpression networks constructed based on case-controlled human brain transcriptomic profiles across five psychiatric disorders: schizophrenia, major depressive disorder, bipolar affective disorder, autism spectrum disorder and alcohol use disorder[84]. In addition to the distinct transcriptomic disturbance in each disorder, the authors identified a shared component of transcriptional dysregulation across all five disorders related to the degree of polygenic overlap. Their results agree with previous findings supporting that a shared causal genetic component underlies all psychiatric disorders[85,86]. Further, the authors identified shared and unique modules across these disorders. In ASD, bipolar affective disorder, and schizophrenia, an astrocyte module with the annotation of glial differentiation is up-regulated; several modules associated with neuronal and mitochondria function are down-regulated in these disorders. A microglia module is uniquely up-regulated in ASD, which is confirmed in another study by Gandal et al., where modules related to microglia and interferon response are significantly up-regulated in ASD but down-regulated in bipolar affective disorder and schizophrenia. There is also a shared upregulation of the NFkB pathway across these three disorders[87].

The conclusions from the Gandal et al. studies are consistent with previous pathway analyses based on genetic risks and studied leveraging a similar network approach. Using pathway analysis, a study by PGC found that common genetic risks of schizophrenia, major depressive disorder, and bipolar affective disorder converge on neural, immune, and histone modification pathways[86]. Kim et al. constructed WGCNA coexpression networks to identify shared modules across schizophrenia, major depressive disorder, and bipolar affective disorder, which were enriched with GABAergic markers, synaptic proteins, and immune functions. Interestingly, genes that compromise immune function-related modules showed no overlap across all three disorders, indicating possible differential response in the immune system[88].

Network applications on treatment response

Besides network applications in studying pathogenesis, networks are also powerful tools to identify driver genes in treatment response. Although many psychopharmacological drugs are available and applied for more than five decades, their action mechanisms remain mostly elusive. Moreover, drug response in individuals differs tremendously. Elucidating the mechanisms of action and identifying biomarkers that predict individual response to drugs can greatly aid precision medicine in psychiatry and the development of novel therapeutics.

Lithium (Li) is a first-line mood stabilizer for bipolar disorder, although its mode of action is not fully elucidated. To reveal the mechanism of Li in treating bipolar disorder and the differential responses across patients, Breen et al. characterized the transcriptomic profiles of subject-derived lymphoblastoid cell lines from Li responders and non-responders[89]. WGCNA gene coexpression modules suggested that Li treatment correlates to upregulated immune response, apoptosis, and protein processing in the endoplasmic reticulum and down-regulated ribosome pathway, translation initiation and phosphatidylserine metabolism. They further discovered that DEGs between Li responders and non-responders are enriched in cell cycle processes and

nucleotide excision repair pathways. To identify psychopharmacological drugs with a similar transcriptomic signature to Li for bipolar disorder patients, the authors then queried the DEGs from Li treatment against DSigDB, a database of drug/compound activated gene expression signatures[90]. Clonidine, an alpha2-adrenoceptor agonist, exhibited a drug-gene signature most reminiscent of Li signature, thus has potential for bipolar disorder treatment. Besides DSigDB, CLUE and Metacore (commercial) also contain drug-gene transcriptomic signatures applicable for studying drug action mechanisms and drug repurposing.

Summary of new insights obtained from network studies of psychiatric disorders

Network methodologies provide us with a perspective beyond the identified dis-order-associated variants. For instance, candidate genes in the network which show high connectivity to many previously identified genes can be prioritized as hub genes or driver genes. Due to their high connectivity, these hub genes may act as the converging points of disorder-related variants and pathways, making them potential targets for therapeutics even if they have not been implicated by genetic evidence yet. Besides revealing hidden novel genes in disorder etiology, network methodologies can elucidate regulatory relationships and coherent biological functions between disorder genes. For example, coexpression networks can identify covariation across modules of genes and differentially regulated modules, while one may fail to identify significant differentially expressed genes.

Many fruitful findings have been made through network methods, which are summarized in **Table 2.3**. Among the six psychiatric disorders discussed in this review, ASD and schizophrenia are the most studied, and independent studies have yielded consistent results. Existing studies exploiting network methods mostly focus on identifying candidate genes and pathways based on transcriptomic studies (**Figure 2.4**). Almost every study on ASD has indicated the pathogenic role of immune and synaptic functions in ASD pathogenesis. Other crucial biological processes

revealed for ASD include chromatin and transcription regulation, early embryonic development, axon guidance, extracellular matrix, and MAPK signaling[46,50,77-78,82,91-95]. Besides the processes affected in general ASD cases, Luo et al. combined electronic health records with genomic and transcriptomic data and identified an ASD subtype with dyslipidemia[96]. In addition, the various studies also implicated key cell types related to ASD, including mid-fetal deep layer cortical projection neurons, superficial cortical layers neurons, cortical interneurons, medium spiny neurons, and microglia[46,50,78,97].

Schizophrenia also engages the immune system, synaptic functions, and neurodevelopmental processes, which fall into the same pathway category as ASD[79, 98]. However, schizophrenia exhibits differential alterations in these pathways. Apart from the differential immune response discussed in 5.5 (upregulated microglia and immune activities in ASD and down in schizophrenia), the candidate genes from de novo CNVs of ASD and schizophrenia showed opposite directions in their biological functions. Most of the schizophrenia candidate genes are associated with synaptic pruning and decreased dendritic spines, while ASD candidate genes are associated with increased dendritic spines, which were also observed in postmortem brain analyses[79]. Besides, genes related to schizophrenia de novo mutations mostly show a characteristic expression pattern: high in the fetal stage, low in childhood, and high again in early adulthood[75], while ASD de novo mutations exhibit high expression in fetal and early post-natal development[46,99]. Another independent study on 22q11.2 deletion identified two hub genes that express during embryonic brain development and adolescence, respectively[100]. This pattern coincides with the typical onset time of schizophrenia, which is around early adulthood. Schizophrenia related genes have also been shown to fail to decrease naturally as control subjects do[101].

The polygenic component of bipolar affective disorder overlaps with schizophrenia significantly, but network application in bipolar affective disorder is very limited. The role of postsynaptic

density in bipolar affective disorder pathogenesis has been indicated by independent studies[102,103]. Hub genes such as MAP4 and ILF2 were also suggested, but due to fewer study numbers and a lack of validation, a consensus can not be reached[66, 104, 105].

Major depressive disorder and PTSD are stress-related disorders and share neuronal and immune dysregulations based on network studies[106-109]. PTSD has been shown to engage immune processes more prominently than major depressive disorder. Dysfunction of multiple immune processes, including innate immunity, interferon responses, cytokine receptor interaction, and glucocorticoid receptor activity, has been implicated[110-115]. Unlike PTSD, for which all network studies identified immune dysregulation, the mechanism behind major depressive disorder seems less coherent across studies similar to the case of bipolar affective disorder. In a study using a mouse model to identify hub genes related to depression susceptibility, several key drivers including Dkkl1, Neurod2, and Sdk1 were validated in vivo, indicating the reliability of network predictions, and implicating synaptic transmission, cell-cell signaling, and oxidative phosphorylation pathways in depression pathogenesis[116].

Lastly, alcohol use disorder is a disorder combining features of addiction and neurotoxicity. Network studies of alcoholism have revealed processes related to mitochondrial dysfunction, synaptic transmission, neuroplasticity, calcium signaling, and immune functions[45, 117-120]. One hub gene Nr3c1 predicted by transcriptional factor network was validated in vivo in a mouse model of alcohol-dependent[63]. More studies are needed to reveal the underlying mechanisms behind alcoholism.

2.6 Conclusions and future directions

In summary, we have introduced and illustrated main network approaches, their strengths and limitations, and how they can complement one another by highlighting relevant studies. Despite

the aforementioned recent discoveries, network applications in psychiatric research are still in their infancy. Networks such as the WGCNA coexpression network and PPI have been extensively applied, while other networks such as BN are rarely adopted despite indications as powerful tools in other research fields. Integrating these network applications may reveal hidden pathogenic mechanisms by capturing underappreciated information from the data. We recommend adoption of diverse types of network approaches in each study to derive comprehensive molecular insights.

Besides leveraging complementary network methods, another future direction would be to have an integrated framework followed by the field to apply a set of benchmarked and well-performing networking methodologies systematically. Such a framework would eliminate technical bias caused by different methods to enable systematic comparison of psychiatric disorders at a network level.

Benchmarked and standardized network methodologies are applicable regardless of disorder types. However, in order to better elucidate trait-specific biology, we recommend careful collection of multiomics data types that reflect the unique aspects of a certain disorder, including specific causal factors (e.g., genetic versus environmental) and the corresponding omics data types (e.g., genetic variants for genetic causes; epigenetic alterations for environmental causes), related brain regions and circuits. In addition to collecting relevant types of data, tissue heterogeneity needs to be addressed as a future direction. As a highly complex organ, the brain consists of numerous subregions and nuclei, each containing various cell subtypes. Previous network application studies in psychiatric disorders are mostly performed at the brain region level using bulk tissue transcriptome. Obviously, with the advancement of scRNA-seq, the opportunity to explore cell-level networks becomes an urgent need. The abundance of single-cell transcriptomic datasets enables researchers to further dissect the pathogenic mechanisms of psychiatric disorders at an increasing granularity of cell type or subtype level. Thus, it is

possible to identify cell subtypes related to a specific condition and pinpoint key driver genes in different cell subtypes.

Appealing as it is, network methods for single-cell datasets are still limited. Due to the challenge of data sparsity, methods applicable to bulk tissue transcriptomics do not perform well on single-cell datasets[121]. However, a few single-cell network methods have been successfully applied widely in studying cell type diversity and non-psychiatric conditions, including ligand-receptor binding network, single-cell gene regulatory network, and single-cell coexpression network. The ligand-receptor binding network is a PPI network emphasizing intracellular interactions. By looking at the ligand and receptor pairs expressed in cell types, we can identify interacting cell types utilizing autocrine, paracrine, and endocrine signaling. For example, CellPhoneDB and iTALK are two standard tools to calculate cell-cell interaction scores[122,123]; SCENIC uses transcriptional factor information and scRNA-seq data to identify regulons at a cell-type-specific level[124]; scLink infers gene coexpression network from sparse gene expression matrix[125]; CytoTalk aims to construct both within cell-type and between cell-type signaling networks[126]. Benchmarking and applying these methods would bring mechanistic research of psychiatric disorders to a finer granularity from the brain region level to cell subtype level.

Besides using scRNA-seq as a resource providing pathophysiological insights with an increasing granularity, single-cell and bulk tissue transcriptomic profiling can also be applied as an approach to validating in silico predictions. Experimental validations have been limited despite the current progress of in silico findings in key drivers and pathways. More experimental validations should be performed to facilitate the transition from in silico predictions to the bench and eventually to the bedside. Current experimental validation methods include RT-PCR and transcriptomics for evaluating possible expression alteration of the key drivers from samples with the disorder[56,47,48]; human genetic studies for identifying risk genes[8,12,15,16]; In vitro and in vivo experiments using appropriate animal model or human subject-derived material for

validating the molecular, cellular and behavioral phenotypes upon disrupting key driver expression[74,127-129] (**Figure 2.3c**).

In conclusion, approaching psychiatric genetics from a network perspective enables researchers to identify the converging pathways in genetic architecture and leverage the abundance of omics databases to yield better understanding of pathophysiology and predictions for therapeutic targets. With this review, we hope to provide a systematic overview of network methodologies, previous network applications, and their findings in psychiatric research. Much remains to be explored – including adopting network approaches from other fields, standardizing a benchmarked and integrated framework, developing single-cell network construction methods, and performing corresponding experimental validations.

2.7 Tables

Table 2.1. Publicly available repositories of multi-tissue multiomics data related to psychiatric research

Omics	Database	Description	URL	Usage in network applications
Genetics	GWAS catalog	Collections of GWAS summary statistics files	https://www.ebi.ac.uk/gwas/	Find trait-related genes, pathways, and subnetwork
	LD-hub		http://ldsc.broadinstitute.org/	
	PGC		https://www.med.unc.edu/pgc/	
Genomics/ Functional genomics/ Transcriptomics	GTEx	Genotype, transcriptome, eQTLs, and sQTLs profiles across 13 brain regions from 948 donors and 2642 samples	https://www.gtexportal.org/home/	"Building bricks" for gene regulatory network
	GEO	A repository for various data types including genotypes, bulk tissue RNA-seq and single-cell RNA-seq datasets	https://www.ncbi.nlm.nih.gov/geo/	
	PsychENCODE	A repository specifically for neuropsychiatric disorders including RNA-seq datasets, QTLs, epigenomic datasets and gene regulatory networks	http://resource.psychencode.org/ https://www.synapse.org/#!Synapse:syn4921369/wiki/235539	
	BrainSpan	Transcriptional profiles of 16 cortical and subcortical regions with a temporal coverage across pre- and post-natal development in both males and females	http://www.brainspan.org/static/download.html	
Epigenomics	ENCODE	Transcriptional regulator and epigenomic factor profiles from 706 brain samples	https://www.encodeproject.org/	Provide regulator target-pair information
	FANTOM	Atlases of transcriptional regulatory networks, promoters, enhancers, lncRNAs, and miRNAs	https://fantom.gsc.riken.jp/	

Table 2.1. *Cont.*

Proteomics	STRING DB	Curated protein interactions including 24.6 million proteins from 5090 organisms	https://string-db.org/	Provide protein-protein interaction information

Table 2.2. Major networks used in psychiatric disorder research

Networks		Relationship Captured	Disadvantages	Example Construction Methods
Gene regulatory network (GRN)	Coexpression network	Covariation and co-regulation among gene clusters	- Not directional - No causal relationships	WGCNA[42], MEGENA[43]
	Bayesian network	Causality of regulation between gene pairs	- High computational cost - Lack of feedback loops - Possibility of failing to find the optimal network structure	RimbaNet[57]
	Regulator-target pair network	Specific regulation of certain transcriptional factors/non-coding RNAs	- Only captures certain types of regulator relationships	From database (FANTOM)[37]; ARACNe[64]; TargetScan[69]
Protein-protein interaction network		Physical interaction affinity between pairs of proteins	- Cannot reflect causality or regulator relationships - Current PPI datasets are not tissue-specific	From database (STRINGDB)[40]
Literature-based network	Background likelihood network	Possibility of gene pairs participating in a similar genetic phenotype	- Limited by current level of knowledge	Gilman et al., 2011[77]
	Phenotypic-linkage network	Gene clusters related with disease-related phenotypes curated from the literature		Ward et al., 2020[80]

Table 2.2. *Cont.*

Hybrid network	General gene-gene interactions, PPIs, and literature co-occurrence	Use premade networks (e.g. PCNet)[83]; Custom script

Table 2.3. Key findings based on network applications in selected psychiatric disorders

Disorder	Networks	Key findings	Ref.
ASD	Coexpression network	Synapse and immune response related modules are affected in frontal and temporal cortex from ASD subjects; ASD rare variants affects early transcriptional regulation and synaptic development pathways and are enriched in superficial cortical layers and glutamatergic projection neurons in developing and adult human cortex.	Voineagu et al. [90] Parikshak et al. [46]
	Protein-protein interaction network	ASD rare variant related protein interactions are enriched in synaptic transmission, cell junction, TGFβ pathway, neurodegeneration, and transcriptional regulation.	de Rubies et al. [92] Sanders et al. [91]
	Bayesian network	Synaptic transmission, MAPK signaling, histone modification, and immune response are the top affected functions in predicted ASD risk genes using a brain-specific network.	Krishnan et al. [54]
	Literature-based network	ASD rare variant genes form a network related to synapse development, axon targeting, and neuron motility; Genes in ASD rare variant and SNV informed network are expressed at the highest level in cortical interneurons, pyramidal neurons and the medium spiny neurons of the striatum.	Gilman et al. [77] Chang et al. [78]
	Hybrid network	The ASD network constructed with peripheral blood transcriptome in children with ASD was enriched for ASD rare mutation genes, as well as their regulatory targets and regulators. RAS–ERK, PI3K–AKT, and WNT–β-catenin signaling pathways are enriched in ASD-specific networks.	Gazestani et al. [81]

ASD: autism spectrum disorder.

Table 2.3. *Cont.*

Disorder	Networks	Key findings	Ref.
AUD	Coexpression network	In prefrontal cortex samples from human ALC subjects, a module functioning in calcium signaling, nicotine response and opioid signaling are down-regulated in ALC, while another module functioning in immune signaling are up-regulated in ALC; In nucleus accumbens samples from human ALC subjects, two neuronal modules enriched for genes in oxidative phosphorylation, mitochondrial dysfunction and MAPK signaling pathways are down-regulated in ALC, while four immune-related modules enriched for astrocyte and microglia markers are up-regulated in ALC.	Kapoor et al.[45] Mamdani et al.[117]
	Transcriptional factor/miRNA regulons	Pathways related to synaptic processes and neuroplasticity are disrupted in a rat ALC model; Nr3c1 acts as a master regulator in multiple brain regions in alcohol-dependent rats.	Tapocik et al., 2013[118] Repunte-Canonigo et al., 2015[63]

AUD: alcohol use disorder

Table 2.3. *Cont.*

Disorder	Networks	Key findings	Ref.
BAD	Coexpression network	BAD common variants are enriched in hippocampus and amygdala across developmental stages. In dorsolateral frontal cortex samples from human BAD subjects, modules enriched for genes related to postsynaptic density, RNA processing and carbon-nitrogen ligase activity are downregulated, while modules enriched for genes related to ion binding and lipid catabolism are upregulated.	Xiang et al.[104] Akula et al.[102]
	Transcriptional factor regulons	EGR3, TSC22D4, ILF2, YBX1 and MADD are predicted as master regulators in human prefrontal cortex with BAD.	Pfaffenseller et al.[66]
	Protein-protein interaction network	CDH4, MTA2, RBBP4, and HDAC2 are the core genes predicted by PPI analysis, involved in early brain development regulation. HP and PC are related to BAD de novo mutations; MAP4, WDHD1, EIF4E and STRN are related to BAD common variant loci.	Xiang et al.[104] Toma et al.[105]

BAD: bipolar affective disorder.

Table 2.3. *Cont.*

Disorder	Networks	Key findings	Ref.
MDD	Coexpression network	CCND3, TXND5, TRI26 are the driver genes for cognitive dysfunction in MDD, validated by plasma protein level in MDD subjects;	Schubert et. al.[130]
		Immune response and protein processing in the ER are disrupted in older adults with recurrent MDD	Ciobanu et al.[106]
	Protein-protein interaction network	The ATP5G1 gene is associated with the pathogenesis of MDD	Zeng et al.[131]
PTSD	Coexpression network	Differential responses to PTSD are observed in correlated modules constructed from peripheral blood transcriptome of PTSD subjects. In men, an IL-12 signaling module is upregulated; In women, a module related to lipid metabolism and mitogen-activated protein kinase is upregulated. Cytokine, innate immune and type I interferon related modules are shared between sexes.	Breen et. al.[110]
	miRNA regulons	Downregulated miRNAs in peripheral blood transcriptome of PTSD subjects are predicted to target IFNG and IL-12.	Bam et al.[68]
SCZ	Coexpression network	Genes related to central nervous system development failed to attenuate with age in SCZ subjects;	Torkamani et al.[132]
		Synaptic protein coexpression was significantly decreased in the auditory cortex of SCZ subjects;	MacDonald et. al.[133]
		SCZ common variants are enriched in negative coexpression genes of C4A	Kim et. al.[134]
	Transcriptional factor regulons	TCF4 is a master regulator identified from postmortem dorsolateral prefrontal cortex of SCZ subjects and cultured olfactory neuroepithelium	Torshizi et. al.[127]
	Protein-protein interaction network	MAPK3/ERK1 is the top hub gene for the 16p11.2 microduplication network	Blizinsky et. al.[74]
	Literature-based network	SCZ rare variant-derived network genes function mainly in axon guidance, neuronal cell mobility, synaptic function, and chromosomal remodeling, and are highly expressed in the brain during prenatal development.	Gilman et. al.[79]

MDD: major depressive disorder; PTSD: post-traumatic stress disorder; SCZ: schizophrenia.

2.8 Figures

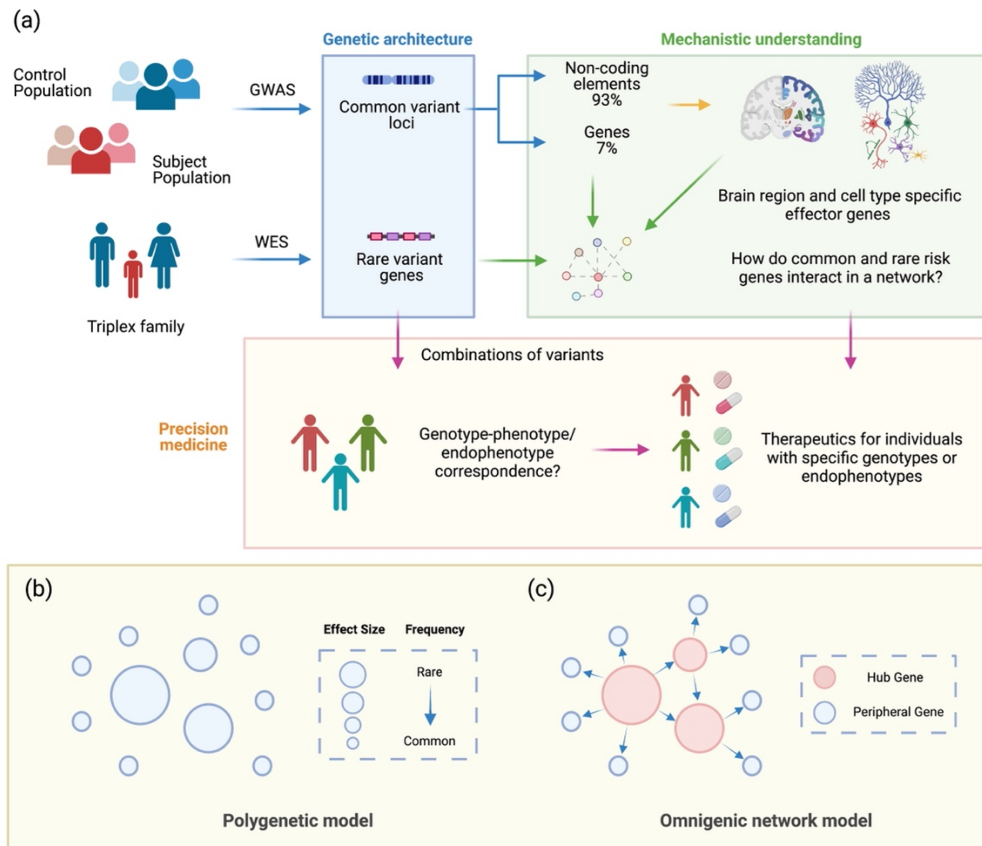


Figure 2.1. Pursuing a network understanding of psychiatric disorders' genetic architectures to advance precision medicine. (a) With the increasing abundance of GWA and WES studies, genetic data for psychiatric disorders are increasingly comprehensive. However, we still lack a mechanistic understanding of the genetic architecture in the pathogenesis of different disorders and symptoms. Establishing such an understanding systematically could enable the development of therapies for subgroups of patients or even on a personalized basis. Network modeling of gene interaction provides a powerful tool to dissect risk gene relationships and pathways affected. The polygenic model and the omnigenic model are proposed for psychiatric disorders. In the polygenetic model (b), a certain trait is determined by a combination of multiple variants with different effect sizes. Common variants have high population frequency and small

effect sizes, while a small number of rare variants have low population frequency but large effect sizes. In the omni-genic network model (c), the regulatory relationships between variants are depicted by the network. A small number of hub genes regulates the majority of other genes (peripheral genes). Rare variants likely reside in core genes and common variants in peripheral genes.

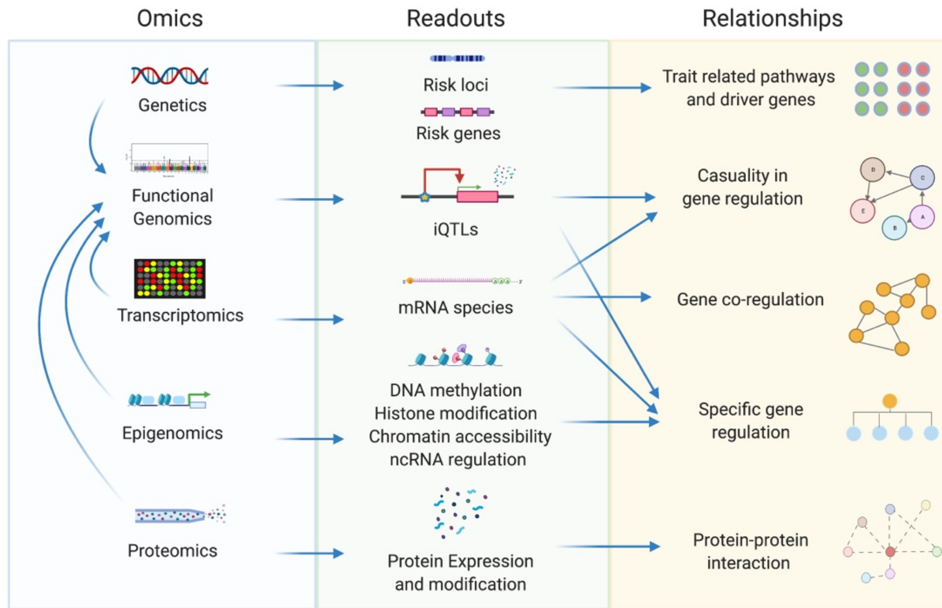


Figure 2.2. Extracting tissue and cell-type specific gene interaction relationships from multiomics data. Genetics, functional genomics, transcriptomics, epigenomics and proteomics are the most commonly used omics in obtaining gene interaction relationships. Genetic readouts can be used to infer trait related pathways and driver genes, while readouts from other omics indicate gene regulatory relationship or protein-protein interaction. Particularly, intermediate phenotype QTLs (iQTLs) such as expression QTLs (eQTLs) or protein QTLs (pQTLs) from functional genomics data act as a bridge linking genetics and other omics by tissue-specific loci-gene regulatory relationship, thus enabling the interpretation of common variant loci in non-coding areas of the genome.

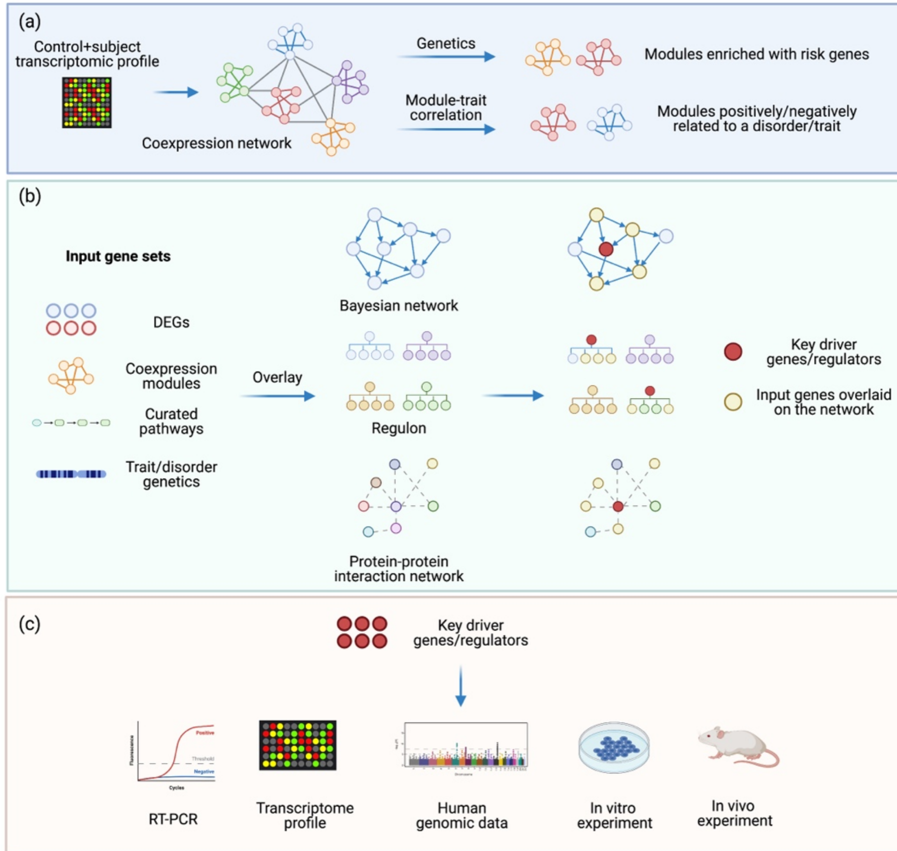


Figure 2.3. Using networks to identify disorder-related networks and key driver genes. (a) Pipeline of identifying disorder-related coexpression modules. Coexpression network is generated from the transcriptomic profiles of subject with a specific disorder and corresponding controls using methods such as WGCNA. By calculating enrichment level of disorder-related risk genes in each module, modules enriched with risk genes can be identified. Alternatively, modules positively or negatively correlated with the disorder can be identified by doing a module-trait correlation analysis. Downstream annotation of these modules' biological functions will reflect pathways affected in the disorder. However, coexpression networks are unable to capture directed, causal relationships, which can be supplemented by Bayesian networks and regulator-target pair networks. (b) Using networks as a 'road map' to identify key driver genes of a specific disorder. Bayesian networks, regulons from regulator-target pair networks, and PPI

networks depict causality, regulation, or direct physical interactions, respectively, and can be used as network models summarizing regulatory or direct gene-gene interactions in a certain tissue. By overlaying disorder-related gene sets, e.g. differentially expressed genes (DEGs), disorder-correlated coexpression modules, related pathways and risk genes, one can pinpoint potential key drivers based on the topology of the networks. (c) A summary of key driver validation approaches. RT-PCR and transcriptomics can evaluate possible expression alteration of the key drivers from samples with the disorder. Key drivers may be also validated if they are identified as risk genes by human genetic studies. In vitro and in vivo experiments using appropriate animal model may help to validate the molecular, cellular, and behavioral phenotypes upon disrupting key driver expression.

Figure 2.4. Comparing shared and distinct pathways across selected psychiatric disorders. Top pathways related to selected psychiatric disorders are shown in the disorder-pathway network. Red lines connecting disorders depicts correlation level between disorders[135]. Synaptic trans-mission-related processes are shared among all six disorders and immune functions are indicated in all disorders except BAD. Pathways including MAPK signaling and transcriptional regulation are shared among AUD, ASD, and SCZ. Each disorder and their associated pathways are annotated with the same node color. Shared pathways between disorders are indicated with multi-color nodes. ASD: autism spectrum disorder; AUD: alcohol use disorder; BAD: bipolar affective disorder; MDD: major depressive disorder; PTSD: post-traumatic stress disorder; SCZ: schizophrenia.

Chapter 3. Chronic adolescent exposure to cannabis in mice leads to long-term sex-biased changes in gene expression networks across brain regions

3.1 Introduction

Numerous studies have reported consistent and clear association between high frequency or dose of cannabis use and adverse long-term health effects, including higher risk of developing psychiatric disorders, dependence, memory deficits, and anxiety[136–140], posing a major public health concern especially for vulnerable populations, such as adolescents.

Adolescence is a critical developmental period for reproductive functions, brain maturation and emergence of sex differences in social, cognitive, and emotional behaviors[141]. The endocannabinoid system (eCB) plays an important role in brain maturation, and, as such, drug-induced alterations of the eCB system during adolescence could lead to substantial sex-specific behavioral alterations and increased risk for neuropsychiatric disorders in adulthood[141–143].

Although animal studies have recapitulated the impact of adolescent cannabis exposure on behavioral outcomes that are relevant to neuropsychiatric disorders, our knowledge of the underlying neurobiological processes remains limited. Long-lasting changes in gene expression and epigenetic marks are likely to be an important mechanism mediating drug-induced neuroadaptations[144]. However, limited data is available concerning sex-specific transcriptional responses to adolescent exposure to cannabinoids across different brain regions[145], limiting our ability to dissect brain region specificity and cross-brain networks in the female and male brain.

Multiple brain regions are involved in cognitive and reward processes or psychiatric disorders that are linked to early and frequent cannabis use. These brain regions included the PFC, which undergoes functional and structural remodeling during adolescence and mediates higher cognitive functions[146]; the nucleus accumbens (NAc), which drives motivation and reward

behaviors[147]; the dorsal medial striatum (DMS), which plays a critical role in goal-directed responses [48]; amygdala (Amy), which is critical for processing emotions and memories[149]; ventral tegmental area (VTA), which is important for a range of learning behaviors related to reward and stressful experiences[150]. However, the analysis of global gene expression changes altered by adolescent exposure to THC is limited to PFC in male rats[145].

Here, we used female and male mice that were chronically administered THC during adolescence and were subjected to behavioral and molecular analysis in adulthood. We assessed long-term recognition memory, social interaction, and anxiety-like behaviors in each mouse. Next, we measured gene expression changes by RNA sequencing (RNA-seq) in 5 brain regions that are involved in cognitive and addiction-related processes. Differential gene expression and gene co-expression network analyses were used to examine gene expression and gene network changes associated with adolescent exposure to THC for each sex and within and between brain regions. Lastly, we performed integrative analysis of coexpression networks affected by THC in mice with human cognition trait-related genes from genome wide association studies (GWAS) to identify links between adolescent THC use and neuropsychiatric disorders.

3.2 Methods

Animals

Forty-eight C57BL/6J male and female mice were purchased from Charles River. Mice were housed (3-4 per cage) under a 12h light/12h dark cycle and provided with food and water ad libitum. All experimental procedures were approved by the institutional animal care and use committee at University of California, San Diego.

Drug treatment protocol and experimental design

THC was provided by the U.S. National Institute on Drug Abuse and was dissolved in a vehicle solution consisting of ethanol, tween, and 0.9% saline (1:1:18) on the day of administration. As shown in **Figure 3.1A**, vehicle or THC (10 mg/kg) was administered daily to mice by intraperitoneal injections for the whole adolescent period in mice, from postnatal day (PND) 28 to PND 48, as previously reported[151,152]. To study the long-term behavioral effects of adolescent exposure to THC, we tested 2 independent cohorts of mice for behavioral assays (n = 24 mice/cohort) two weeks after the last injection, starting at PND 63. Behavioral assays were conducted on separate days and all behavioral tests were performed once on each mouse. To prevent bias due to olfactory cues, the behavioral apparatus was cleaned in between mice. At the end of the behavioral protocol, brains from one cohort (n = 24 mice) were collected in dry ice and stored at -80ºC before dissections and molecular analysis.

Elevated Plus-Maze (EPM) Test

The EPM was performed as described in Iemolo et al.[153]. Briefly, mice were placed individually onto the center of the maze for a 5-min period. The percent of total arm time [i.e.,100 × open arm/(open arm + closed arm)] was measured and used as an index of anxiety-related behavior[154].

Six different objects test (6-DOT)

We used the 6-DOT to study recognition memory in mice, as previously described[152] with minor modifications. Mice were left free to explore an empty arena during a 10 min habituation trial. Afterwards, mice were exposed to six different objects for 10 min (familiarization trial). After 24 hours, mice were exposed for 5 min to 5 familiar objects and a novel object (test trial). The exploration time for each trial was measured with Anymaze (Ugo Basile, Varese, Italy). Absolute discrimination index D1 = (time spent exploring novel object – average time spent exploring

familiar objects), and a relative discrimination index D2 = [(time spent exploring novel object – average time spent exploring familiar objects) / Total time spent exploring novel + familiar objects]. The D2 index also takes into account differences in exploration time between mice[155]. The discrimination indexes were used as measures of recognition memory. A longer time spent exploring the novel object is also interpreted as a sign of memory reconsolidation[156] or novelty seeking behavior[157]. During the habituation trial, total distance traveled (m) was measured to assess basal locomotor activity.

Three-chamber social approach test

The three-chamber social approach test was performed as previously described[152]. The target mouse was first placed in the center chamber and allowed to explore the apparatus for 15 min. After introducing the novel mouse and the novel object in the side chambers, the mouse was allowed to explore for 10 min. The placement of the novel mouse or novel object in the left or right chambers was systematically alternated in between trials. The time spent actively sniffing the novel mouse or the novel object was manually scored. Longer time exploring the novel mouse versus the novel object was considered an index of sociability. Social preference was calculated as [(Time sniffing novel mouse/(total time sniffing novel mouse and novel object))*100].

Body weight measurements

Body weight (g) was measured through the course of the drug administration protocol and when each behavioral assay began.

Statistical analysis of behavioral measures

To evaluate how factors (treatment, sex) influenced mice behavior, we used linear mixed models (LMM) in JMP pro v. 16.0 (SAS Institute, Inc). LMMs allow both fixed and random effects, which includes repeated measures[158]. We modeled the categorical predictor variables for the overall effect of treatment and sex, and we included sex x treatment interaction to study sex-specific effects. We used mouse cohorts and individual subjects as random effects (when appropriate) to account for possible non-independence of the data. Tukey HSD post hoc pairwise comparisons identified differences among specific groups. A $p < 0.05$ was considered significant. Outliers were detected using the Huber M-estimation method[159] in JMP pro 16.0 and removed when appropriate ($n = 1$ in absolute discrimination index D1 and $n = 1$ in social preference).

Brain punching

Brains were sliced on a cryostat, and 5 brain regions were collected using 1-2mm punches, starting at the following bregma coordinates: +2.68 (PFC), 1.78 (NAc), 1.10 (DMS), -1.34 (Amy), and -2.92 (VTA), for a total of 120 samples.

RNA extraction

Brain punches (n = 120) were homogenized in Trizol Reagent (Invitrogen, Cat, num. 15596018) and Zirconium Beads RNase Free (Next Advance, Cat. num. ZrOB05-RNA 0.5mm) using the Bullet Blender Blue (Next Advance, Model. num. BBX24B) at speed 6 for 1 min. RNA was then purified using the Direct-Zol kit (Zymo, Cat. num. R2052). RNA quality was assessed using the Agilent 2200 TapeStation system and samples with RIN above 7.5 were used for library preparations. One sample for a VTA vehicle-treated male mouse with low RIN was excluded.

RNA-seq library preparations

An average of 200ng of RNA was used for each sample. The NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs, Cat. Num. E7760S) was used following manufacturer's instructions. A total of 119 libraries were sequenced on the Illumina NovaSeq 6000 system (2 x 100 bp reads).

RNA-seq raw data processing and identification of differentially expressed genes (DEGs)

Raw sequencing reads were cleaned to remove adapter sequences and low-quality sequences using cutadapt v2.9[160] with the paired-end trimming mode and the minimum 18-base setting. The cleaned reads were aligned to mouse genome (mm10) using STAR v2.7.3a[161] following ENCODE parameters (https://github.com/ENCODE-DCC/long-rna-seq-pipeline/blob/master/DAC/STAR_RSEM.sh). The aligned reads were quantified at the gene level using RSEM v1.3.1[162]. The quantified gene-level counts were analyzed with DESeq2 v1.28.1[163] for differential gene expression analysis by comparing the THC- versus vehicle-treated groups for each brain region and sex. Significant DEGs were identified using FDR <5% or 10% and absolute log (fold change) > 0.4 as the cutoff. To identify sex x THC treatment interaction DEGs, we performed DEG analysis using the following formula for each brain region: gene expression ~ sex+treatment+sex:treatment. Significant sex:treatment interaction DEGs were identified using FDR < 10% as the cutoff.

Rank Rank Hypergeometric Overlap (RRHO) Analysis

To assess consistency or disagreement in the gene expression changes, we performed RRHO analysis using the RRHO2 R package[164, 165]. RRHO analysis detects the overlap of transcriptional profile change in the same or opposite directions between two datasets using a threshold-free approach. Full differential expression signatures were used to generate a ranking

score. RRHO heatmaps were generated using the RRHO2_heatmap function in the RRHO2 R package.

Weighted gene coexpression network analysis (WGCNA)

RNA-seq data was normalized with DEseq2 median of ratios normalization and then log-transformed, and genes with a standard deviation greater than 0 were chosen for WGCNA analysis. The WGCNA R package was used to generate ten signed coexpression networks for the PFC, NAc, DMS, Amy, and VTA for males and females separately[42]. An adjacency matrix was generated to capture pair-wise biweight midcorrelation between genes, using dataset-specific soft power for the best fit of a scale-free topology. A topological overlap measure (TOM) matrix was then calculated based on the adjacency matrix to measure relative interconnectedness between each pair of genes. Next, a gene dendrogram was built using the flashClust function based on hierarchical clustering. Branches of the dendrogram correspond to clusters of positively correlated genes, which are termed modules. The cutreeDynamic function based on a branch-cutting algorithm were used to identify modules using the following parameters: method=hybrid, minModuleSize = 30, pamStage = F, pamRespectsDendro = T. DeepSplit and cutHeight value were chosen on a per-network basis to optimize module detection. After module detection, module eigengenes (MEs) were calculated as the first principal component of genes in a module to summarize each module's expression profile. Modules with ME correlation higher than 0.85 were considered highly similar modules and merged using the mergeCloseModules function. To examine the similarity of each gene's expression profile with its module's ME, biweight midcorrelation to the ME was calculated for each gene as its module membership score. Hub genes were defined as genes with an intramodular connectivity score among the top 10% in the module and a gene module membership score higher than 0.8. To identify module-trait associations, Pearson correlation between module MEs and treatment, percentage of time on the open arm of the elevated plus

maze, social preference score, object discrimination indexes D1 and D2, and new object interaction time were performed. Correlation p value of 0.05 were considered significant.

Pathway annotation of DEGs, WGCNA coexpression modules, and subnetworks

Pathway annotation for the DEG sets and WGCNA modules from individual brain regions was performed using the enrichR R package[166]. General databases including KEGG, Reactome, GO Biological Process, BioCarta and WikiPathways from enrichR's database library were used. Pathway enrichment was calculated based on the deviation from the expected rank for terms, and P-values were adjusted for multiple testing corrections using Benjamini-Hochberg correction. Adjusted P values of 0.05 were considered significant.

DEG and cell type marker enrichment in WGCNA modules

To explore the potential cell type source for the coexpression modules, we used cell type markers curated from Werling and coathors[97] to perform enrichment analysis, including pyramidal neurons, interneurons, oligodendrocytes, microglia, activated microglia, astrocytes, mural cells, endothelial cells, and ependymal cells. Between each cell type marker set and each WGCNA module, a fold enrichment score and enrichment p-values were calculated using the phyper R function. We also assessed the overlap between the WGCNA modules and DEGs. DEGs with an FDR value lower than 0.05 and the absolute value of logFC equal to or higher than 0.4 were extracted for overlap analysis. DEGs were further split into upregulated and downregulated DEGs based on whether the corresponding logFC was positive or negative. Each DEG set was assessed for overlap with WGCNA modules using the method described for cell-type marker overlap above.

Weighted Key Driver Analysis (wKDA)

wKDA from the Mergeomics R package was used to identify key drivers of selected modules or DEGs based on a gene interaction network[167]. We used a brain-specific Bayesian network capturing causal gene-gene regulatory relationships constructed using the RimbaNet package[57]. Besides human and mouse brain genetic and transcriptomic datasets as previously described, we also integrated human brain-region specific RNA-seq and QTLs from GTEx version 8, and transcriptional factor data from FANTOM v5[36, 37, 168]. In wKDA, hub genes were first identified based on the input network's topology. Subsequently, the input module genes or DEGs were overlaid onto the network. The hub genes whose downstream network neighbors were enriched with the input module genes or DEGs were highlighted as key drivers for the trait-related genes or modules. 10000 permutations were performed and top 10 key drivers from each module were selected based on FDR value. Networks of prioritized key drivers were visualized using Cytoscape version 3.8.2[169].

Module-module interaction inference

To examine possible module-module interactions across brain regions, Pearson correlations between module MEs were calculated among modules from all five brain regions in males and females, respectively. Module-module correlations with a p-value < 0.05 and $|r| > 0.5$ were used for visualization and the following analysis. Cytoscape 3.8.2 and sankeyNetwork function from the networkD3 R package were used for visualization.

Associating DEGs and WGCNA modules with human GWAS traits

To test whether the THC-correlated WGCNA modules are enriched for genes implicated in related human phenotypes, we used the Mergeomics R package that integrates gene sets with GWAS summary statistics through functional genomics. The following GWAS summary statistics were acquired: (1) a GWAS meta-analysis of cannabis use disorder (CUD) by Johnson

et al.[170] from https://www.med.unc.edu/pgc/download-results/; (2) a GWAS of social interaction by Day et al.[171], from the NHGRI-EBI GWAS CatalogGWAS Catalog[172] (study accession GCST006920, GCST006921, and GCST006922); (3) a GWAS of cognitive aspects of educational attainment by Demange et al.[173] from the NHGRI-EBI GWAS CatalogGWAS Catalog (study accession GCST90011875); (4) a GWAS of memory performance by Davies et al.[174], from the NHGRI-EBI GWAS CatalogGWAS Catalog (study accession GCST003497); and (5) a GWAS of schizophrenia[175] from https://www.med.unc.edu/pgc/download-results/.

SNPs analyzed in each GWAS were adjusted for linkage disequilibrium with a cutoff of 0.5 to remove redundant SNPs. GWAS SNPs were mapped to genes using the following mapping strategies: (1) genes within 20kb distance of SNPs and (2) genes under regulation of tissue-specific expression and splicing quantitative trait loci (eQTLs/sQTLs) from GTEx v8 for brain regions matching or semi-matching those we investigated, including amygdala, nucleus accumbens, frontal cortex, caudate and putamen (representing striatum), and substantia nigra (close to VTA). GWAS SNPs, their mapped genes, and the corresponding GWAS p values were then intersected with coexpression modules for each brain region using the Marker Set Enrichment Analysis (MSEA) function in Mergeomics to assess whether coexpression modules, compared to random gene sets, are enriched for genes with low p value SNPs in each GWAS. Modules with an FDR < 0.05 are considered as significantly enriched for GWAS related genes.

Key driver genes from highlighted GWAS-associated modules were identified using wKDA as described above. To assess the enrichment significance of schizophrenia (SCZ) common variant genes and rare variant genes, permutation tests were performed by subsampling equal-sized subnetworks with the network of interest for 10000 times. Genes mapped to SCZ common variants were acquired from the NHGRI-EBI GWAS Catalog[172] (accession EFO_0004609). SCZ rare variant genes identified using whole exome sequencing (WES) were acquired from a curated list from SZDB2.0[176].

3.3 Results

THC administration during adolescence impairs cognitive behaviors in a sex-specific manner

We used 6-DOT to assess the long-term effects of adolescent exposure to THC on object recognition memory[152, 177]. THC exposure during adolescence significantly reduced the absolute (D1) and relative (D2) discrimination indexes by 36% and 30%, respectively, compared to vehicle control group (treatment effect $F(1, 41) = 7.1$, $p = 0.011$ for D1, and $F(1, 42) = 5.3$, $p = 0.026$ for D2, **Fig 3.1B-C**). Despite the lack of a significant interaction of sex with treatment ($F(1, 41) = 3.9$, $p = 0.056$ for D1, and $F(1, 42) = 1.9$, $p = 0.176$ for D2), the effect tended to be stronger in females than males ($t = 3.35$, $p = 0.009$ for D1, and $t = 2.64$, $p = 0.055$ for D2). Moreover, only female mice previously exposed to THC showed a significant 40% decrease in the exploration of the novel object compared to the vehicle control group (sex x treatment interaction, $F(1, 42) = 4.6$, $p = 0.038$, **Fig. 3.1D**).

The behavioral alterations triggered by THC were specific for the cognitive components of the assay, as treatment had no detectable effect on the distance traveled during habituation phase (effect and interaction $p > 0.1$, **Fig. 3.2A**) nor on the total amount of exploratory activity towards objects in familiarization or test phase (effect and interaction $p > 0.1$, **Fig. 3.2B-C**).

When we assessed social behaviors, social preference was significantly decreased by 12% in both female and male mice exposed to THC during adolescence compared to the vehicle group (treatment effect $F(1, 41)$, $p = 0.016$**, Fig. 3.1E**).

Next, we examined anxiety-like behaviors and did not observe significant differences in the willingness of mice to explore open environments, which was measured by the time spent in the open arms (treatment and interaction $p > 0.1$, **Fig. 3.2D**).

Finally, treatment was not associated with changes in body weight at the time of behavioral testing, excluding potential confounding effects on exploratory activity in any behavioral task (treatment and interaction $p > 0.1$, **Fig. 3.2E**).

Overall, these results demonstrate that chronic exposure to THC during adolescence leads to long-term deficits in recognition memory and social interaction, with stronger memory deficits observed in female mice.

Identification and functional annotation of gene expression changes associated with chronic adolescent exposure to THC

We profiled the transcriptome of PFC, DMS, NAc, Amy and VTA from vehicle and THC-treated mice. PCA on the raw RNA-seq data showed that the studied brain regions can be distinguished based on their transcriptome (**Fig. 3.3A**).

We identified DEGs from each of the brain regions in each sex by comparing THC and vehicle groups (**Table S1**). We found the largest number of significant DEGs in the Amy of female mice (n = 549 DEGs) and in the NAc of male mice (n = 22 DEGs) using a cutoff of FDR < 0.05 and logFC >0.4 (**Fig. 3.3B**). At a less stringent statistical cutoff of FDR < 0.1 and logFC > 0.4, we observed the same sex- and brain-region specificity, and in general we identified more DEGs in females in most brain regions except NAc, which has a larger number of DEGs in males. To explore the influence of sex on transcriptional responses to THC, we compared expression changes induced by THC treatment between males and females. At a statistical cutoff of FDR < 0.1, this analysis yielded significant sex x treatment interactions for DEGs in Amy (n = 106), DMS (n = 7), and NAc (n= 13), but not for PFC and VTA (**Fig. 3.3C, Fig. 3.4**), indicating that females and males responded differently to THC.

Based on the DEGs, we assessed the enrichment for biological pathways altered by THC (**Fig. 3.3D**, **Table S2**). In female Amy and DMS, DEGs showed enrichment for overlapping pathways, including addiction-related pathways, opioid signaling and GPCR ligand binding. Moreover, DEGs in female Amy were related to neurotransmission, axon guidance and retrograde cannabinoid signaling. Genes that were up-regulated only in females were linked to presynaptic SNARE complex formation and several signal transduction pathways (e.g. mTOR, Wnt, oxytocin signaling). In contrast, pathways associated with DEGs in male NAc did not overlap with those identified in females and were related to interferon signaling and ubiquitin-mediated proteolysis.

In agreement with DEG analysis, threshold-free RRHO analysis showed that there was minimal overlap in DEGs when we compared gene expression changes between most pairs of brain regions or between sexes (**Fig. 3.5**).

Together, these data provide strong evidence for sex-specific regulation of transcriptional responses by THC across several brain regions.

Identification of gene coexpression networks correlated with THC administration and cognitive traits

Because DEG analysis found limited significant changes at the level of individual genes across brain regions and sexes, we reasoned that gene network modeling approaches would be a more powerful method to provide a broader characterization of the biological changes induced by THC treatment in the brain.

Therefore, we constructed WGCNA gene coexpression networks for each sex within each brain region. Module membership and significance for each gene are reported in **Table S3**. Next, we performed trait-module correlation analysis to identify gene coexpression networks correlated with THC treatment and/or cognitive traits (correlation values and significance for each module

and trait are reported in **Table S4** and visualized in **Fig. 3.6**. As shown in **Fig. 3.6A**, this analysis identified 29 modules significantly correlated with THC treatment (referred to as "THC-correlated modules") and 12 modules significantly correlated with memory traits from the 6-DOT ($p < 0.05$). Consistent with the DEG analysis, a larger number of THC-correlated modules was observed in female Amy (n = 9) and male NAc samples (n = 7), while male PFC and Amy modules showed no correlation with THC treatment or memory traits. Social preference was correlated with 9 modules (**Table S4**); however, there was no overlap with THC-correlated modules.

Next, we performed pathway enrichment analysis of THC-correlated modules. We report the enriched terms for each module in **Table S5** and highlight top enriched terms in **Fig. 3.6B-C**. This analysis showed that some pathways were uniquely enriched in specific THC-correlated modules, while others were shared among several modules. Among uniquely regulated pathways, we identified glutathione metabolism in female Amy (*white* module), p53 signaling in female PFC (*tan* module), pregnane X receptor pathway in female DMS (*violet* module), glial differentiation in female VTA (*red* module), and phosphonate metabolism in male VTA (*cyan* module). Among the shared pathways, the *greenyellow* module from female Amy and the *blue* module from male NAc were enriched for genes implicated in dopaminergic neurotransmission and addiction-related pathways and were both positively correlated with THC treatment. In addition, the *tan* module from female DMS was related to dopamine signaling but negatively correlated with THC. Other THC-correlated modules in female Amy (*black, yellow*) and male NAc (*orange*) were related to similar pathways, such as regulation of glutamatergic synapse, but displayed positive correlation in females and negative correlation in males with THC.

Of note, we identified modules that were simultaneously correlated with THC treatment and memory traits, thus revealing gene networks that are directly associated with cognitive deficits observed in mice after adolescent exposure to THC. Among these modules, the *saddlebrown*

module (enriched for genes involved in the metabolism of endogenous cannabinoids) in the female DMS, the *bisque4* (enriched for interferon signaling genes) and lightsteelblue1 modules (enriched for differentiation processes) in the female VTA were negatively correlated with THC treatment but positively correlated with memory traits (**Fig. 3.6C**). Finally, in the male NAc, the *darkgrey* module (no pathways enriched) showed positive correlations with both THC and memory traits but the *orange* (enriched for synaptic transmission) module showed a negative correlation with both THC and memory traits (**Fig. 3.6B**).

Comparison between the THC-correlated modules and DEGs revealed that the majority of THC-correlated modules did not overlap with DEGs, with the exception of a few cases in which we observed an enrichment of DEGs in specific modules from the same tissue (**Fig. 3.7**). These results indicate that network analysis captures additional information about responses of coordinated gene sets that goes beyond what changes in individual DEGs reflect.

Taken together, WGCNA indicates that distinct gene coexpression networks are influenced by THC in a brain region- and sex-specific manner.

Characterization of cellular specificity of THC correlated gene coexpression networks

To identify cell type contributions to co-expression modules, we performed cell-type marker enrichment analysis within each module. We observed strong cell type specificity for gene coexpression networks associated with THC from several brain regions (**Fig. 3.8**).

THC treatment in female Amy, female NAc and male NAc presumably involved neuronal cell types: modules positively correlated with THC showed enrichment of pyramidal neuron markers, while modules negatively correlated with THC showed enrichment for interneuron markers (**Fig. 3.8A**). This result was consistent with their association with pathways related to dopamine signaling, addiction and glutamatergic synapse (**Fig. 3.3D**).

In contrast, 3 out of 4 THC-correlated modules from female VTA were negatively correlated with THC and enriched for markers of glial cells, including astrocytes, oligodendrocytes, and microglia (**Fig. 3.8B**), which was supported by the immune functions revealed by the pathway analysis (**Fig. 3.3D**). In addition, one module was positively correlated with THC and enriched in neuronal markers.

Finally, both neuronal and non-neuronal cells contributed to cell type enrichment in female DMS and male VTA THC-correlated modules (**Fig. 3.8C**). In female DMS, the 3 THC-correlated modules showed negative correlation with treatment and were enriched in both neuronal and glial markers, including pyramidal neurons, interneurons, astrocytes, and oligodendrocytes. In male VTA, one THC-correlated module showed negative correlation with treatment and was enriched for oligodendrocytes markers, while one module was positively correlated with THC and enriched for both neuronal and non-neuronal (mural and ependymal) cell markers.

Together these results suggest that cell type-specific mechanisms are triggered by THC administration in a sex- and region-specific manner.

Identification of potential regulators of THC-correlated modules

To better understand the mechanisms underlying changes in transcriptional responses to THC, we performed network analyses on coexpression modules and identified “hub genes" based on intra-modular connectivity and “key driver genes” based on causal gene regulatory relationships. Hub genes and key driver genes represent potential regulators of THC-correlated coexpression networks. We identified a total of 104 hub genes with high degree of intra-module connectivity across modules that were significantly correlated with THC treatment (**Table S6**). In **Figure 3.9A-D**, we highlight gene interaction networks that show correlation with both THC and memory traits, including female DMS *saddlebrown*, female VTA *bisque4* and *lighsteelblue1*,

male NAc *orange* and *darkgrey* modules. Among these hub genes, several were involved in the regulation of neuronal plasticity and memory processes. These hub genes included *Lynx1* (*saddlebrown*, **Fig. 3.9A**) which encodes for a protein that suppresses memory processes by inhibiting nicotinic receptors-induced neuronal plasticity [178, 179]; Cyp46a1 (saddlebrown, **Fig. 3.9A**) which is the rate-limiting enzyme for cholesterol degradation and is linked to neuroprotective effects[180]; Sgpl1 (bisque4, **Fig. 3.9B**) which is implicated in lipid metabolism and cognitive functions[181]; Gng7 (lightsteelblue1, **Fig. 3.9C**) which encodes for a synaptic heterotrimeric G protein with a role in dopamine receptor stimulation and cognitive abilities[182, 183].

We also used the wKDA analysis in Mergeomics coupled with a brain-specific Bayesian gene regulatory network to predict key driver genes regulating the THC-correlated coexpression networks[168]. In contrast to coexpression module hub genes which are purely based correlation-based connectivity, Bayesian gene regulatory network reveals gene regulatory relations and pinpoint potential causal regulatory relationships. This analysis identified 141 key drivers for the modules correlated with THC treatment (**Table S6**). These key driver genes included *Drd1* and *Gpr88*, which were shared between female Amy *greenyellow* and male NAc blue modules (**Fig. 3.9E**). These genes are known regulators of addition processes, which agrees with the pathway enrichment analysis of Amy greenyellow and male NAc blue modules. *Drd1* encodes the dopamine receptor subtype 1, which is highly expressed in NAc and is a key regulator of addiction behavior[184, 185]. *Gpr88* encodes an orphan G-protein-coupled receptor implicated in drug taking behavior[186].

These analyses identify new genes that are potential regulators of the long-term behavioral effects of adolescent exposure to THC, including cognitive impairments and addictive behaviors.

Cross-brain region module-module interactions affected by THC administration

Next, we analyzed the correlations between modules within and between brain regions to identify potential intra- or inter-region molecular circuitries that are disrupted by THC. Thus, we define "THC-interconnected modules" as those gene coexpression networks that are significantly correlated with THC-correlated modules with $|r| > 0.5$. We identified numerous THC-interconnected modules in both females (**Fig. 3.10A**) and males (**Fig. 3.10B**), indicating that additional gene networks in the same region or across brain regions are influenced by THC-correlated modules. Sankey diagrams illustrate the negative or positive correlations of THC-interconnected modules (**Fig. 3.10C-D**). In both sexes, positive and negative correlations were relatively balanced across regions. In females, higher levels of connectivity were observed between Amy-Amy modules, followed by Amy-VTA, VTA-VTA, DMS-DMS and Amy-DMS (**Fig. 3.10E**). In males, higher levels of connectivity were observed between NAc-NAc, followed by NAc-VTA, NAc-PFC, VTA-NAc, and VTA-VTA (**Fig. 3.10F**). Consistent with the DEG analysis, the module-module interaction analysis revealed a significant sexual dimorphism with a broader impact of THC on module-module connection patterns in Amy in females and in NAc in males (**Fig. 3.10E-F**).

Overall, these findings suggest that chronic exposure to THC during adolescence leads to long term changes in molecular circuitries across different brain regions in a sex-specific manner.

Genetic association of THC-related coexpression modules for human cognitive traits and disorders

Next, we examined the relationship between the coexpression networks altered by THC in mice and human cognitive phenotypes related to cannabis use disorders, such as those examined in GWAS for CUD, SCZ, memory performance, cognitive aspects of educational attainment, and social interaction. Using Mergeomics (**Fig. 3.11A**) [168], we generated a list of "GWAS-associated genes" by integrating GWAS summary statistics, distance mapping and eQTLs/sQTLs from

human brain regions that were matching those examined in mice for THC response. We then defined “GWAS-associated modules” as those WGCNA modules enriched in GWAS-associated genes for each sex within each brain region. We provide a detailed list of module associations for each human trait in **Table S7**. THC-correlated modules only overlapped with 11.3% and 6.1% GWAS-associated modules in females and males, respectively (**Fig 3.11B**). However, when we included THC-interconnected modules into the analysis, the overlap increased to 79% and 54% in females and males, respectively (**Fig. 3.11C**). This suggests a potential role of THC-interconnected modules in THC-induced behavioral abnormality, despite the fact that they are not directly correlated with THC treatment from our animal model.

To evaluate the biological significance of GWAS-associated modules that respond to THC, we focused on the functional annotations for gene networks that are significantly enriched for genes from at least 3 studied human cognitive traits or disorders, plus significant correlation with THC treatment in mice (inferred from either from THC-correlated or interconnected modules). These modules and their functional annotations are highlighted in **Figure 3.12A-B**. Our analysis not only confirmed known pathways relevant to cognitive processes and psychiatric disorders, such as glutamatergic transmission (female NAc *blue*, Amy *yellow*, and male NAc *orange*, DMS darkred and Amy *darkorange*), dopaminergic transmission (female NAc *darkmagenta* and VTA *blue*), ubiquitin mediated proteolysis (female Amy *darkorange*), axon guidance (female DMS *darkorange/paleturquoise* and male VTA blue), and immune function (female VTA *darkorange*, male MAc *royalblue*), but also pinpointed the brain region and specificity for these pathways. Importantly, our analysis also revealed relatively new pathways and their specific tissue and sex contexts, such as glycosaminoglycan metabolism (female PFC *black*), inositol metabolism (male DMS *pink*) and PPARalpha signaling (male DMS *greenyellow*).

To identify potential regulatory components of the gene networks altered by THC and enriched in GWAS-associated genes, we focused on SCZ associated genes given the potential link

between SCZ vulnerability and adolescent cannabis use, and the availability of both common and rare variants from well-powered GWAS and whole exome sequencing (WES) studies in humans. Here, we report key driver genes of the female Amy *yellow* and *darkorange* modules which were selected because the female Amy transcriptome is more potently altered by THC compared to other brain regions. We found that both yellow and darkorange networks were significantly enriched for common and rare variant genes for SCZ as demonstrated by a permutation test (**Fig. 3.12C**). As shown in **Figure 3.12D**, 10 out of 20 key drivers also overlapped with SCZ variant genes. The vast majority of key driver genes encode membrane proteins and are localized at the synapse and vesicles. Besides, several key driver genes engage in brain development, including *Plxna4*, *Syngr3*, *Agap2*, *Camk1d* and *Atp6v0d1*.

Subnetwork pathway analysis revealed that 10 out of the 20 key driver genes (*Jph4, Plxna4, Rimbp2, Rnf187, Syp, Syngr3, Tmem130, Agap2, Apba1* and *Camk1d*) orchestrate tissue-specific subnetworks of genes implicated in synaptic transmission processes, including chemical synaptic transmission, neurotransmitter release cycle, GABA transmission, synaptic vesicle cycle, and calcium signaling. 5 out of the 20 key drivers (*Agap2, Rimbp2, Tmem130, Camk1d, Lonrf2*) regulate central nervous system development related subnetworks. Three key drivers (*Atp6vod1, Rnf187, and Ube20*) regulate glucose metabolism subnetworks. We also identified key drivers (*Pi4ka, Rnf187, Ldoc1*) regulating mitochondrial biogenesis, antigen processing and protein deubiquitination subnetworks.

Taken together, this integrative analysis supports a strong mechanistic connection between genes and pathways altered by THC and those associated with schizophrenia vulnerability.

3.4 Discussion

Our work provides the first comprehensive, tissue- and sex-specific view of molecular processes perturbed by adolescent THC treatment in mice. Our findings suggest that females are more sensitive than males to the long-term detrimental effects of THC on recognition memory, which coincides with larger transcriptional responses in the female brain. The gene network analysis identified potential regulators and biological pathways altered by THC, including cognitive and addiction processes. Lastly, by intersecting our mouse transcriptomic data with human GWAS of neuropsychiatric disorders and cognitive behavioral traits, we found a connection between gene networks affected by THC and human traits and disorders known to be influenced by cannabis use.

Our behavioral analysis demonstrated that adolescent exposure to THC in mice led to long-term impairments in object recognition memory and social interaction, but not in anxiety-like behaviors. The results concerning memory and social interaction are in line with previous reports in rodent models[152, 187–193]. However, conflicting results have been reported concerning anxiety-like phenotypes likely due to varying mice genetic background and experimental conditions among different research groups (e.g., THC dose, mice age)[194–196]. Our study also showed sex differences in the effects of THC on recognition memory, which was impaired more in female compared to male mice. While the influence of sex on the effects of THC on memory has not been evaluated in animal models, female rats have been reported to be more susceptible than males to the effects of THC on locomotor activity, nociception, and reward processes[197–201]. Importantly, the greater sensitivity of females to the harmful effects of THC on spatial memory is evident also in humans[202–206]. Overall, our behavioral data confirms the harmful effect of THC on cognitive functions and highlights the importance of addressing sex differences when studying the long-term impact of cannabis exposure.

Sex differences in gene expression patterns may elucidate the mechanisms underlying sexual dimorphism in behavioral phenotypes in response to drugs[207]. In agreement with this

hypothesis, our study demonstrated that adolescent exposure to THC caused sex-specific gene expression changes in the brain. When we identified DEGs or THC-correlated modules separately for each sex, female mice showed major transcriptional alterations in the Amy, while male in the NAc. In support of sex-specific responses to THC, RRHO analyses showed limited overlap in gene expression changes induced by THC between males and females for each brain region. Further, when we conducted a statistical analysis that explicitly tested for sex differences in DEGs, we found significant sex x treatment interaction in Amy, NAc and DMS. Although only a fraction of DEGs showed a significant sex x treatment interaction (FDR < 0.1), presumably due to lack of statistical power for specific datasets, this is the first study to provide strong statistical evidence for sexual dimorphism in the transcriptional responses to THC, which may underlie sex-specific behavioral alterations. These sex-specific gene expression differences could also influence other mechanisms known to play an important role in determining THC effects on females and males, including sex-specific effects of hormonal changes on the endocannabinoid system during adolescence[208], differential density of cannabinoid receptors in the brain[209], and pharmacokinetic factors[210]. Taken together, these results suggest that female mice with a history of adolescent exposure to THC exhibit more profound cognitive behavioral deficits and larger transcriptional alterations than males.

It is unknown what particular brain region drives the behavioral abnormalities induced by adolescent exposure to THC. Our work that simultaneously investigates 5 brain regions, for the first time, suggests an extensive brain region specificity in the gene signatures and networks altered by THC. The analysis of sexually dimorphic DEGs and THC-correlated modules revealed that female Amy and male NAc may be sites of particular importance for the persistent changes induced by adolescent exposure to THC. In line with these findings, brain morphological studies of human cannabis users have shown that marijuana use may be associated with the disruption of neural organization of the Amy and NAc[211]. Moreover, previous

studies have documented that female teenagers who use marijuana are more susceptible than males to structural abnormalities of the Amy, which were correlated with worse internalizing symptoms[212]. Consistent with these observations, animal studies also reported perturbation of synaptic transmission in the Amy and NAc following administration of exogenous cannabinoids[213–215]. However, previous analysis of transcriptomic changes induced by adolescent exposure to THC have focused only on PFC of male rats 10. Miller et al reported that adolescent exposure to THC was associated with long-term gene expression changes in the PFC, which were related to cytoskeleton and chromatin regulation[145]. While we did not identify any DEGs or THC-correlated modules in the male PFC in mice, we found 2 THC-interconnected modules in the male PFC, which were linked to the same biological pathways. Between-species differences may explain the discrepancy.

Our multiple brain region studies also allowed us to uniquely infer network connections within and between brain regions. The THC-interconnected modules are likely indirectly influenced by THC, as they are inferred from the module-module interaction network. We speculate that the inter-region interaction network could predict how THC directly influences one brain region which then cascades down to other brain regions. Specifically, our analysis predicts that adolescent exposure to THC alters neural circuits that connect Amy with VTA and DMS in females, and neural circuits that connect NAc with VTA and PFC in males. Future experiments perturbing the THC modules using animal models will be necessary to validate these predictions.

A major strength of this study is that we can directly correlate the transcriptional responses to THC in different brain regions with the cognitive traits altered by THC in the same mice. The trait-module correlation analysis suggested that the disruption of endogenous cannabinoid signaling in the DMS and inflammatory pathways in the VTA may drive the deficits in recognition memory observed in female mice. We also showed that neuronal cell types in the DMS and glial

cells (astrocytes and microglia) in the VTA may contribute to the transcriptional response to THC underlying memory deficits. These results are in line with previous reports on the effect of chronic administration of cannabinoids. The endocannabinoid system plays an essential role in learning and memory processes, with the engagement of the dorsal striatum specifically in encoding habit-related memories[216–218]. Moreover, repetitive exposure to synthetic cannabinoids led to inflammatory phenotypes including astrogliosis in the VTA[219].

Moreover, we also identified modules that may underlie behavioral alterations not measured in our study, such as addictive behavior. For instance, the neuronal-specific coexpression modules in female Amy (*greenyellow*) and male NAc (*blue*) were positively correlated with THC and shared enrichment for genes implicated in dopamine signaling and addiction processes. Different key drivers were identified as key regulators of the combined gene network including *Drd1* and *Gpr88*, which are known to regulate drug seeking behaviors[184-186].

We conclude that adolescent exposure to THC leads to different behavioral abnormalities by reprogramming cell type-, brain region-, and sex-specific biological pathways.

The frequent use of cannabis during adolescence has been linked to deleterious long-term consequences in humans, including an elevated likelihood of earlier onset of schizophrenia, cannabis use disorders and addiction[220–228]. We found coexpression modules that both respond to THC treatment and are enriched in genes relevant to human cognitive traits, schizophrenia, and CUD. These modules are involved in pathways already reported to be implicated in the development of psychiatric disorders, such as synaptic transmission, dopaminergic transmission, axon guidance, MAPK regulation, chromatin organization and immune functions[220–226]. We have also identified novel pathways that link chronic adolescent THC exposure to SCZ, such as sumoylation and glycosaminoglycan metabolism in females and inositol metabolism in males. This analysis also revealed that three key driver genes (*Atp60vd1,*

Rnf187, Ube20) regulate glucose metabolism, suggesting a novel link between THC use and schizophrenia, which is also supported by evidence in human studies[229].

Taken together, our results corroborate previous findings on the correlation of cannabis use with negative mental outcomes, and pinpoint potential coexpression modules, pathways and genes driving pathological mechanisms.

Our results should be considered in light of certain limitations. First, we focus on probing transcriptomic changes that occur during early adulthood and we correlate these gene expression patterns with behaviors measured at the same time. This approach has the advantage of capturing persistent changes associated with a history of adolescent exposure to THC. However, a limitation of this study design is that it cannot directly assess the earlier transcriptional changes and the concomitant behavioral correlates during the adolescent period, which we hypothesize to be particularly vulnerable to THC exposure. Moreover, we use a single dosage of THC (10mg/kg) and do not examine overall dose response curves. This dosage was selected as it leads to plasma levels of THC similar to those detected in humans using cannabis[230,231]; hence, it can mimic heavy chronic cannabis intake. The second limitation of our study is that we focus on behavioral phenotypes of interest limited to cognitive behaviors, such as recognition memory and social interaction. However, other behaviors are likely to be influenced by THC, including addiction-like phenotypes. It is important to note, however, that addictive properties of THC are not well modeled in mice. Moreover, our study is limited to 5 brain regions and other additional gene regulatory networks can be missed. Thus, it would be important to expand this study to other brain regions, such as the hippocampus given its critical role in recognition memory.

In spite of these limitations, our study is the first to unveil the potential underlying molecular mechanisms underpinning the persistent behavioral alterations linked to cannabis use in a sex-

and brain region-specific manner. Our study also provides, for the first time, a comprehensive database of molecular events associated with THC including individual genes, gene networks, pathways, and cell types for further study.

3.5 Figures

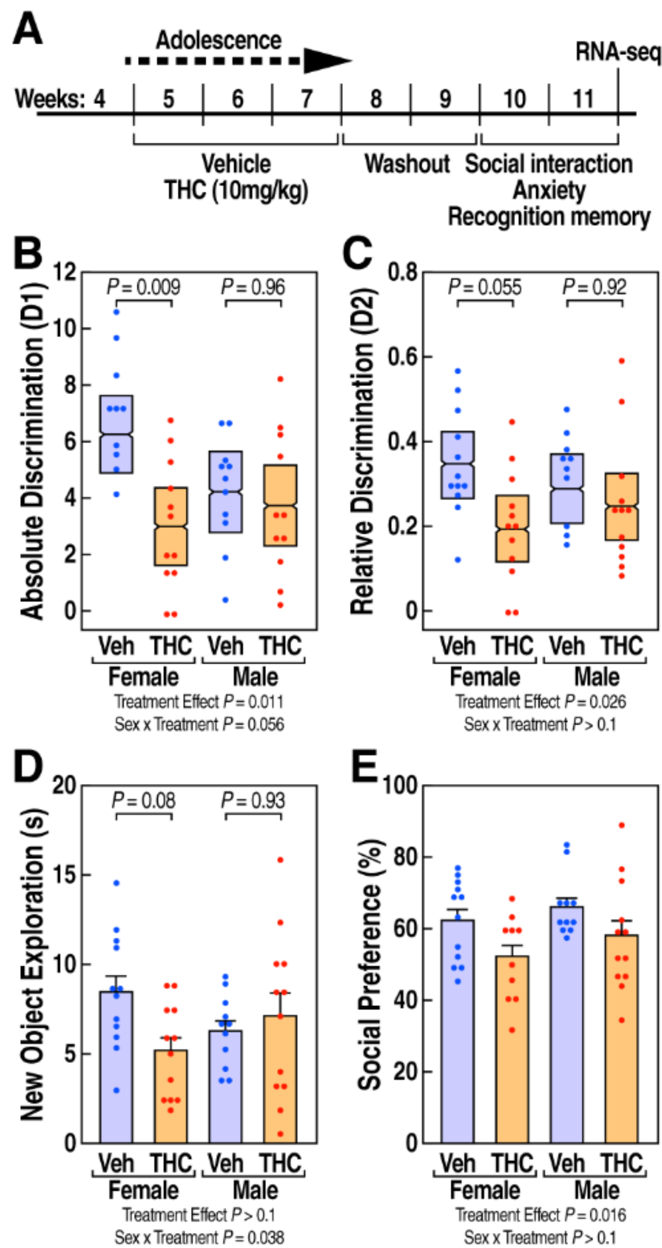


Figure 3.1. Adolescent exposure to THC reduced recognition memory and social interaction in a sex-specific manner.

(A) Timeline of the study design. (B) D1 and (C) D2 are shown as mean ± 95% confidence intervals showing decreased recognition memory in THC-treated groups compared to vehicle

controls. Treatment effect $F_{1, 41} = 7.1$, $p = 0.011$ for D1, and $F_{1, 42} = 5.3$, $p = 0.026$ for D2. Sex x Treatment interaction $F_{1, 41} = 3.9$, $p = 0.056$ for D1, and $F_{1, 42} = 1.9$, $p = 0.176$ for D2. (D) Exploration time (s) of the novel object is expressed as mean ± SEM showing a decrease only in female mice. Sex x treatment interaction, $F_{1, 42} = 4.6$, $p = 0.038$. (E) Social preference (%) is expressed as mean ± SEM and does not change across groups. Treatment effect $F_{1, 41}$, $p = 0.016$. P values from Tukey HSD post-hoc analyses are shown in panel B-D.

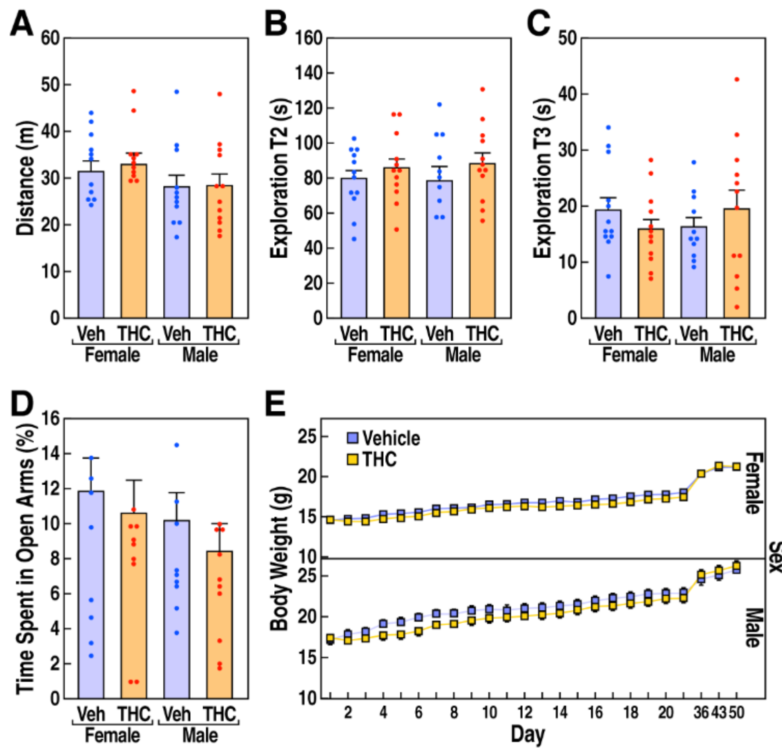


Figure 3.2. Behavioral characterization of female and male mice following adolescent exposure to THC.

(A) Locomotor activity is expressed as mean distance traveled (m) ± SEM. Exploration (s) in familiarization phase T2 (B) and test phase T3 (C) are expressed as mean ± SEM. (D) Body weight (g) is expressed as mean ± SEM for the THC administration course (day 1 to 21), and for day 36, 43, and 60 when the three behavioral assays started. All effect and interaction p values > 0.1.

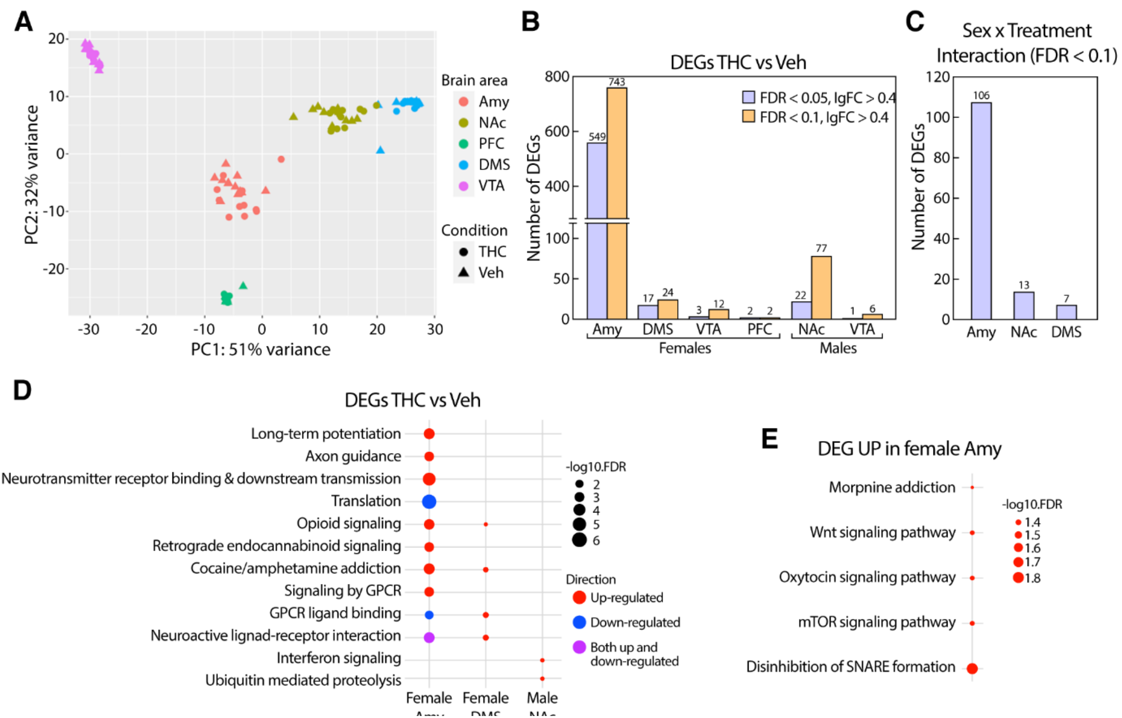


Figure 3.3. Adolescent THC exposure induced long-term sex-specific transcriptional changes.

(A) PCA visualization of male and female samples across brain regions and treatment conditions. (B) Bar plot of treatment affected DEG numbers across brain regions and sexes using different FDR cutoffs in analyses within each sex and brain region. (C) Bar plot of DEG numbers for treatment by sex interaction across brain regions in analyses including both sexes for each brain region. (D) Dot plot of treatment affected DEG pathway enrichment in female Amy, DMS, and male NAc. Dot color depicts the direction of regulation and dot size illustrates the significance. (E) Dot plot of upregulated treatment:sex interaction DEG pathway enrichment in female Amy. Dot size illustrates the significance. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area; Veh, vehicle; DEG, differential expression gene.

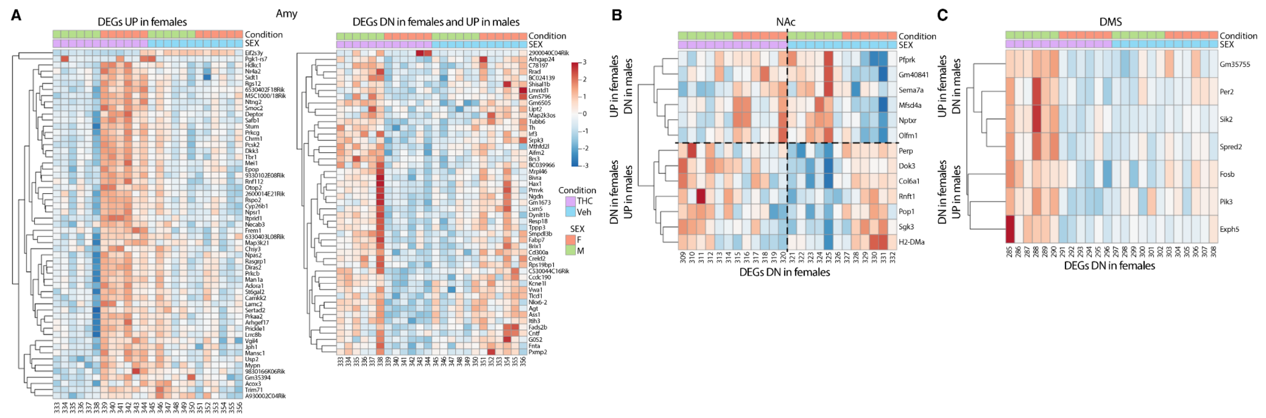


Figure 3.4. Adolescent THC administration induced sex-specific transcriptional changes.

Heatmaps of treatment by sex interaction DEGs in Amy (A), NAc (B), and DMS (C). DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; Veh, vehicle; DEG, differential expression gene; UP, upregulated; DN, downregulated.

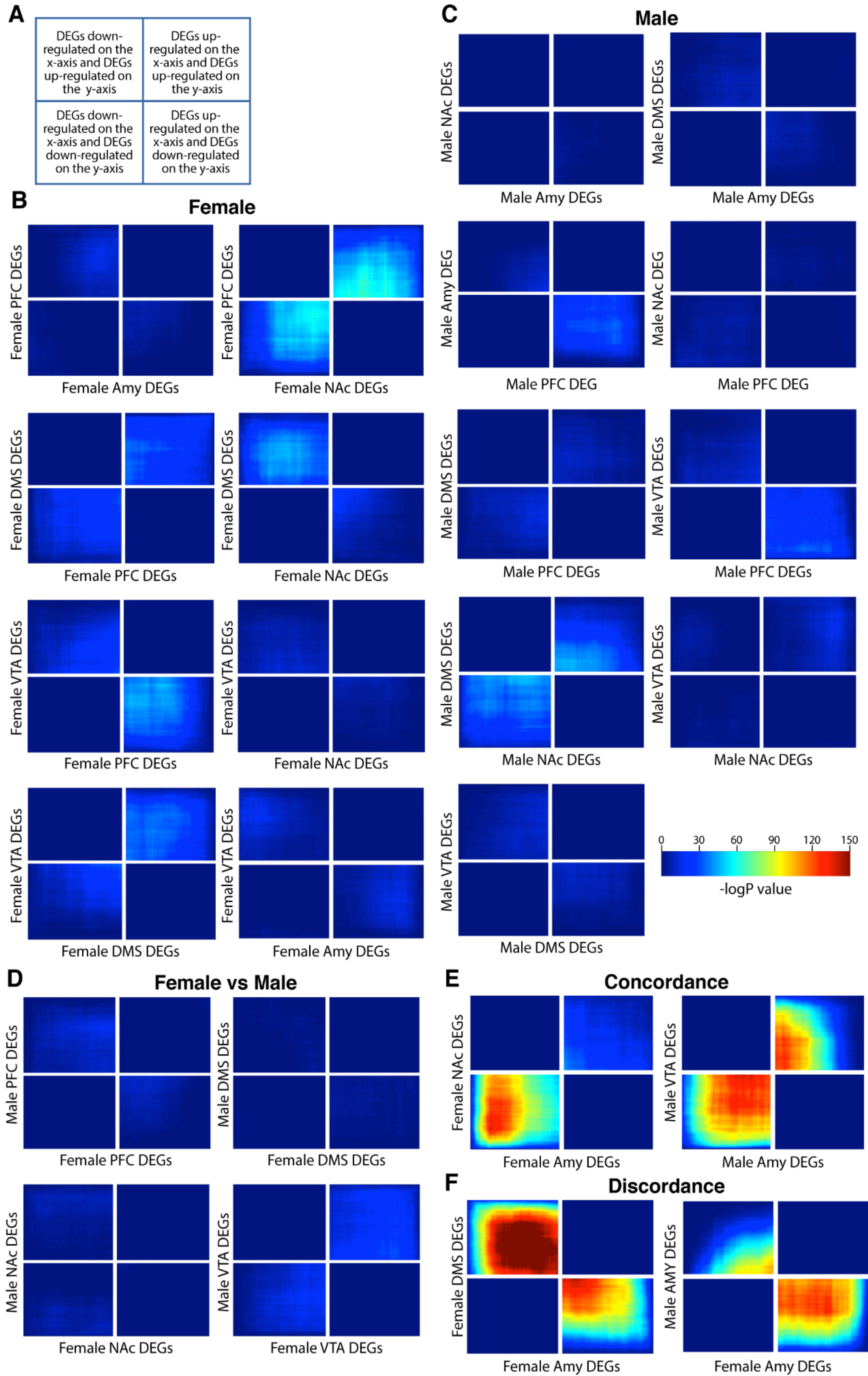
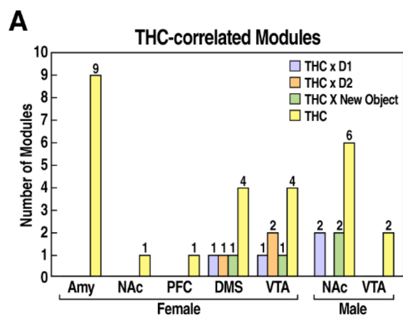
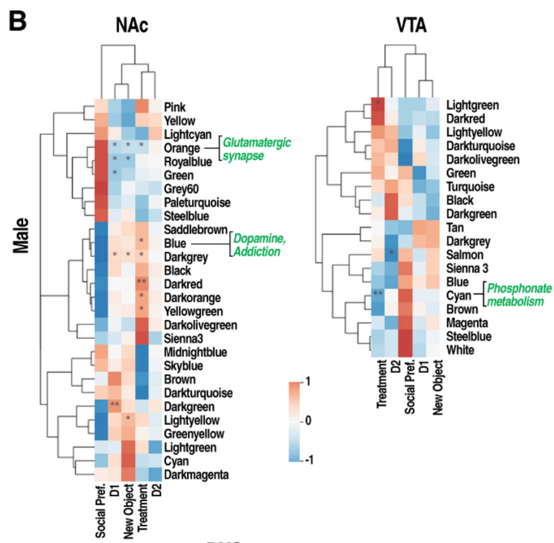

A
DEGs down-regulated on the x-axis and DEGs up-regulated on the y-axis
DEGs up-regulated on the x-axis and DEGs up-regulated on the y-axis
DEGs down-regulated on the x-axis and DEGs down-regulated on the y-axis
DEGs up-regulated on the x-axis and DEGs down-regulated on the y-axis
B
Female
Female PFC DEGs
Female Amy DEGs
Female NAc DEGs
Female DMS DEGs
Female PFC DEGs
Female VTA DEGs
Female DMS DEGs
Female Amy DEGs
C
Male
Male NAc DEGs
Male Amy DEGs
Male DMS DEGs
Male Amy DEG
Male PFC DEG
Male NAc DEG
Male PFC DEGs
Male VTA DEGs
Male NAc DEGs
Male DMS DEGs
0
30
60
90
120
150
-logP value
D
Female vs Male
Male PFC DEGs
Female PFC DEGs
Male DMS DEGs
Female DMS DEGs
Male NAc DEGs
Female NAc DEGs
Male VTA DEGs
Female VTA DEGs
E
Concordance
Female NAc DEGs
Female Amy DEGs
Male VTA DEGs
Male Amy DEGs
F
Discordance
Female DMS DEGs
Female Amy DEGs
Male AMY DEGs
Female Amy DEGs

Figure 3.5. Minimal transcriptional overlap was detected in most comparisons across sex and brain regions.

(A)Schematic of RRHO heatmap interpretation. (B-D) RRHO heatmaps show minimal overlap in transcription between pairs of brain regions within females (B), males (C), and between sexes (D). (E-F) RRHO heatmaps show concordance (E) and discordance (F) in transcription between pairs of brain regions. We define concordant as positive correlation and discordant as negative correlation in DEG overlap patterns. Amy, amygdala; DMS, dorsomedial striatum; VTA, ventral tegmental area; PFC, prefrontal cortex; NAc, nucleus accumbens; DEG, differential expression gene.


A
THC-correlated Modules
Number of Modules
THC x D1
THC x D2
THC X New Object
THC
Amy
NAc
PFC
DMS
VTA
NAc
VTA
Female
Male


B
NAc
VTA
Male
Pink
Yellow
Lightcyan
Orange
Royalblue
Green
Grey60
Paleturquoise
Steelblue
Saddlebrown
Blue
Darkgrey
Black
Darkred
Darkorange
Yellowgreen
Darkolivegreen
Sienna3
Midnightblue
Skyblue
Brown
Darkturquoise
Darkgreen
Lightyellow
Greenyellow
Lightgreen
Cyan
Darkmagenta
Glutamatergic synapse
Dopamine, Addiction
Social Pref.
D1
New Object
Treatment
D2
Lightgreen
Darkred
Lightyellow
Darkturquoise
Darkolivegreen
Green
Turquoise
Black
Darkgreen
Tan
Darkgrey
Salmon
Sienna 3
Blue
Cyan
Brown
Magenta
Steelblue
White
Phosphonate metabolism
Treatment
D2
Social Pref.
D1
New Object

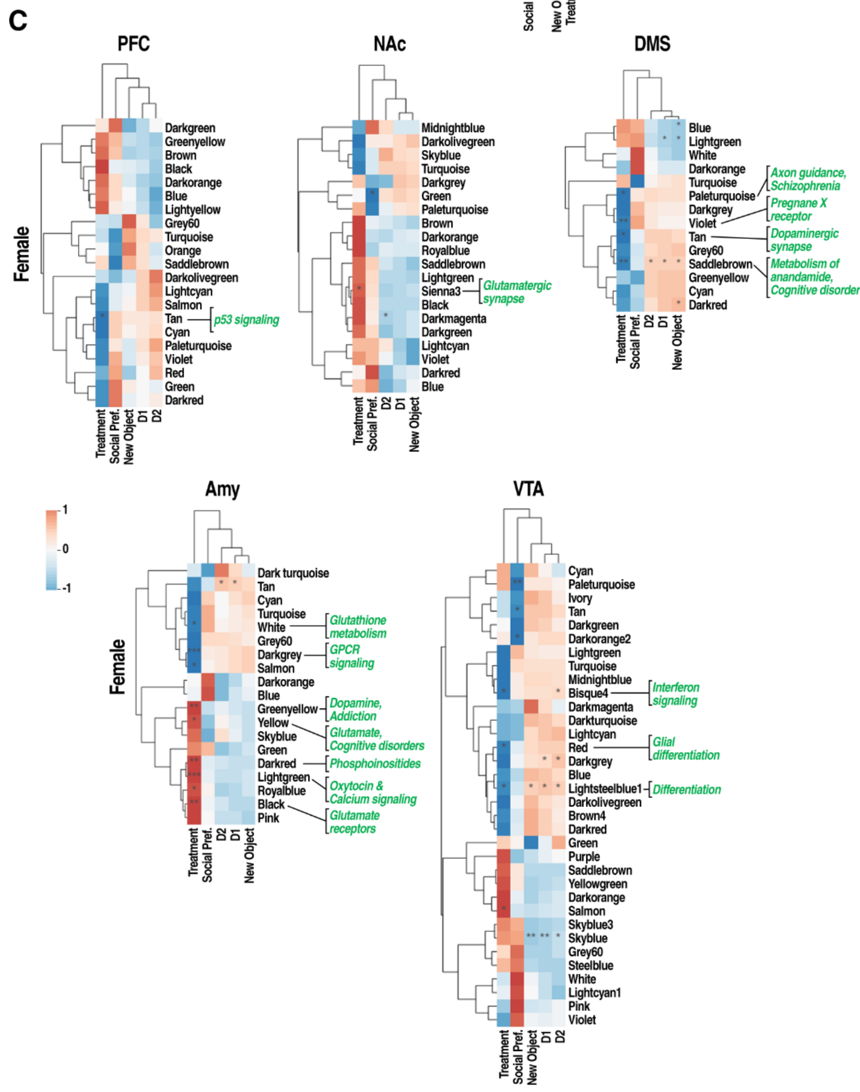

C
PFC
NAc
DMS
Female
Darkgreen
Greenyellow
Brown
Black
Darkorange
Blue
Lightyellow
Grey60
Turquoise
Orange
Saddlebrown
Darkolivegreen
Lightcyan
Salmon
Tan
Cyan
Paleturquoise
Violet
Red
Green
Darkred
p53 signaling
Treatment
Social Pref.
New Object
D1
D2
Midnightblue
Darkolivegreen
Skyblue
Turquoise
Darkgrey
Green
Paleturquoise
Brown
Darkorange
Royalblue
Saddlebrown
Lightgreen
Sienna3
Black
Darkmagenta
Darkgreen
Lightcyan
Violet
Darkred
Blue
Glutamatergic synapse
Treatment
Social Pref.
D2
D1
New Object
Blue
Lightgreen
White
Darkorange
Turquoise
Paleturquoise
Darkgrey
Violet
Tan
Grey60
Saddlebrown
Greenyellow
Cyan
Darkred
Axon guidance, Schizophrenia
Pregnane X receptor
Dopaminergic synapse
Metabolism of anandamide, Cognitive disorder
Treatment
Social Pref.
D2
D1
New Object
Amy
VTA
Female
Dark turquoise
Tan
Cyan
Turquoise
White
Grey60
Darkgrey
Salmon
Darkorange
Blue
Greenyellow
Yellow
Skyblue
Green
Darkred
Lightgreen
Royalblue
Black
Pink
Glutathione metabolism
GPCR signaling
Dopamine, Addiction
Glutamate, Cognitive disorders
Phosphoinositides
Oxytocin & Calcium signaling
Glutamate receptors
Treatment
Social Pref.
D2
D1
New Object
Cyan
Paleturquoise
Ivory
Tan
Darkgreen
Darkorange2
Lightgreen
Turquoise
Midnightblue
Bisque4
Darkmagenta
Darkturquoise
Lightcyan
Red
Darkgrey
Blue
Lightsteelblue1
Darkolivegreen
Brown4
Darkred
Green
Purple
Saddlebrown
Yellowgreen
Darkorange
Salmon
Skyblue3
Skyblue
Grey60
Steelblue
White
Lightcyan1
Pink
Violet
Interferon signaling
Glial differentiation
Differentiation
Treatment
Social Pref.
New Object
D1
D2

Figure 3.6. Coexpression modules correlated with chronic THC administration and mouse cognitive phenotypes.

(A) Bar plot of the number of coexpression modules significantly correlated with THC treatment and cognitive phenotypes ($p < 0.05$). (B-C) Heatmaps of module correlation with THC treatment and cognitive phenotypes across brain regions in males (B) and females (C). Color depicts direction and strength of correlation. Pathway annotations of selected modules were shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area; D1, absolute discrimination index 1, D2, relative discrimination index 2.

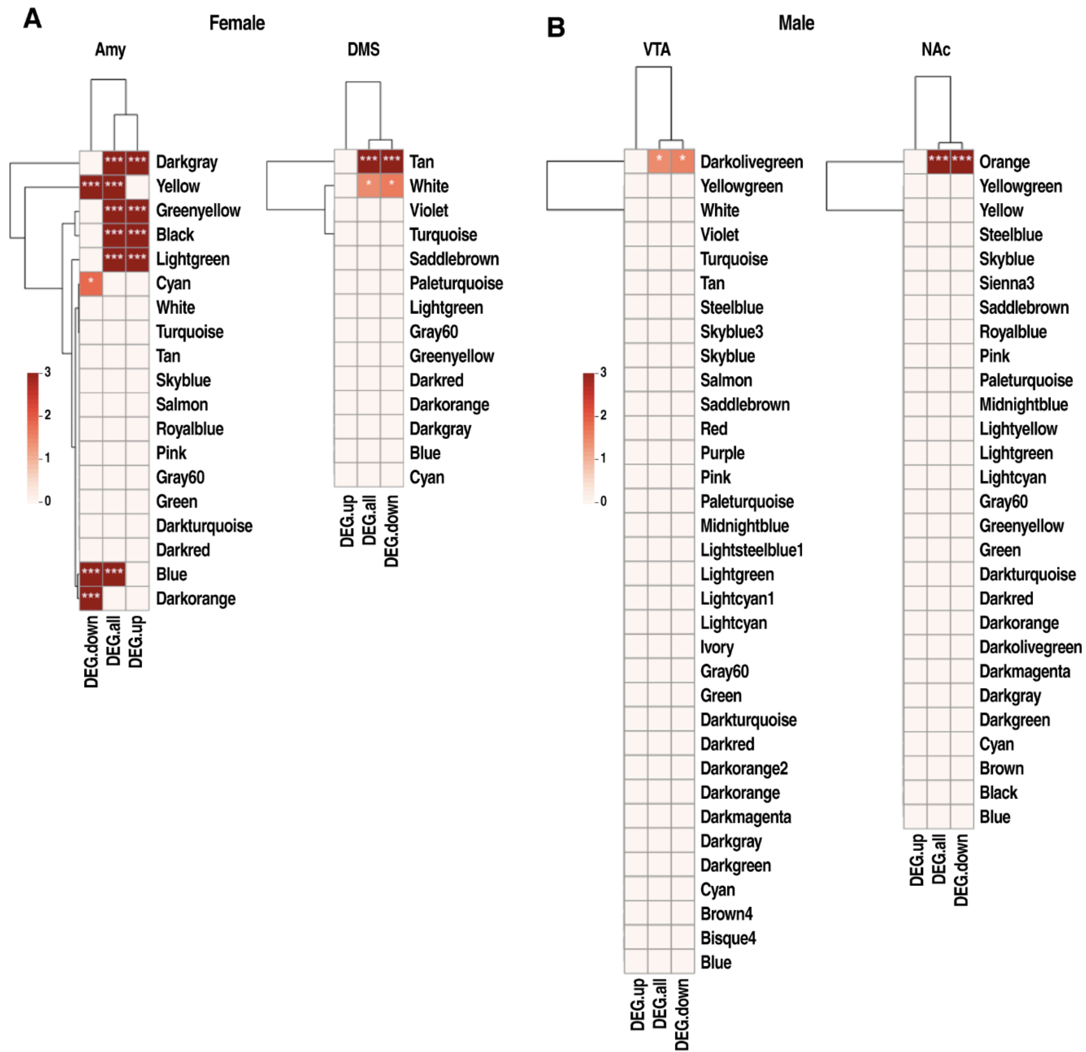


Figure 3.7. DEG enrichment in coexpression modules was detected in a few brain regions.

(A-B) Heatmaps of DEG enrichment in brain regions. Only female Amy, DMS and male VTA, and NAc showed significant enrichment in DEGs. Color depicts -log10P value of the enrichment and only significant enrichments were illustrated. *p < 0.05; **p < 0.01; ***p < 0.001. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area; DEG.all, all the differential expression genes of a certain brain region; DEG.uo, upregulated differential expression genes of a certain brain region; DEG.down, downregulated differential expression genes of a certain brain region.

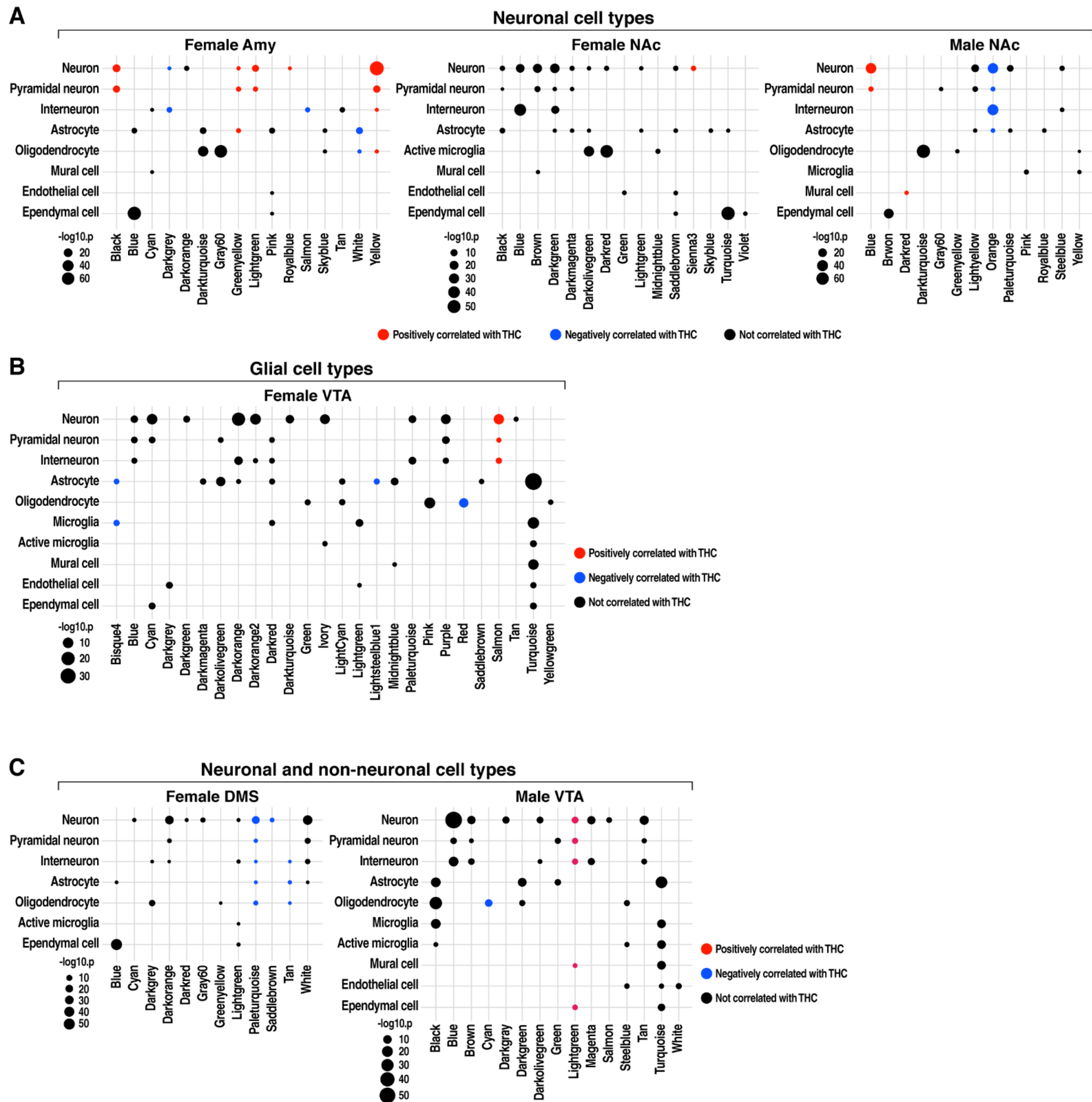


Figure 3.8. THC-correlated coexpression modules exhibit cell-type marker enrichments.

(A) Dot plots of brain regions with THC-correlated modules enriched in neuronal cell markers. (B) Dot plots of brain regions with THC-correlated modules enriched in glial cell markers. (C) Dot plots of brain regions with THC-correlated modules enriched in both neuronal and non-

neuronal cell markers. Dot color depicts the direction of correlation with chronic THC administration. Dot size depicts the significance of the enrichment pattern. Modules not shown are not enriched in cell-type markers. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

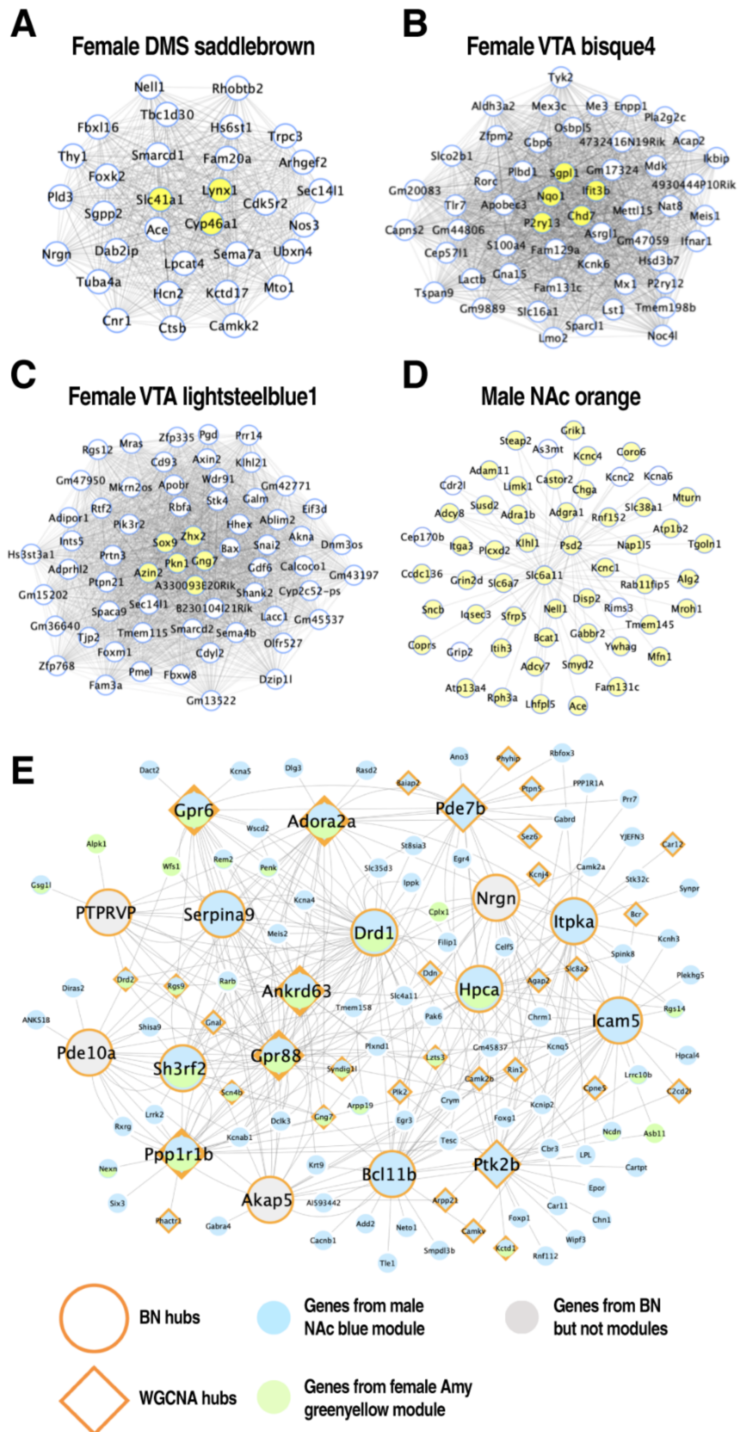


Figure 3.9. Hub genes and key driver genes were identified in THC-correlated coexpression modules.

(A-D) Visualization of coexpression modules correlated with both THC administration and recognition memory traits. The edges denote positive correlations between pairs of genes by the definition of signed coexpression networks. Hub genes are highlighted in yellow. Only the top 100 edges based on topological overlap weight were visualized due to the large size of the male NAc orange module. (E) Visualization of Bayesian network of the male NAc blue module and female amygdala greenyellow module. Hub genes and key driver genes are labeled with orange borderlines. Key driver genes are illustrated by enlarged circles, overlaps between key driver genes and hub genes are illustrated by enlarged diamonds, and hub genes are illustrated by diamonds. Blue or green color denotes the origin module of the genes and grey color denotes genes from the BN but not in the WGCNA modules. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area; BN, Bayesian network.

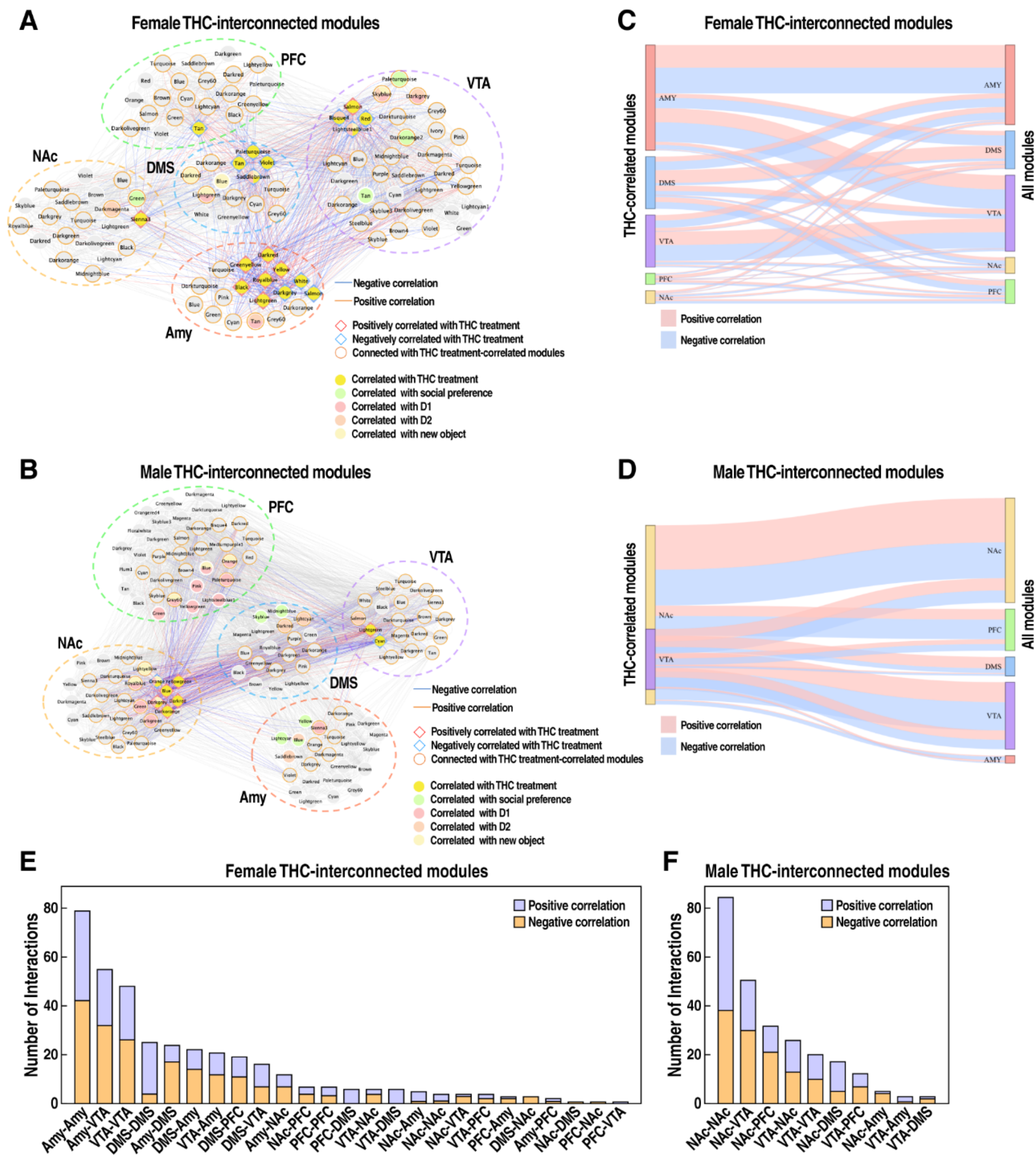


Figure 3.10. Construction of THC-interconnected module map reveals potential intra- or inter-region molecular circuitries disrupted by THC.

(A-B) Visualization of female (A) and male (B) THC-interconnected modules with $|r| > 0.5$ and $p < 0.05$. Edge and node borderline colors denote positive or negative correlation between pairs of modules. Node fill colors denote module correlation relationships. (C-D) Sankey plots of female (C) and male (D) THC-correlated module interactions. Link colors denote the direction of

correlation. (E-F) Bar plots of the number of intermodular interactions in THC-interconnected modules. Color denotes the direction of correlation. DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

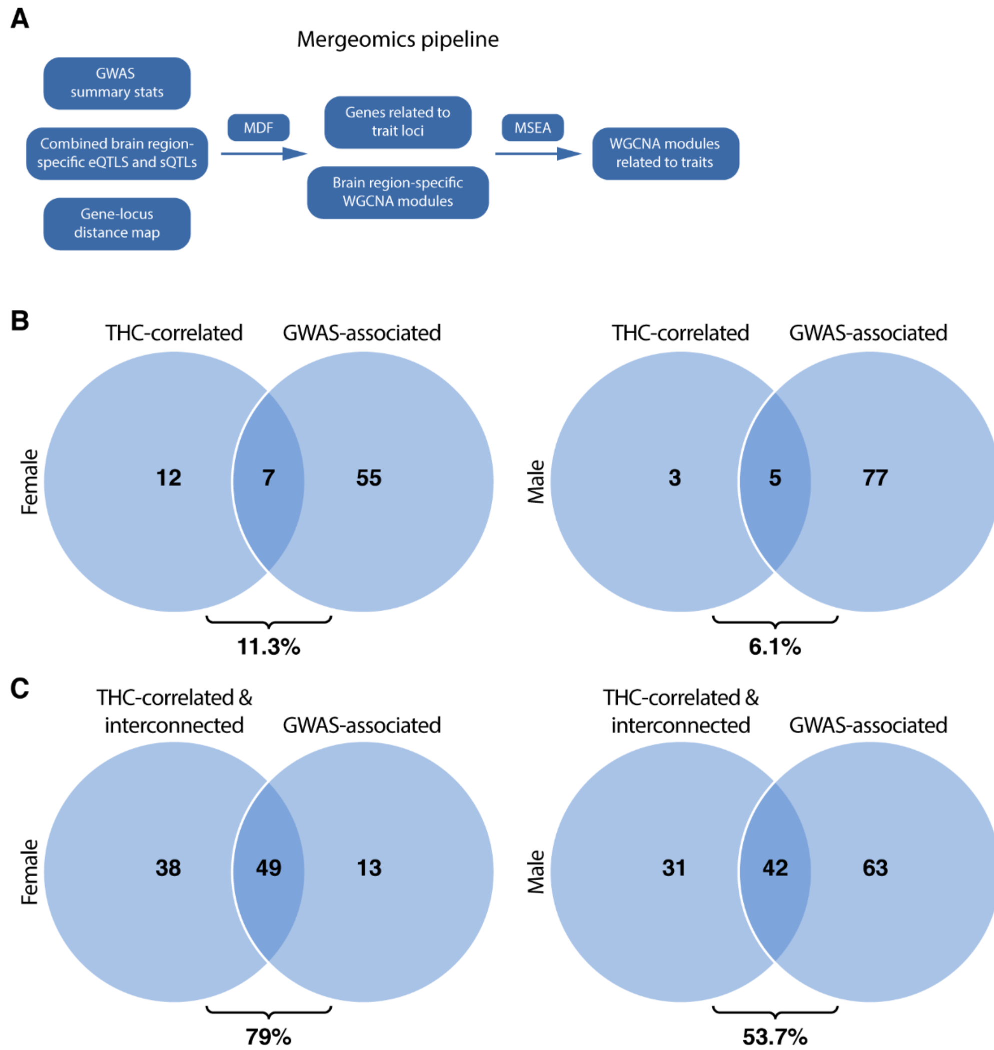


Figure 3.11. Identification of GWAS-associated modules using Mergeomics.

(A) Schematic of Mergeomics pipeline. (B) Percentage of overlap in GWAS-associated modules with THC-correlated modules. (C) Percentage of overlap in GWAS-associated modules with THC-correlated and interconnected modules. Percentage of overlap is calculated as the number of overlapped modules divided by the total number of GWAS-associated modules. MDF, marker dependency filtering; MSEA, marker set enrichment analysis; QTL, quantitative trait loci.

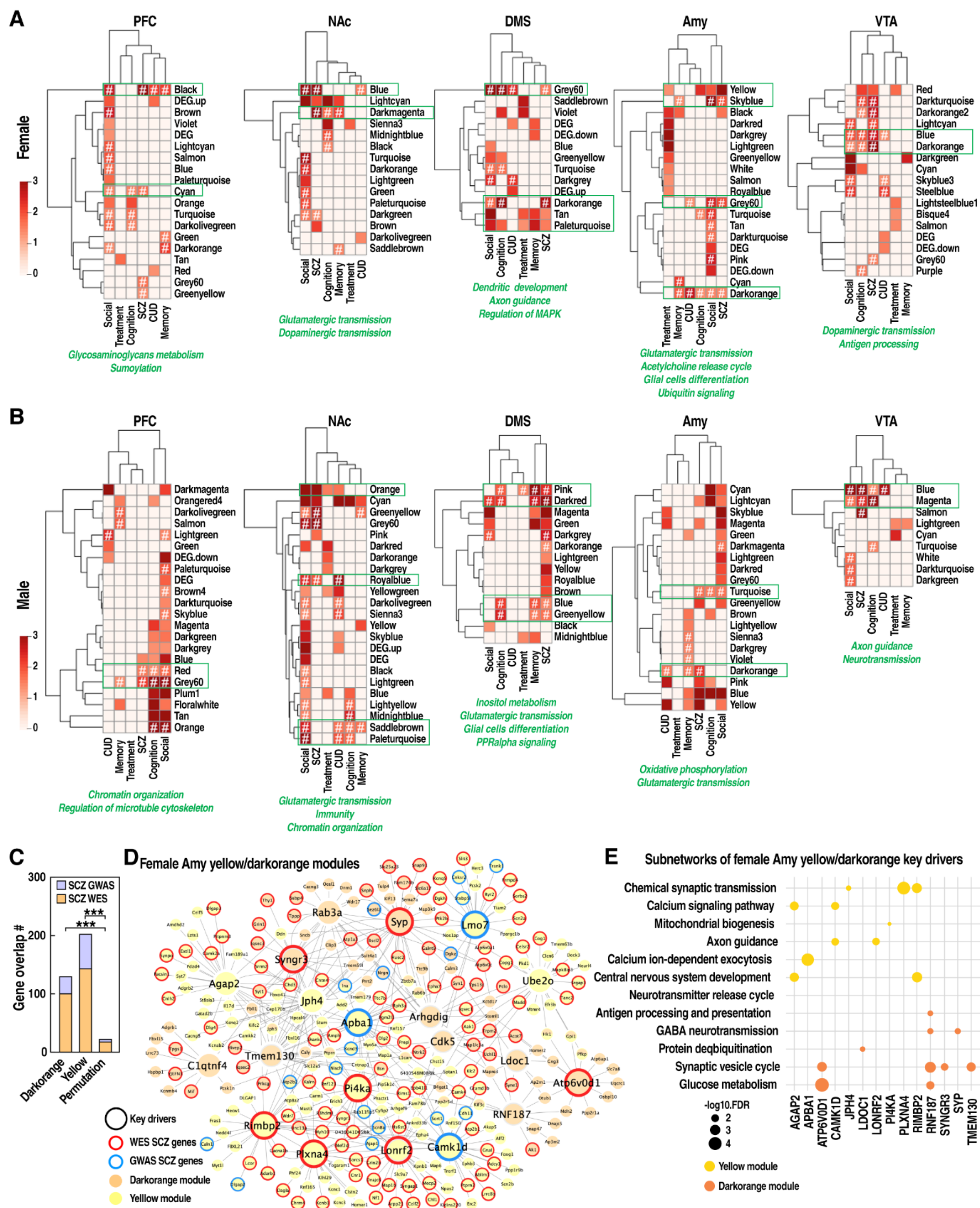


Figure 3.12. THC-correlated and interconnected modules are associated with human cognitive traits and disorders.

(A-B) Heatmaps of the modules that are significantly associated with GWAS traits (cognition, memory, social, CUD, and SCZ) in females (A) and males (B). # denotes THC-interconnected

modules. Color depicts significance in terms of -log (FDR). (C) Bar plot of SCZ variant gene overlap numbers with yellow and darkorange module genes from the female amygdala. *** $p < 0.001$. (D) Visualization of Bayesian network of the female Amy yellow module and darkorange module. Key driver genes are represented by large size nodes. SCZ rare variants discovered by WES are labeled with red borderline and SCZ common variants discovered by GWAS are labeled with blue borderline. Yellow or orange color denotes the origin module of the genes (yellow and darkorange, respectively). (E) Dot plot of subnetwork pathway enrichment. Dot size depicts -log (FDR) value of the enrichment and color depicts yellow or darkorange module.DMS, dorsomedial striatum; Amy, amygdala; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area; CUD, cannabis use disorder; SCZ, schizophrenia; WES, whole-genome sequencing.

Chapter 4. Gene network perturbations and drug candidates for autism spectrum disorders based on multi-omics integration

4.1 Introduction

ASD is a neurodevelopmental disorder characterized by social and communication deficits and repetitive and stereotype behaviors[232, 233]. Currently there is no approved drug targeting these two core symptoms due to a lack of comprehensive understanding of the pathogenic mechanisms. ASD has a heritability of more than 80% according to twin studies and is genetically heterogenous[232]. Recent advancement in ASD genetics have identified 136 ASD rare mutations through exome sequencing studies and 12 common polygenic risk loci via genome-wide association studies (GWAS)[8, 234, 235]. Based on rare mutations, a number of mouse models have been established to study the underlying mechanisms[99, 236]. Despite the success in identifying numerous genetic risk variants, the heterogeneous nature of ASD genetics and the complexity of the brain anatomy make it challenging to fully understand the target genes and pathways driving ASD pathogenesis.

ASD is clearly polygenic in nature and may even follow an omnigenic disease model[23], which states that genes in highly interconnected networks together contribute to disease development, with core hub network genes exhibiting stronger effects and peripheral network genes showing subtle to moderate effects. Building on this model and the recent support for the use of network approaches for psychiatric research[3, 237], we hypothesize that rare and common genetic variants affect genes interacting in tissue- and cell-type specific gene networks in different brain regions. With the recent surge of both bulk and single-cell transcriptome profiling data derived from many human and mouse brain regions, it is possible to map the network perturbations caused by ASD risk variants by incorporating the functional diversity of brain regions and cell types and by modeling gene regulatory cascades in a tissue and cell-type specific manner.

In addition to utilizing network approaches to understand complex diseases, network-based medicine has emerged as a powerful approach to guide drug repositioning[238-242]. In general, this approach utilizes the matching patterns of molecular networks between diseases and drug targets, where both disease and drug networks can be derived using different data sources and methodologies. The recent success of repurposing the FDA-approved drug bumetanide for improving social interaction in children with ASD supports the possibility of repurposing other drugs for potential ASD treatments[243]. However, more efforts are needed to address the scarcity of ASD therapeutics.

Here, we applied Mergeomics, an integrative genomics pipeline to identify cell subtypes and construct Bayesian perturbation network of ASD common variants. We identified potential regulator genes of these networks and characterized the physiological functions of their subnetworks. We found that cortical inhibitory neurons of the caudal ganglionic eminence (CGE) lineage are highly enriched in ASD common variant related genes, and their corresponding perturbation networks displayed enrichment in ASD rare variants. We have also predicted potential drugs using the perturbation network signature, which are evident to influence ASD phenotype.

4.2 Methods

Construction of brain-region specific Bayesian networks (BNs)

We constructed BNs from GTEx version 8 bulk tissue RNA-seq data for 14 central nervous system (CNS) tissues using RimbaNet[36, 57]. To improve causal inference, we used two types of prior information: brain-region specific expression quantitative trait loci (eQTLs) from GTEx version 8 and brain-region specific transcription factor to target relationships from FANTOM version 5[37]. For each brain region, 1000 networks were generated with random seeds, and a consensus network containing the shared edges across the 1000 reconstructions was used as

the final BN for the given brain region. The numbers of edges and nodes in each network are shown in Table 4.1.

Construction of brain-region specific WGCNA coexpression networks

We constructed region-specific WGCNA coexpression networks based on the GTEx version 8 RNA-seq data for the CNS tissues[42]. Signed WGCNA coexpression networks were generated for each tissue using optimized parameters which yield modules each enriched for specific biological functions. Total module counts for each tissue are in **Table 4.1**.

Construction of cell-type specific WGCNA coexpression networks

We further constructed cell-type specific WGCNA gene coexpression networks using single-cell RNA-seq (scRNA-seq) data from 356 cell subtypes in 7 brain regions across developmental stages and species (**Table 4.2**). scRNA-seq data were first filtered based on total RNA counts, and high-quality cells with total RNA counts above 40% among all cells were used as the input for network generation. Signed WGCNA coexpression networks were generated for each cell subtype using optimized parameters. Pathway enrichment analysis was performed on each network to assess the biological functions of each module.

Inferring ASD-related brain regions, cell subtypes, networks, and pathways

Mergeomics, a multi-omics data integrative genomic pipeline developed in our lab, was used to identify which networks and pathways from which brain regions and cell subtypes are related to ASD common variants. The GWAS full summary statistics of ASD common variants was integrated with brain region-specific eQTLs and splicing QTLs (sQTLs) from GTEx version 8 to connect common SNPs with potential target genes[234]. Genes within 20 kb distance to GWAS SNPs were also used for SNP-to-gene mapping. The brain-region and cell-subtype specific coexpression networks were used to assess which coexpression modules from which brain

regions and cell subtypes harbor genes whose potential regulatory SNPs mapped via eQTLs/sQTLs or chromosomal distance showed stronger ASD GWAS associations compared to random gene sets.

PharmOmics prediction of drug candidates

We intersected the ASD common variant-informed Bayesian perturbation networks and used the consensus network as the input to PharmOmics. Overlap based drug reposition mode was used, and candidate drugs were ranked based on Benjamini-Hochberg procedure adjusted p value.

4.3 Results

To identify top brain regions enriched in ASD common variant related genes, we firstly performed marker set enrichment analysis using bulk-tissue gene coexpression modules. Among the 14 CNS regions, 11 showed significant enrichment of ASD common variant-related genes in one or more modules. Importantly, most of the significant tissues and modules were identified when tissue-specific eQTLs/sQTLs were used as the SNP-to-gene mapping methods, supporting the functional regulation of the modules by ASD GWAS SNPs. Pathways enriched in the ASD-associated modules include immune system pathways, mRNA processing, metabolism, and neuronal processes (**Table 4.3**).

To further dissect ASD pathogenesis with increased granularity, we identified cell subtypes related to ASD common variants informed by GWAS. Cell-type specific WGCNA modules were used as input to Mergeomics and modules significantly enriched in ASD common variant-related genes informed by common variants were identified. We firstly surveyed the frontal cortex, striatum, and hippocampus from DropViz, which is a scRNA-seq database for adult mouse brains. We hypothesized that more excitatory neuron subtypes are enriched in ASD common variant-related genes based on previous studies[46, 50]. However, among the 15 neuronal cell

subtypes whose modules are significantly enriched in ASD common variant-related genes, 9 are interneurons (**Table 4.4**). Pathways over-represented in the ASD-associated modules from these neuronal subtypes also included metabolic pathways, neuronal processes, and RNA processing, which are in general agreement with those from the tissue-level module analysis (**Table 4.3**).

We further examined networks from all cell types across developmental stages and species and found that inhibitory interneurons from the CGE lineage ranked the top among neuronal subtypes in terms of ASD common variant-related gene enrichment. Such enrichment was found consistently in the human mid-gestation developing cortex, adult mouse frontal cortex, and adult human frontal cortex (**Figure 4.1**). The conservation across species and developmental stages highlights the critical role of inhibitory interneurons in ASD pathogenesis.

To identify potential regulators of the significant ASD coexpression modules in BNs which capture regulatory gene-gene relations, we used modules significantly enriched for ASD common variants as the inputs to weighted key driver analysis (wKDA) in Mergeomics. We hypothesized that ASD common and rare variants follow the omnigenic model, with the rare variants serving primarily as the hub genes in a biological network, while common variants acting in the peripheral pathways. Consistent with this hypothesis, the hub genes showed a ~3-fold enrichment for ASD rare variants compared to all genes in ASD-associated BNs across brain regions surveyed (**Figure 4.2**). As exemplified in **Figure 4.3**, several top hub genes contain ASD rare variants (larger colored nodes). Additional hub genes (diamond nodes) were also predicted.

To date, no drug has been approved targeting ASD-related phenotypes. To identify possible drug candidates that can affect ASD phenotypes, we adopted PharmOmics, a tissue- and species-specific drug signature database developed in our lab for drug repurposing[242]. **Table**

4.5 illustrates example top drug candidates that have been shown to promote or suppress ASD-related phenotypes. In addition to the candidate drugs supported by literature, numerous additional drugs were also predicted and warrant further investigation.

4.4 Conclusion

Our study has, for the first time, investigated the pathogenic mechanism of ASD common variants. Using a combination of gene coexpression network, Bayesian causality network and integrative genomic pipeline Mergeomics, we identified brain regions and cell types enriched in ASD common variants. Specifically, we found cortical inhibitory neurons of the CGE lineage to be the most enriched neuronal cell type. Further, Bayesian perturbation networks of ASD common variants are enriched in ASD rare variant genes, supporting the omnigenic hypothesis of complex disorders. Taken together, our study offers a database of networks, potential regulator predictions and drug candidate predictions to aid future research.

4.5 Tables

Table 4.1. BrainNet Bayesian networks and WGCNA module information

GTEx Tissue	BN edge counts #	BN node counts #	WGCNA module counts #
Amygdala	6277	5437	88
Anterior cingulate cortex BA24	6725	5505	34
Caudate	6885	4503	24
Cerebellar hemisphere	6790	5353	25
Cerebellum	6465	5131	25
Cortex	7924	5410	10
Frontal Cortex BA9	7087	5391	33
Hippocampus	7580	5379	9
Hypothalamus	7656	5479	19
Nucleus accumbens	7924	5410	15
Putamen	7439	5419	58
Spinal cord	6403	5334	55
Substantia nigra	5280	5254	29
Pituatary	7339	5018	30

Table 4.2. Cell subtypes used for single-cell WGCNA network generation

Brain region	Species	Cell subtype #	Ref.
Frontal cortex	Mouse	81	Saunders et al.[244]
Hippocampus	Mouse	103	Saunders et al.[244]
Striatum	Mouse	52	Saunders et al.[244]
Medial amygdala	Mouse	8	Chen et al.[245]
Mid-gestation cortex	Human	64	Polioudakis et al.[246]
Frontal cortex	Human	23	Lake et al.[247]
Dorsolateral prefrontal cortex	Human	25	Nagy et al.[109]

Table 4.3. Brain region-specific modules enriched in ASD common variant related genes

Brain region	Module	FDR	Mapping	Top annotations
Frontal cortex	FC-gray60	7.77E-19	sQTL	Adaptive immune system; Spliceosome; Neuronal system
Cortex	Cortex-blue	6.63E-15	eQTL	Olfactory signaling pathway
Amygdala	AMY-black	9.98E-13	eQTL, sQTL	Oxidative phosphorylation; mRNA processing; Adaptive immune system
Putamen	Putamen-tan	3.84E-07	eQTL	Proximal tubule bicarbonate reclamation; Transmembrane transport of small molecules
Cerebellum	CB-black	6.29E-05	eQTL, sQTL	Spliceosome; Oxidative phosphorylation; Adaptive immune system
Caudate	Caudate-pink	1.79E-04	eQTL	Immune system
Frontal cortex	FC-black	4.19E-04	eQTL	Immune system
Frontal cortex	FC-pink	3.20E-03	eQTL	Fatty acid metabolism; Metabolism of amino acids and derivatives
Cerebellum	CB-purple	5.13E-03	Distance (20 kb)	Glycolysis
Hypothalamus	Hypo-turquoise	5.78E-03	eQTL, sQTL	mRNA processing; Adaptive immune system

Table 4.4. Neuron subtypes enriched in ASD common variant related genes

Brain region	Cell subtype	Cell type annotation	Significant module annotations
FC	1-8	CGE-derived interneuron	ARAP pathway
	1-10	CGE-derived interneuron	Protein secretion; Metal ion transporters; Activation of chaperone genes
	2-3	MGE-derived cortical interneurons--Martinotti cells	ECM pathway; Glycolysis; Metabolism of RNA; mTORC1 pathway
	1-1	CGE-derived interneuron	Metabolism of amino acids and derivatives; Fatty acid metabolism; GABA pathway
	1-7	CGE-derived interneuron	Oxidative phosphorylation
	7-2	Deep layer pyramidal cells--layer 5	Neurotrophin signaling pathway; Protein secretion; Intergration of energy metabolism
Hippocampus	1-2	Interneuron, Neurogliaform1	Spliceosome; ECM pathway; Transcription
	3-4	Entorhinal cortex, Slc17a7	Host interaction of HIV factors; Kainate receptor activation; Oxidative phosphorylation; mTOR pathway
	1-21	Interneuron, candidate CGE-derived	Spliceosome; Glycolysis; Oxidative phosphorylation; mTOR pathway
	13-2	Neurogenesis (SGZ)	E2F targets; Oxidative phosphorylation; mTOR pathway; Spliceosome
	3-11	Entorhinal cortex, Slc17a7	MAPK pathway; LTP; Endosome
	6-8	CA3 Principal cells	Myc targets; Spliceosome; GABA receptor activation
	5-5	CA1 Principal cells	Splicing; Peroxisome; PI metabolism
	5-13	Postsubiculum	Myc targets; Proteasome; Spliceosome; MAPK pathway; Respiratory chain
Striatum	14-1	Fast-spiking interneuron, Pvalb+	Regulation of autophagy; Neuvous system; Apoptosis

Table 4.5. Evidence of drugs identified using PharmOmics on modulating ASD phenotypes

Drug	FDR	Evidence on modulating ASD phenotypes	Ref.
Valproic	5.96E-16	Maternal exposure is associated with significant increased risk of ASD in offspring	Christensen et al.[248]
Trichostatin-a	6.55E-15	In utero exposure induced ASD phenotypes in mice	Moldrich et al.[249]
Acetaminophen	1.15E-14	In Utero Acetaminophen Exposure is associated with increased ASD risk	Ji et al.[250]
Vorinostat	6.27E-10	Rescued abnormal GTF2I expression in 7q11.23 microduplication autism spectrum disorder patient-derived cortical neurons	Cavallo et al.[251]
Curcumin	1.74E-09	Neonatal curcumin treatment rescued ASD-related behaviors	Zhong et al.[252]
Quinidine	1.77E-09	Decreased irritability in adult ASD patient	Chez et al.[253]
Estradiol	3.35E-09	Rescued ASD phenotypes in mice	Macrì et al.[254]
Panobinostat	1.15E-08	Rescued abnormal GTF2I expression in 7q11.23 microduplication autism spectrum disorder patient-derived cortical neurons	Cavallo et al.[251]
Memantine	1.15E-08	May improve symptoms in ASD children	Hardan et al.[255]
Roscovitine	1.47E-08	Rescued abnormalities from Timothy syndrome patient iPSC-derived neurons	Paşca et al.[256]

4.6 Figures

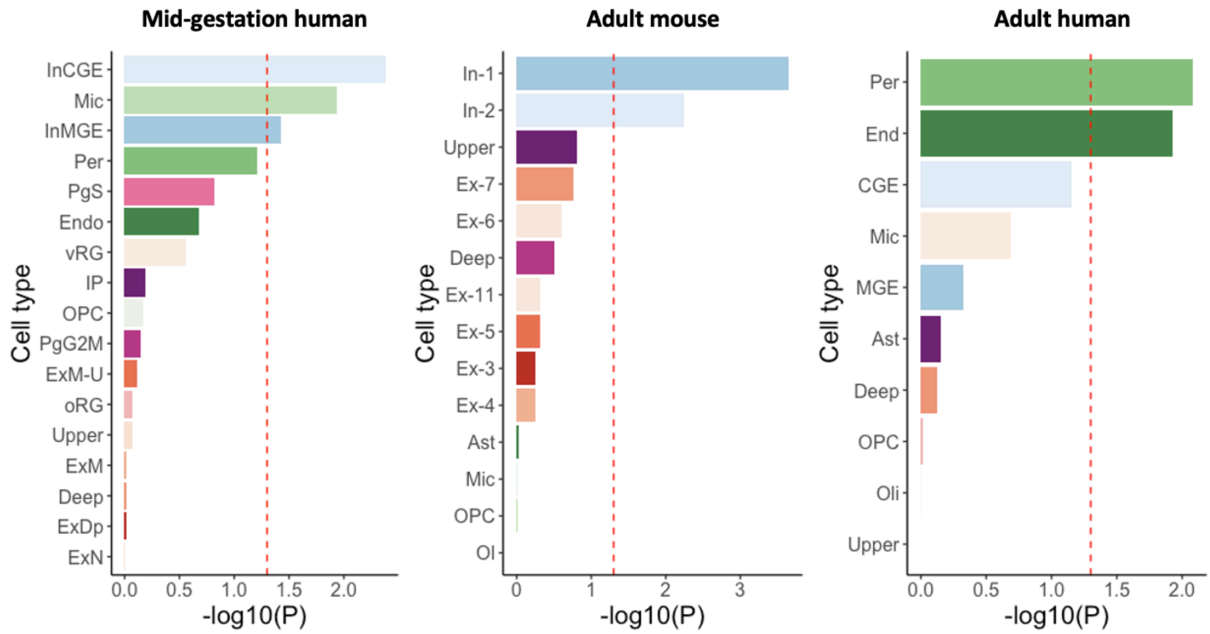


Figure 4.1. CGE-derived interneurons have the highest level of ASD common variant related gene enrichment among neurons across species and developmental stage. In the adult mouse panel, In-1 denotes interneurons of the CGE lineage and In-2 denotes interneurons of the MGE lineage.

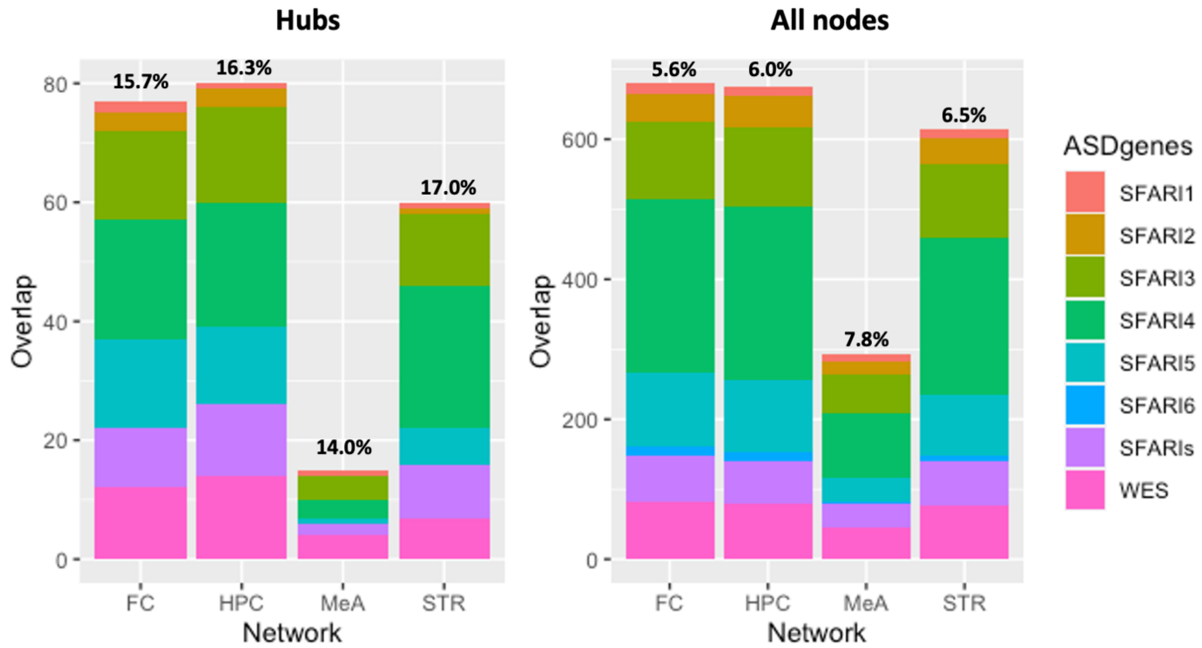


Figure 4.2. The hub genes of ASD common variant-informed perturbation networks are enriched in ASD rare variants. Percentage of enrichment are displayed above each bar. SFARI1-6: ASD-related genes in SFARI database Level 1-6; SFARIs: syndromic ASD genes in SFARI database; WES: ASD genes identified by whole exome sequencing in Ruzzo et al.[235] and Satterstrom et al.[8]

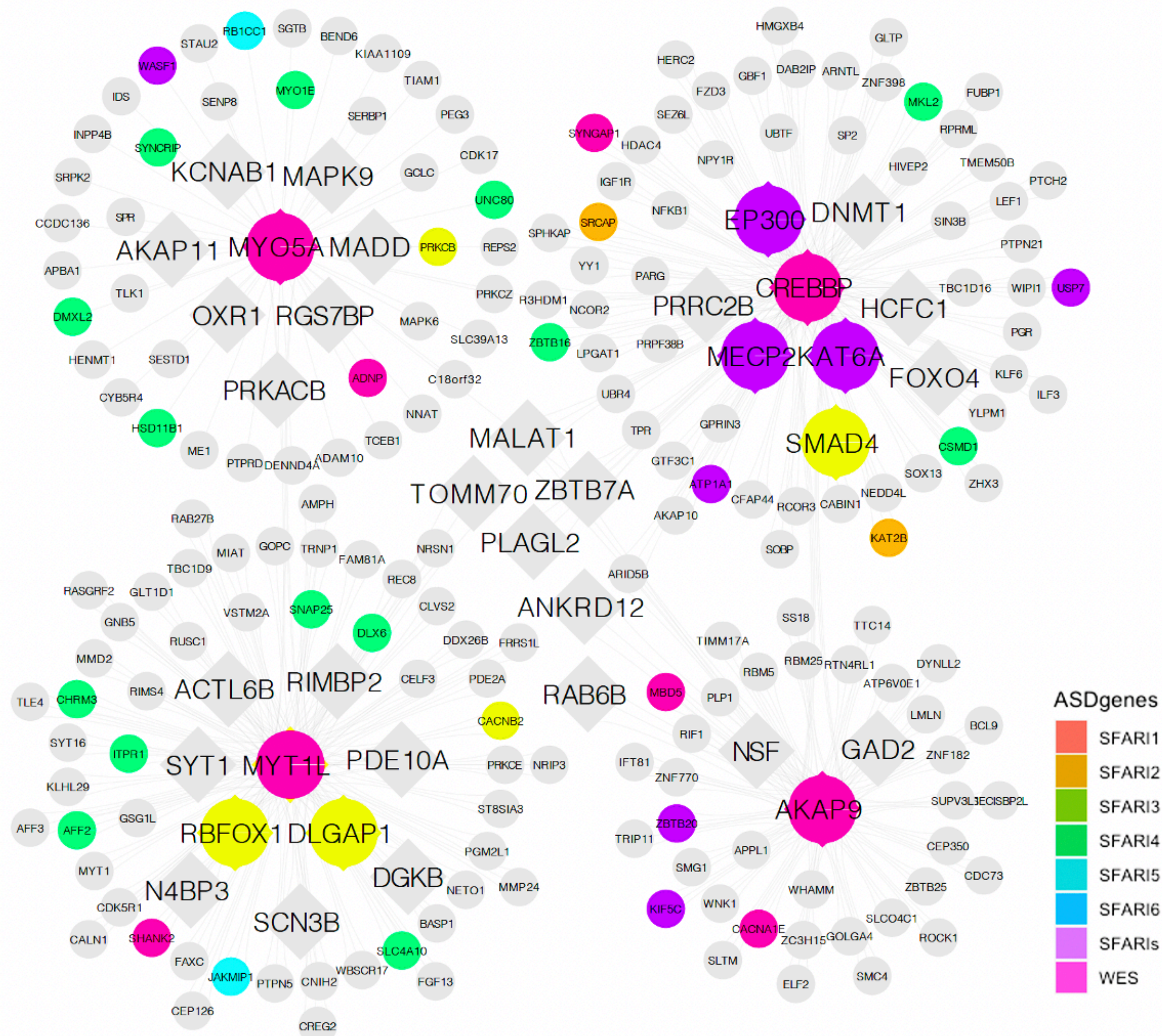


Figure 4.3. Representative ASD common variant-informed perturbation Bayesian network from the frontal cortex. Documented ASD-related genes including rare variants are labeled in colors. SFARI1-6: ASD-related genes in SFARI database Level 1-6; SFARIs: syndromic ASD genes in SFARI database; WES: ASD genes identified by whole exome sequencing in Ruzzo et al.[235] and Satterstrom et al.[8]

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