Prenatal Δ9-tetrahydrocannabinol exposure in males leads to motivational disturbances related to striatal epigenetic dysregulation

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Abstract

Background: Cannabis remains one of the most widely abused drugs during pregnancy. *In utero* exposure to its principal psychoactive component, Δ9-tetrahydrocannabinol (THC), can result in long-term neuropsychiatric risk for the progeny. The current study investigated epigenetic signatures underlying these enduring consequences.

Methods: Rat dams were exposed daily to THC (0.15mg/kg) during pregnancy and adult male offspring were examined for reward and depressive-like behavioral endophenotypes. Using unbiased sequencing approaches, we explored transcriptional and epigenetic profiles in the nucleus accumbens (NAc), a brain area central to reward and emotional processing. An *in vitro* CRISPRa model coupled with RNA-sequencing was also applied to study specific consequences of epigenetic dysregulation and altered molecular signatures were compared to human major depressive disorder (MDD) transcriptome datasets.

Results: Prenatal THC-exposure induced increased motivation for food, heightened learned helplessness and anhedonia, and altered stress sensitivity. We identified a robust increase specific to males in the expression of Histone-Lysine N-Methyltransferase 2A (*Kmt2a*) that targets lysine 4 on histone H3 (H3K4me) in cellular chromatin. Normalizing *Kmt2a* in the NAc restored the

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motivational phenotype of prenatally THC-exposed animals. Comparison of RNA and H3K4me3 sequencing datasets from the NAc of rat offspring with the *in vitro* model of *Kmt2a* upregulation revealed overlapping, significant disturbances in pathways that mediate synaptic plasticity. Similar epigenetic alterations were detected in human MDD.

Conclusions: These studies provide direct evidence for the persistent effects of prenatal cannabis exposure on transcriptional and epigenetic deviations in the NAc via *Kmt2a* dysregulation and associated psychiatric vulnerability.

Keywords

cannabis; pregnancy; motivation; depression; epigenetics; chromatin

Introduction

Cannabis remains the most abused illicit drug during pregnancy, with an estimated 4% of pregnant women reporting past-month use, and 7% in the past 2 to 12 months. Among pregnant women reporting exposure the past year, 16.2% used cannabis daily (1–3). These women cited nausea, pain, and low appetite as reasons for their use, with the erroneous perception that cannabis is innocuous to the developing fetus (1,4,5). However, the main psychoactive component, ^{9}-tetrahydrocannabinol (THC), crosses the placental barrier and can affect the developing fetus, with enduring neurobiological repercussions (6–8). Epidemiological evidence indicates that prenatal cannabis exposure is associated with an elevated risk of neuropsychiatric disorders later in life (9–12). Multiple studies report that progeny exhibit increased impulsivity (13,14) and risk for depression, anxiety, and substance use disorders (10,11,15), particularly when exposed to traumatic experiences (16). While many factors (e.g., environment and genetics) may account for some of the negative consequences ascribed to prenatal cannabis exposure in humans (17–19), controlled animal experiments demonstrate a direct link between cannabinoid exposure and neuropsychiatric related pathologies (20,21,22).

THC directly targets the cannabinoid CB_1 receptor within the endocannabinoid system, which mediates cell fate decisions, axonal guidance, and synaptic wiring under highly specific temporal and spatial conditions during prenatal development (23–27). Given the long-term molecular and behavioral effects seen particularly in males with prenatal cannabis/THC exposure (28), we hypothesized underlying epigenetic disturbances as the cellular context by which environmental factors influence gene expression and related behaviors. To that end, we leveraged several behavioral paradigms and identified phenotypic differences in adult male offspring with prenatal THC exposure (PTE) as well as gene expression and epigenetic abnormalities in systems related to synaptic function within the nucleus accumbens (NAc), a brain region mediating reward and emotional processing (29–31). Importantly, we demonstrated a direct relationship to motivational behavior via Kmt2a (histonelysine N-methyltransferase 2A), an epigenetic regulator, as well as novel overlapping transcriptomic and epigenomic signatures common to PTE and human major depressive disorder (MDD).

Methods and Materials

Animals

Long-Evans female rats (44–50 days old; Charles River Laboratories) were carried through PTE as described previously (32) (Figure 1A). All experiments and procedures were approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

THC exposure paradigm

Descriptions of drug preparations and animal models are provided in Supplement 1. THC (0.15 mg/kg; in 0.3% tween) was intravenously injected into pregnant dams (via jugular catheter) from gestational day 5 to postnatal day (PND) 2, equivalent to the early-to-midgestational period in humans (33). At PND2, male offspring were cross-fostered to vehicle-exposed dams and THC-treated birth mothers removed from the study. No dams raised their own pups. Approximately 12 pups were randomly assigned to each dam until pups were weaned at PND21 and housed in groups of 3 or 4.

Behavioral Testing

Palatable Food Self-administration.—To assess hedonic food reinforcement, animals were placed in operant chambers where lever pressing resulted in the release of a chocolate pellet. Rats were food restricted (10–18 g/day) 1 day before and throughout testing. Self-administration behavior was studied at fixed ratio 1 (FR1) (one food pellet in response to one active lever press) to monitor reward sensitivity, or progressive ratio (PR) reinforcement schedules (34,35) to assess motivational effort. Animals that never acquired the FR1 task were excluded (1 saline and 1 THC animal).

Forced Swim Test.—Behavioral despair paradigm was used to determine learned helplessness behavior (36). Immobility score reflects time spent floating versus active.

Sucrose Preference.—The sucrose preference test was used to evaluate hedonic state in the preference for sucrose over water. Anhedonia-like behavior is operationalized as a reduction in sucrose intake.

Data Analysis

Groups of 7 to 9 rats were used for behavioral tasks to achieve 80% statistical power with an alpha level of 0.05. Using SAS, JMP, and Prism 8 (GraphPad Software Inc.) statistical software, independent t tests were used when treatment was the sole group variable. When multiple variables existed, analysis of variance statistical models were applied, including appropriate covariates (e.g., treatment, body weight, litter) and/or repeated behavioral measures, to evaluate the influence of PTE on behavioral outcomes. Outliers were detected using Grubbs' test and removed from the analysis. All data were tested for normality and transformed if not.

Gene and Protein Expression Analysis

Quantitative reverse transcription polymerase chain reaction, NanoString, and Western blotting are described in Supplemental Methods and Materials in Supplement 1. Assay details are shown in Tables S1 and S2 in Supplement 2.

Kmt2a knockdown

One day prior to the behavioral test, 1 μL of a 20-pmol small interfering RNA ON-TARGETplus SMART pool (rat *Kmt2a*, L-100995-02-0005; Horizon Discovery) or nontargeting pool was injected into the NAc via infusion needles through indwelling bilateral cannulae (37–39) as described in Supplemental Methods and Materials in Supplement 1. Knockdown efficiency is shown in Figure S1 in Supplement 1.

RNA Sequencing, Chromatin Immunoprecipitation Sequencing, and *In Vitro* CRISPR-Mediated *Kmt2a* Activation

Sequencing strategies were used as discovery tools for genes and ontology networks associated with PTE. Technical and analytic procedures, as well as data comparisons, are provided in Supplemental Methods and Materials in Supplement 1.

Gene Ontology

All gene ontology enrichment analyses were conducted using GOrilla (40): http://cbl-gorilla.cs.technion.ac.il.

Gene Network Analysis

Networks were constructed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (41) and analyzed with NetworkAnalyzer via Cytoscape (42,43) to quantify node degrees. More details are provided in Supplemental Methods and Materials in Supplement 1.

Differential Gene Correlation Analysis

Differential gene correlation analysis (44) was conducted on each dataset to identify gene-gene regulatory relationships that were increased or decreased between experimental groups in our sequencing datasets (RNA sequencing [RNA-seq] and chromatin immunoprecipitation sequencing [ChIP-seq] from the rat NAc and RNA-seq from the CRISPR [clustered regularly interspaced short palindromic repeats] model).

Comparison of Rat NAc and CRISPR Models With Human MDD Datasets

Gene expression datasets from human studies of MDD (45,46) in the NAc; anterior insula; Brodmann areas 11, 25, and 89; and subiculum were used for comparison with our data from rat NAc after PTE and CRISPR activation models.

Results

PTE Increases Motivation for Reward and Depressive-like Behaviors in Adult Offspring

We first explored the direct contributions of PTE to rewardrelated behaviors in adult male progeny because we had previously demonstrated such long-term increase on drug reward (47,48). To assess effects on natural reward, we investigated the response of the offspring to palatable reward in a food selfadministration paradigm. There was no THC-related difference in food self-administration behavior when examined in an FR1 schedule (Figure 1B). In contrast, there was a significant effect in the PR motivational model, as evident with repeatedmeasures analysis of variance on the breakpoint threshold (Figure 1C) ($F_{1,12}$ = 8.682, p = .0122). *Post hoc* analysis indicated group differences in the breakpoint in each session, suggesting that PTE consistently increased the motivation to obtain reward.

Depression and altered stress response are reported in the progeny of women who use cannabis while pregnant (10,11). To assess potential depressive-like behaviors in the animal model, rats underwent the forced swim test (FST) to measure learned helplessness (49,50). THC-exposed rats showed significant increases in immobility ($F_{1,15}$ = 3.8372; p = .0490) (covaried for body weight) (Figure 1D), reflective of a depressive-like endophenotype. These results were replicated in a separate cohort of rats (Figure S2 in Supplement 1). The FST also presents an unexpected, highly stressful situation, and rats with or without this stress experience subsequently showed different sucrose intake behavior, a measure of hedonic state. A significant interaction was evident between PTE and postnatal FST experience for sucrose preference ($F_{1,28}$ = 4.511; p = .0426) (Figure 1E). PTE animals experiencing FST had reduced sucrose intake, suggesting an anhedonic response, although the stressful event had occurred 4 weeks prior to the sucrose test.

PTE Increases Expression of *Kmt2a* in the NAc, Linked to Reward-Related Motivation

To gain insight into underlying epigenetic mechanisms relevant to PTE, we evaluated the expression of 80 genes (Table S1 in Supplement 2) encoding various epigenetic regulators involved in chromatin and DNA modification in the NAc, which plays a critical role in reward, motivation, and emotion processing (29–31). This analysis identified significant increases in the messenger RNA (mRNA) expression of Setdb1 (set domain, bifurcated 1) (p = .0114), Kat2b (lysine acetyltransferase 2B) (p = .0274), Kmt2a (p = .0298), and Ncoa6 (nuclear receptor coactivator 6) (p = .0358) (Table S3 in Supplement 2; Figure 2A). Of these genes, Kmt2a upregulation was the most reproducible across male cohorts and detected by quantitative reverse transcription polymerase chain reaction even at a later developmental time point (PND117, p = .007) (Figure 2B). Kmt2a in the NAc of PTE-exposed adult female rats was unchanged (Figure S3 in Supplement 1). An increase in Kmt2a was also observed at the protein level in adult male offspring at PND62 (p = .03) (Figure 2C). Kmt2a is a histone-H3-lysine-4 (H3K4) methyltransferase known to mediate chromatin modifications often associated with transcriptional activation (51–54) and has a documented role in the regulation of genes that are critical for brain development (55).

To investigate the functional consequences of *Kmt2a* dysregulation in the NAc, we used a small interfering RNA–mediated knockdown strategy to alter *Kmt2a* levels (Figure S1 in

Supplement 1). Four months after the initial PR food self-administration behavioral testing (Figure 1B, C), rats with intra-NAc infusions of the negative control small interfering RNA retained their prior PR behavior, with those exposed to prenatal THC showing higher PR breakpoint ($F_{1,9} = 9.8756$, p < .01) (Figure 2D). Interestingly, in the THC group, *Kmt2a* knockdown resulted in a significant reduction in PR breakpoint ($F_{1,10} = 5.6382$, p < .05) (Figure 2D). *Kmt2a* manipulation modulated motivational behavior and did not affect general locomotion (data not shown). In addition, in a separate study, NAc *Kmt2a* knockout mice showed altered FR5 responding in males (Figure S4 in Supplement 1). These results suggest that abnormal *Kmt2a* levels induced by PTE are directly related to the observed motivational disturbances.

PTE Is Associated With Enduring Transcriptomic Alterations Relevant to Synaptic Plasticity and Neuropsychiatric Disorders

In view of the pivotal role of NAc in emotional processing and the known role of Kmt2a, we conducted a larger screen, using the NanoString nCounter technology, to measure mRNA levels of 400 genes relevant to various neuropsychiatric phenotypes and neurobiological processes (Table S2 in Supplement 2), yielding 42 upregulated and 63 downregulated genes altered by PTE (Figure 2E; Table S4 in Supplement 2). Functionally, the affected genes related to neurotransmitter systems including glutamate and GABAergic (gamma-aminobutyric acidergic) receptors, multiple ion channels, zinc transporters, calcium sensors, and genes encoding cytoarchitectural and other cellular components related to structural integrity. This screen also confirmed, in another independent animal cohort, the significant upregulation of *Kmt2a*.

To gain an unbiased insight into the long-term dysregulation of NAc due to PTE, RNA-seq was used to explore the transcriptome (Figure 3). Principal component analysis (Figure S5A in Supplement 1) was performed on the RNA-seq counts, and some separation by treatment was observed. As expected, clustering based on treatment was more pronounced when only differentially expressed genes (DEGs) were included in a heatmap (Figure 3A). To ensure rigor, we used DEGs shared at p < .05 by both DESeq2 (Figure 3B; Table S5 in Supplement 2) and Voom-limma (Figure 3C; Table S6 in Supplement 2) in all downstream analyses, given that the two approaches showed good agreement (Figure S5B, C in Supplement 1). This combined approach identified 600 upregulated and 808 downregulated genes. Fisher's exact test showed statistically significant overlap between the RNA-seq and NanoString datasets, both for upregulated (odds ratio [OR] = 6.47, p = .0002) and downregulated (OR = 4.73, p = 3.95 3 1027) genes.

To address the biological significance of the observed transcriptome alterations, gene ontology analysis was performed on all 1408 DEGs, revealing an impact of PTE on synaptic function and neuronal communication (Figure 3D; Table S7 in Supplement 2). Genes in the top 10 enriched ontologies included cannabinoid receptor 1 (Cnr1), glutamate receptors (Grin3b, Grin2c, Gria3, Grm2, Grm4), dopamine receptor 2 (Drd2), and proteins crucial for neurotransmitter release (Snap25, Syn1, Syn2) (Figure 3E). The NanoStringbased assay in a different cohort of animals similarly showed a tendency for THC-related decrease of gene expression (Figure 2E), suggesting repressive effects of PTE on the NAc transcriptome.

PTE Is Associated With Complementary Epigenomic Alterations Relevant to Synaptic Plasticity and Psychiatric Disorders

To identify genomic loci directly relevant to *Kmt2a*, we conducted a ChIP-seq assay in the NAc of adult rats with PTE for H3K4me3 (H3K4-trimethylation), the methylation target of Kmt2a present at active promoters contributing to the recruitment of transcriptional machinery. Significant differences in H3K4me3 enrichment were identified at 2813 loci (Table S8 in Supplement 2). The majority of affected loci fell within proximal promoter regions of protein coding genes located <1 kb from the transcription start site (Figure 4A). No major shift in the location of H3K4me3 peaks was observed (Figure 4B), indicating that the THC-mediated chromatin modification likely affects the density of histones within a particular region rather than changing their specific positions. Figure 4C provides examples of differential ChIP-seq peaks within several genes related to cannabis and synaptic neurobiology (*Cnr1*, *Camk2d*, *Gabbr2*, *Shank1*, *Kcnj3*) in individual animals. The specificity of H3K4me3 enrichment in proximal promoter regions of *Cnr1* and *Camk2d* is illustrated in Figure S6 in Supplement 1.

To examine genes where mRNA expression and H3K4me3 enrichment are affected by PTE, we identified genes that contained a differential H3K4me3 enrichment site and were differentially expressed, yielding 224 genes (Figure 4D). We stratified these overlapping genes by direction in either dataset (H3K4me3-up/RNA-up, H3K4me3-down/RNA-up, H3K4me3- up/RNA-down, H3K4me3-down/RNA-down), with counts of genes whose differential H3K4me3 sites were contained in the gene promoter or gene body (Figure 4E; Table S9 in Supplement 2). Interestingly, the highest number of overlapping genes were associated with increased H3K4me3 enrichment and decreased mRNA expression (i.e., H3K4me3-up/RNA-down) at promoter regions. Fisher's exact test further confirmed that out of the four overlaps, only H3K4me3-up/RNA-down showed statistical significance (OR = 2.004, p = 5.53 3 10212). Gene ontology analysis of this particular subset of genes identified strong enrichment of synaptic regulatory mechanisms (Figure 4F; Table S10 in Supplement 2). This extends our prior findings from the NanoString and RNA-seq assays showing reduced expression by PTE of genes involved in regulation of synaptic plasticity by demonstrating that these same genes are also differentially enriched for H3K4me3 binding (Figure 4G). We further investigated this H3K4me3-up/RNA-down set of genes by conducting a gene ontology analysis of all genes with H3K4me3 enrichment (Table S16 in Supplement 2) and identified enriched ontologies (false discovery rate-corrected) such as negative regulation of transcription by RNA polymerase II (p = .0441), negative regulation of nucleic acid–templated transcription (p = .0447), and negative regulation of transcription, DNA templated (p = .0457). These ontologies included 118 unique genes, 14 of which were also downregulated in the CRISPR model (Supplement). This demonstrates that H3K4me3 is enriched in genes that *repress* transcription, giving a functional explanation for H3K4me3-up/RNA-down being the largest and only statistically significant overlapping gene set. There were no significant ontologies identified in the H3K4me3-up/RNA-up list, which was the next most abundant category, but many genes falling in this group relate to functions such as skeletal muscle satellite cell activation, extracellular matrix, splicing, and transcription.

Specific *Kmt2a* Upregulation in Neuronal Cells Results in Molecular Disturbances Similar to PTE Effects

The neurobiological consequences of PTE develop over the period between conception and adulthood. As such, molecular mechanisms can be challenging to untangle, even using wellcontrolled *in vivo* animal models. To explore specific associations to *Kmt2a* dysregulation strictly in a neuronal context without the influence of environmental variables, we developed a CRISPR activation–based overexpression model of endogenous *Kmt2a* upregulation in human neuronal progenitor cells from male patients (Figure 5A) and identified specific changes in the cellular transcriptome (Figure S7A, B in Supplement 1; Tables S11 and S12 in Supplement 2), reflected in strong hierarchical clustering by treatment (Figure 5B). Comparison of our PTE-related RNA-seq dataset with the consequences of specific *Kmt2a* upregulation in the CRISPR model revealed, again, robust alterations of pathways related to synaptic mechanisms and neurotransmission (Figure S7C, D in Supplement 1; Table S13 in Supplement 2), demonstrating that *Kmt2a* indeed plays a direct role in these functional impairments. On a global level, Fisher's exact test showed that for a given gene, differential expression in the rat NAc due to PTE is associated with 38% higher odds of also being a DEG in the CRISPR activation RNA-seq data (OR = 1.38, p < .0001).

PTE and *Kmt2a* Upregulation Are Associated With Common Differential Gene Correlations and Hubs Within Gene Regulatory Networks

To address more complex gene-gene regulatory relationships in relation to *Kmt2a* and PTE, differential gene correlation analysis was conducted separately on the PTE RNA-seq, ChIP-seq, and CRISPR RNA-seq datasets (Table S14 in Supplement 2). To highlight overlapping regulatory relationships perturbed in these three datasets, we identified genegene pairs that were differentially correlated in more than one dataset. There were 1213 differential correlations common to two datasets, representing 591 unique genes. Gene ontology analysis on these 591 genes showed enrichments associated with glutamatergic synapse, postsynaptic density, synaptic membranes, and neuron part (Figure 5C). Of the 1213 differential correlations, 829 overlapped between the PTE ChIP-seq and CRISPR RNA-seq datasets, 146 between the PTE RNA-seq and CRISPR RNA-seq datasets, and 238 between the PTE ChIP-seq and PTE RNA-seq datasets. Of the 238 overlapping differential correlations between the PTE ChIP-seq and PTE RNA-seq datasets, 116 were between pairs of genes that were both part of the H3K4me3-up/RNA-down subset previously mentioned. These 116 gene pairs show robust THC-induced increases in correlation magnitude in both the RNA-seq and ChIP-seq datasets, demonstrating common, multiscale gene regulatory alterations by THC on both the gene expression and H3K4me3 enrichment levels (Figure 5D). These analyses demonstrate significant overlap of gene-gene regulatory relationships between experimental contexts (i.e., PTE and *Kmt2a* upregulation) and scales of analysis (i.e., gene expression, H3K4me3 enrichment).

To gain a deeper understanding of the molecular dynamics of *Kmt2a* in the contexts of PTE and *Kmt2a* upregulation, we conducted network analyses using the STRING protein-protein interaction database (Figure 6A). First, a *Kmt2a* reference network was built based on known protein-protein interactions between *Kmt2a* and its interaction partners.

Second, we constructed networks based on DEGs from the CRISPR *Kmt2a* overexpression dataset composing the top 10 enriched gene ontology categories for Biological Process and Cellular Component. Third, we constructed a network based on the DEGs from the PTE RNA-seq dataset in the NAc. These three data-driven networks collectively included 24 proteins overlapping with the *Kmt2a* reference network (Table S15 in Supplement 2), demonstrating that both the PTE and *Kmt2a* upregulation contexts recruit many known interaction partners of *Kmt2a* that are part of its molecular complex. The overlapping nodes as they appear in the *Kmt2a* reference network are shown in Figure 6B (for full network, see Figure S8 in Supplement 1). Several of the 24 overlapping proteins between the *Kmt2a* and two data-driven networks have well-known functions in the regulation of chromatin modification (e.g., Kmt2b, Hdac1, Ncoa3, Kat2b, Wdr5, histone variants) or regulate different steps of the transcriptional process in concert with *Kmt2a* (e.g., Mta2, Tp53, Ruvbl2, Cited2). Many genes altered by PTE and *Kmt2a* overexpression showed comparable and consistent direction of change (Figure 6C), emphasizing the central role of *Kmt2a* in the network of developmental epigenetic processes influenced by PTE. This analysis also highlighted several genes with consistent expression alterations involved in well-characterized repressive transcriptional processes (H2afx, Ncoa3, Ago2, Mef2a, Cited2, Mta2), which might explain the prevalence of transcriptional downregulation by PTE (Figure 6C). To identify potential mechanisms of how these epigenetic and transcriptional regulators affect synaptic regulation and plasticity, we examined their interaction partners in our data-driven networks in relation to the gene ontology categories enriched for the overlapping DEGs between the PTE and CRISPR datasets (Figure S7C, D in Supplement 1). Several genes related to synaptic plasticity were interaction partners of the epigenetic/transcriptional regulators: *Bcl11a*, *Dhcr24*, *Insig1*, *Klf5*, *Mef2c*, *Nup210*, *Smad3*, *Stat6*, and *Thbs1*. This analysis demonstrates thatknown interaction partners of *Kmt2a* are recruited in both the PTE and *Kmt2a* upregulation contexts and interact with genes with known involvement in the regulation of synaptic plasticity.

Gene Dysregulation Patterns Associated With PTE and *Kmt2a* Overexpression Are Present in Human MDD

We explored whether transcriptional networks induced by PTE or *Kmt2a* overexpression were also evident in human MDD because key behavioral effects observed in PTE animals included increased depressive-like phenotypes and altered hedonic state. Using publicly available RNA-seq and microarray datasets from human patients with MDD (the NAc, anterior insula, subiculum, and Brodmann areas 11, 25, and 89), we measured the overlap of DEGs in these datasets with the 591 genes mentioned previously that composed 1213 differential correlations that occurred in two of our sequencing datasets (i.e., PTE ChIP-seq and RNA-seq, *Kmt2a* upregulation RNA-seq). We filtered DEGs from the human datasets and calculated the percentage of DEGs overlapping with our set of 591 genes. In the PTE and *Kmt2a* upregulation RNA-seq datasets, 19.8% and 12.2% of DEGs overlapped with our set of 591 genes, respectively. Thus, there appears to be a core subset of genes related to synaptic plasticity that are differentially expressed *and* differentially correlated in PTE, *Kmt2a* upregulation, and MDD. Moreover, in relation to genes potentially involved in transcriptional repressive mechanisms (Figure 6C), there was consistency in the direction of

their expression in the NAc between our PTE and *Kmt2a* conditions and the human MDD datasets (Figure S9 in Supplement 1), along with some cortical regions.

Discussion

We demonstrated a relationship between PTE and long-term perturbations in natural reward–related motivation and anhedonia, endophenotypes that can be present in depression and other psychiatric disorders, and stress sensitivity concomitant with disturbances in the epigenomic regulation of the NAc. Our findings revealed that PTE-induced epigenomic dysregulation relates to histone methyltransferase Kmt2a and downstream synaptic plasticity–related transcriptional impairments that altogether converge on a network of repressive transcriptional processes implicated in MDD.

The observations that PTE leads to increased motivation for natural reward in adulthood are consistent with the literature that emphasizes long-term influence of developmental THC on reinforcement and motivation for drug and natural rewards (56–58). Our study now provides sex-specific molecular evidence that PTE increases expression of *Kmt2a* in the NAc (Figure S3 in Supplement 1) that persists into adulthood and that normalization of *Kmt2a* levels can normalize motivation for natural reward in adult male offspring. NAc *Kmt2a* knockout mice showed sex-specific altered FR5 responding (Figure S4 in Supplement 1). Similar to previous findings (10,59–61), we observed that PTE induced learned helplessness and anhedonic phenotypes in males lasting into adulthood. Moreover, stress is known to negatively affect individuals depending on compromised physiological and emotional states, and our findings suggest that PTE, in combination with stressful experiences, shifts the normal functioning toward distress, resulting in increased vulnerability to endophenotypes such as anhedonia present in depression and other psychiatric disorders. Thus, consistent with a "double-hit" hypothesis (62), PTE (first hit) and postnatal stressful conditions (second hit) may exacerbate negative affect and psychiatric vulnerability. Important for future studies is in-depth interrogation of sex and brain region specificity of Kmt2a regulation.

Various lines of evidence emphasize synaptic dysregulation in psychiatric disorders, and our study revealed robust longterm effects of PTE on the regulation of synaptic plasticity that directly related to the Kmt2a network. Kmt2a is an epigenetic regulator that affects multiple forms of histone methylation and is required for both neurogenesis (54) and normal synaptic plasticity in the adult brain (63). Moreover, the CB1 receptor, which mediates the initial cascade of events induced by PTE, is well known to regulate synaptic transmission by inhibiting calcium channels and activating potassium channels (64–67). Accumbal expression of these ion channels were altered not only after PTE but also by *Kmt2a* upregulation, implicating this epigenetic factor in the long-term synaptic plasticity alterations evident with PTE. Interestingly, the subset of genes strongly associated with synaptic plasticity within the differential gene correlation analysis for the PTE, and *Kmt2a* upregulation conditions also constituted a significant portion of the DEGs in subjects with MDD. These findings suggest common synaptic disturbances in PTE and MDD that are regulated by Kmt2a.

The unbiased data-driven networks also identified consistent alterations of genes (e.g., Ago2 and Cited2 in the NAc) involved in repressive transcriptional processes, which is intriguing, given the predominant decrease in gene expression associated with PTE and *Kmt2a* upregulation. Our data suggest that transcriptional repressors (e.g., H2afx, Mta2; increased), transcriptional activators (e.g., Cited2, Ncoa3; reduced), and posttranscriptional repressive mechanisms involving small RNA processing by Ago2 are perturbed by PTE. Importantly, these have important roles in synaptic regulation. For example, Mta2 (metastasis-associated 1 family member 2) is a subunit of NuRD histone deacetylase complex involved in synaptic development connectivity (68). Cited2 (Cbp/P300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2) is a transactivator involved in neuronal activity–dependent long-term potentiation (69). Ncoa3 (nuclear receptor coactivator-3) is a histone acetyltransferase that influences neuroplasticity in a microRNAdependent manner via regulating Ago2 (70). Importantly, many key Kmt2a network components were also dysregulated in human MDD, emphasizing a translational connection between PTE and depressive-like phenotypes.

Overall, our findings support a direct role of PTE to enhance neuropsychiatric risk as identified in human longitudinal studies (16) including motivational disturbances, stress sensitivity, and depressive-like phenotypes. We emphasize a role of Kmt2a dysregulation to induce behavioral changes underlying long-term neuropsychiatric vulnerability and demonstrate an epigenetic signature linked to molecular disturbances of synaptic plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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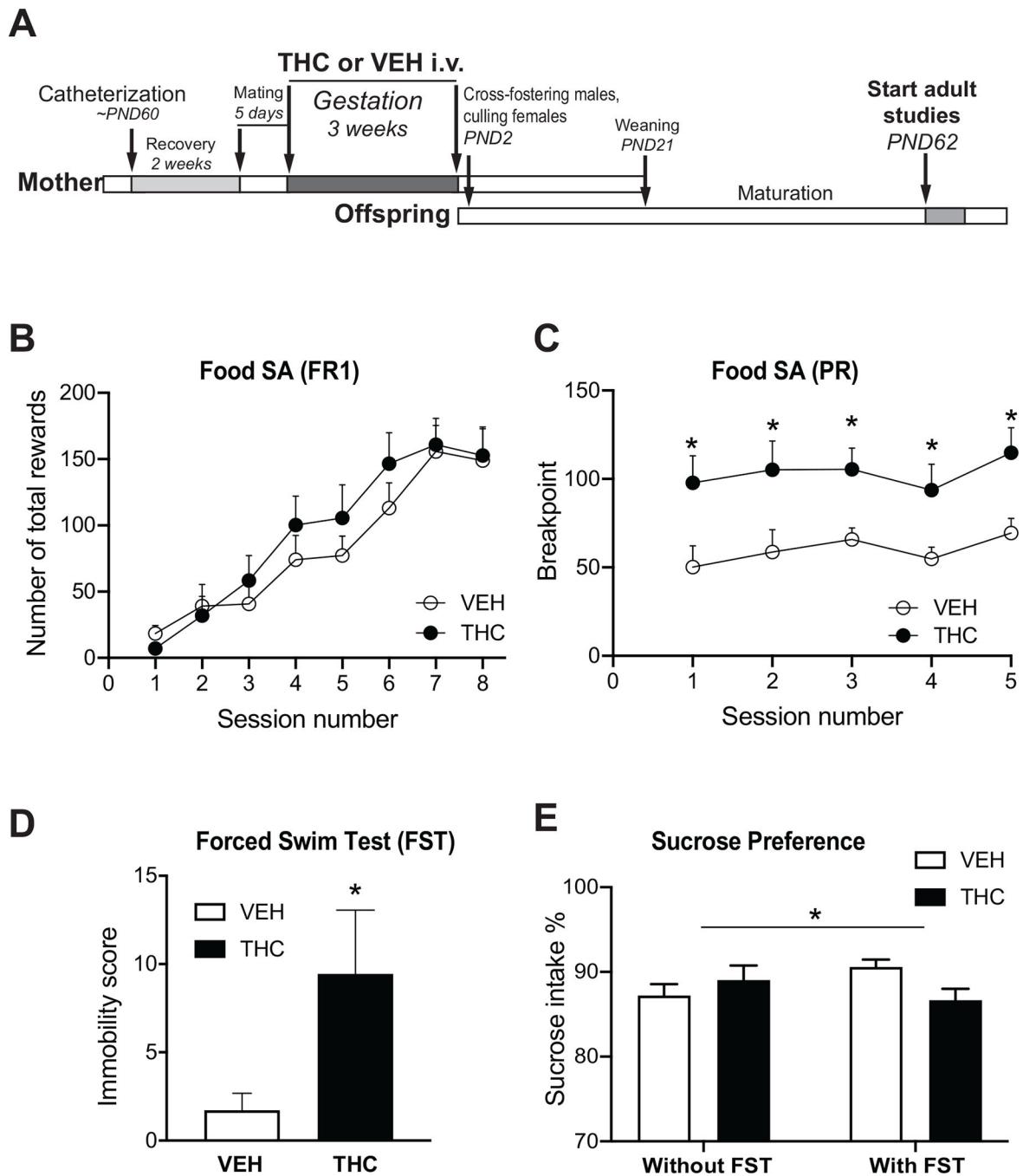
A

Figure 1. THC treatment *in utero* leads to enduring behavioral alterations in adult offspring.
(A) Prenatal THC Paradigm. Pregnant females were treated with low dose THC or VEH and offspring were studied at adulthood. Food Self-administration under (B) Fixed Ratio 1 (FR1) and (C) Progressive Ratio (PR) schedules. (D) Immobility behavior in the Forced Swim Test (FST). (E) Preference of 1% sucrose solution over water. Horizontal line and asterisk indicate a significant interaction effect between PTE and FST. N=7–9/group in all behavioral tests. Data are expressed as mean ± SEM. *:p<0.05.
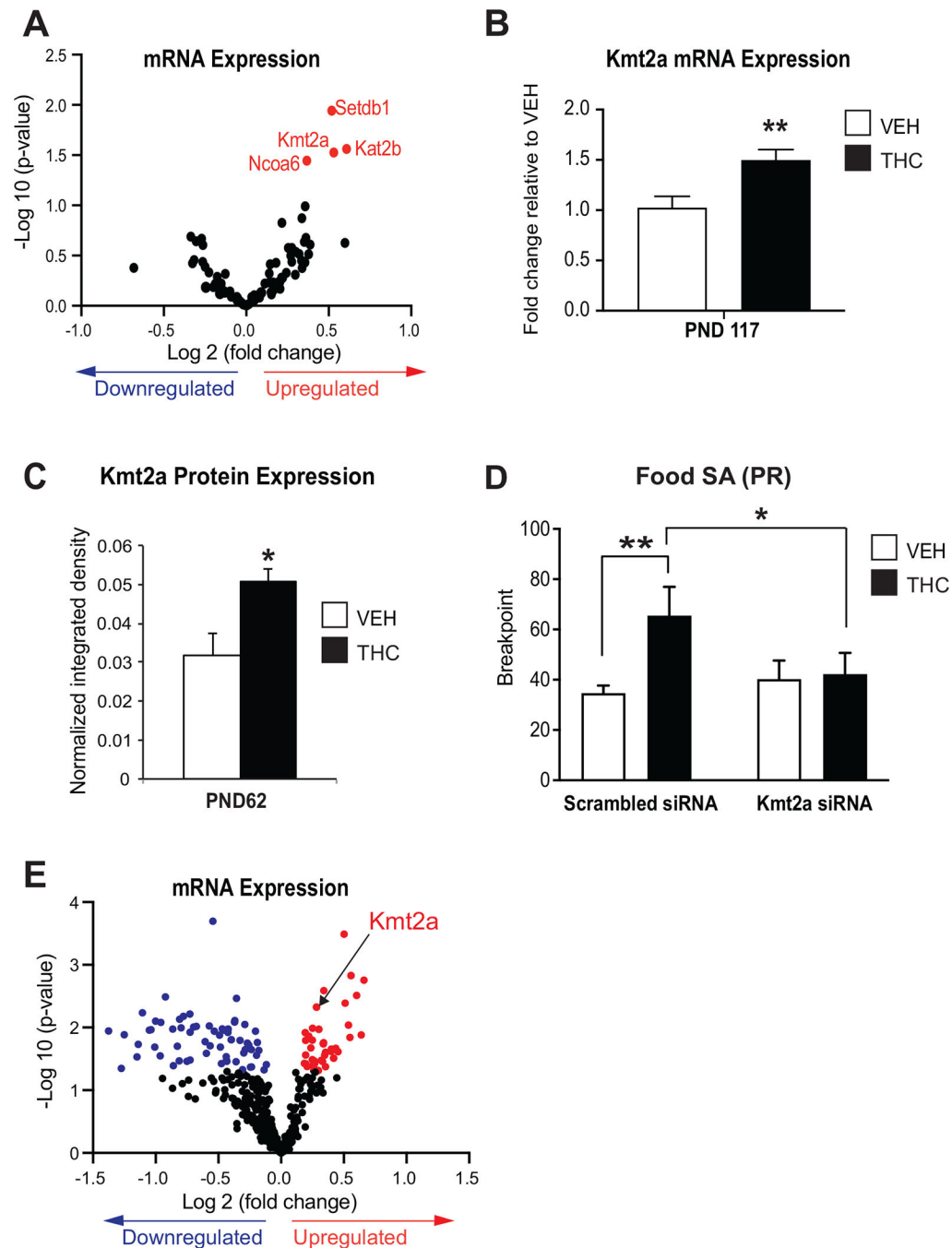
Figure 2. Increased NAc *Kmt2a* expression due to PTE is associated with impaired motivational behavior and dysregulation of genes functionally related to neuronal development and plasticity. (A) Changes in the mRNA levels of epigenetic regulators. Red dots: upregulated genes at p<0.05. N=5–6/group, PND62. (B) *Kmt2a* mRNA expression throughout development and (C) protein level in young adult offspring. (D) PR food SA breakpoint in siRNA-infused animals, 24h post-infusion. N=4–6/group. Data represented as mean ± SEM. *:p <0.05. (E) Gene expression alterations in adult rats with prenatal THC. Blue dots: downregulated genes, red dots: upregulated genes at p<0.05. N=6–10/group, PND62.
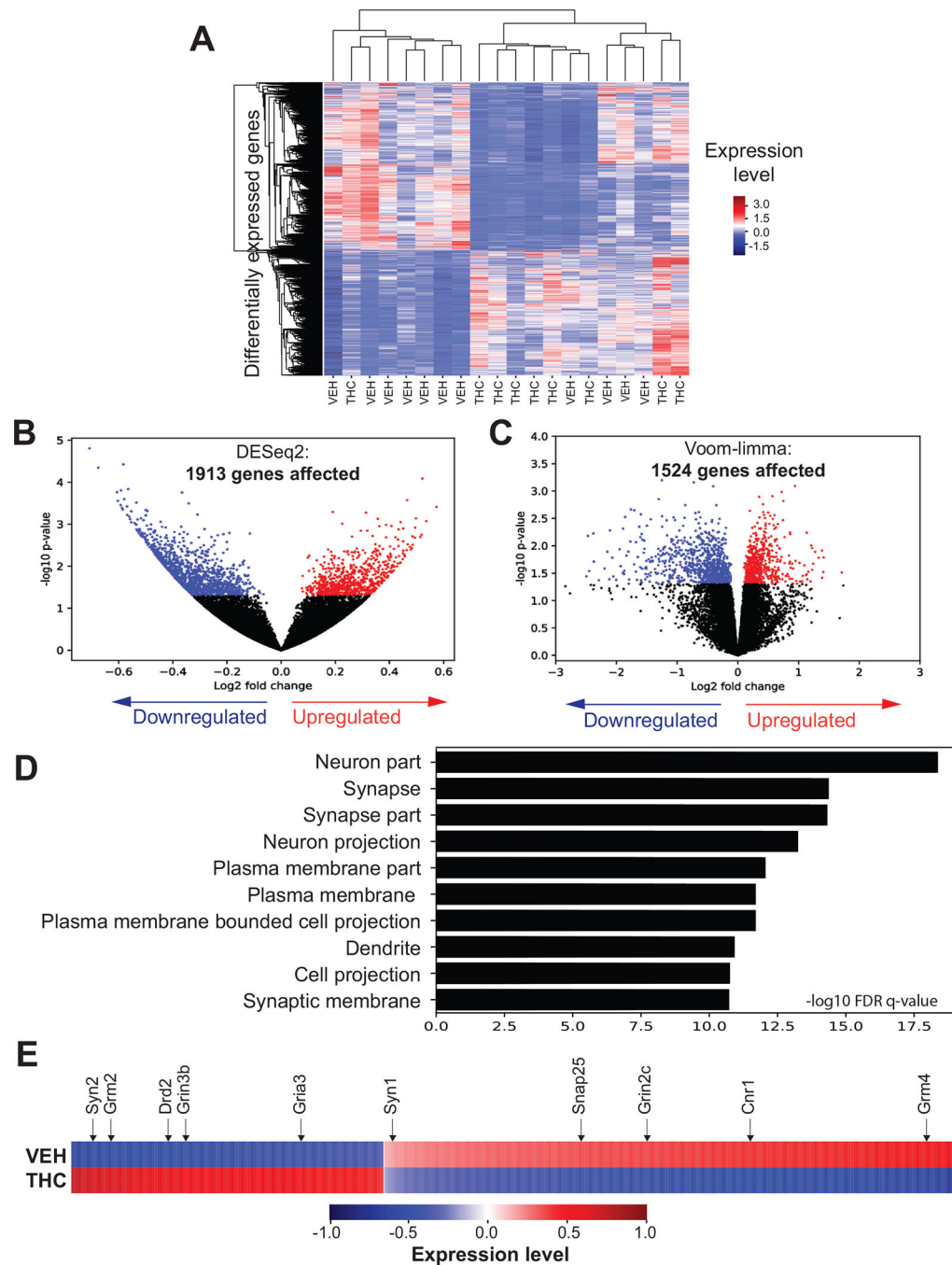
Figure 3. Consequences of PTE on the NAc transcriptome and affected biological pathways.
(A) Hierarchical clustering of RNA-seq reads for all DEGs (vertical dimension) and samples (horizontal dimension), PND62. (B, C) Distribution of DEGs obtained using the DESeq2 and Voom-limma analysis tools. Each dot corresponds to a single gene; colors indicate significant up-(red) or down-(blue) regulation. (D) Gene ontology analysis using GOrilla on DEGs shared by the DESeq2 and Voom-limma analyses; graph represents the top 10 categories within "cellular component". (E) Heatmap showing changes in the RNA

expression level of DEGs within the above top 10 categories. Arrows point to several interesting genes involved in synaptic regulation.
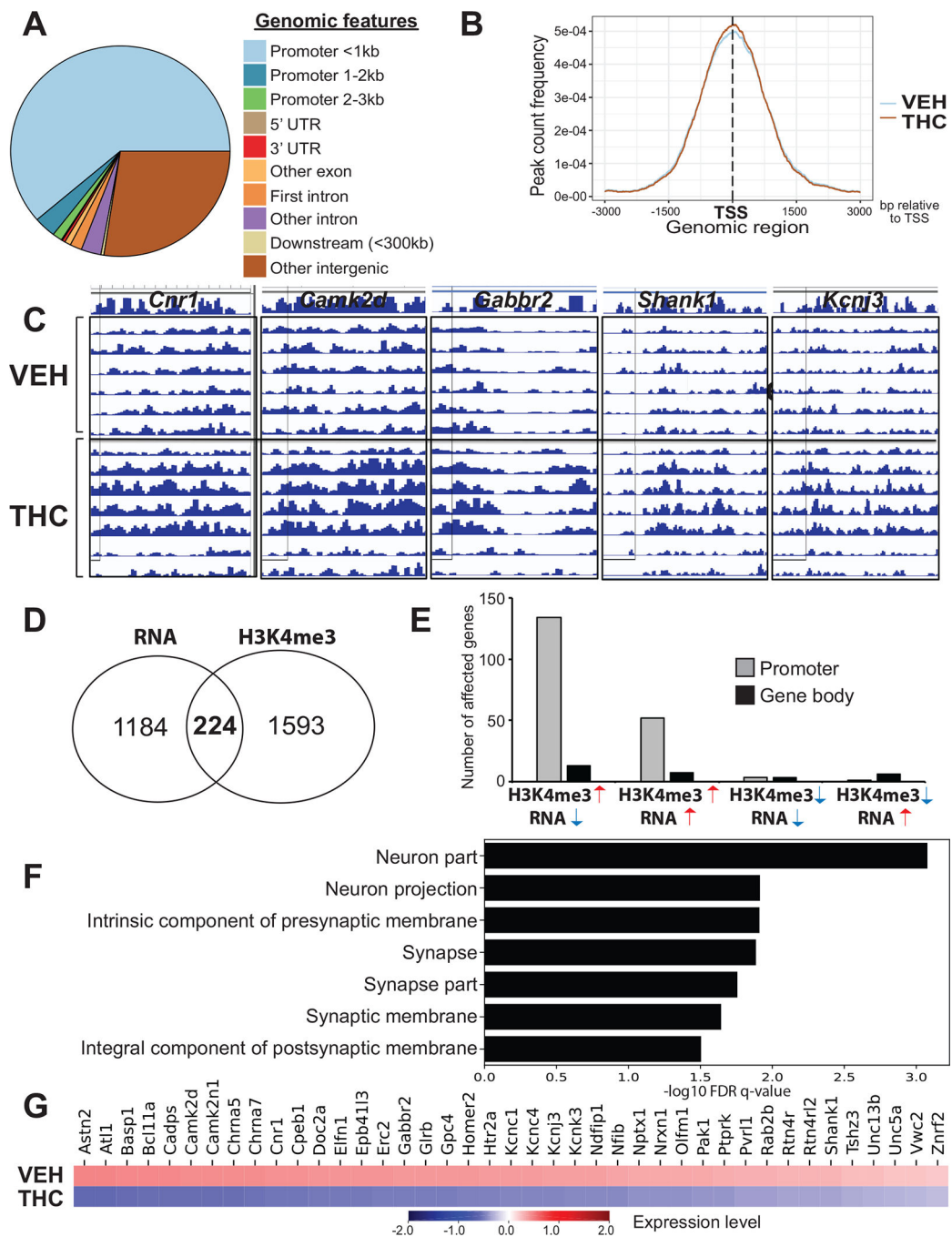
Figure 4. PTE changes the epigenomic profile of the NAc at gene loci functionally relevant to the regulation of synaptic plasticity.

(A) Enrichment of different genomic features within chromatin domains affected by THC. N=6–7/group. (B) No significant THC-related shift in the distribution of H3K4me3 peaks relative to the transcriptional start sites of affected genes. (C) ChIP-seq peaks for five genes related to cannabis and synaptic neurobiology. Individual rows correspond to samples within the VEH and THC groups. (D) Comparison between genes affected on the mRNA (RNA-seq) and H3K4me3 (ChIP-seq) level. Numbers inside the Venn diagram indicate altered expression regardless of direction of change. (E) Relationship between mRNA transcript

and H3K4me3 level changes at different gene loci. Note the higher abundance of affected promoters vs. coding regions. (F) Gene ontology analysis on "H3K4me3 up/RNA down"; graph represents the top 10 "cellular component" categories and heatmap (G) showing the affected genes.
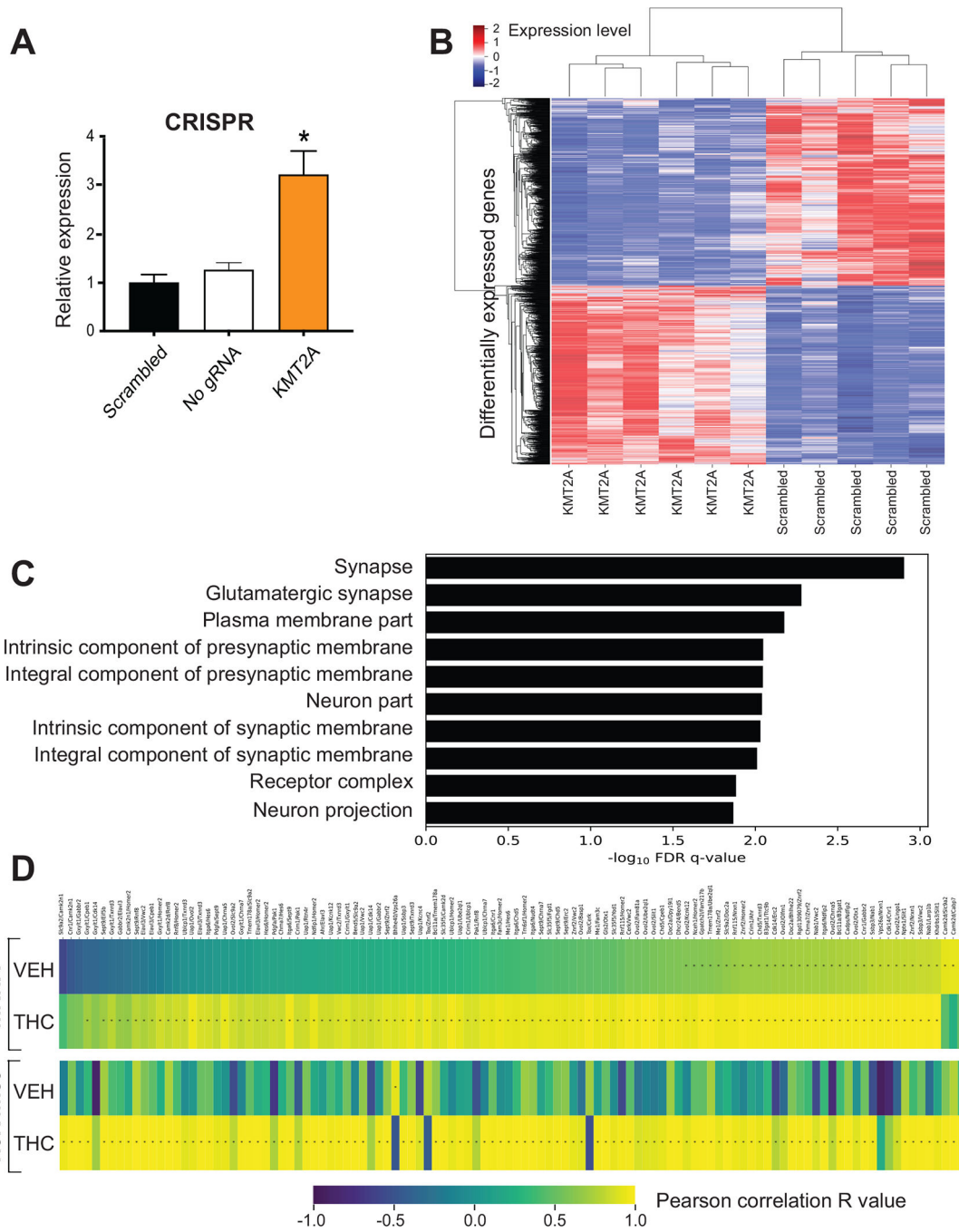
Figure 5. Specific KMT2A upregulation is associated with similar complex alterations to PTE.
(A) KMT2A upregulation achieved using CRISPR in Neuronal Progenitor Cells. Normalized relative mRNA levels of *Kmt2a* (orange) compared to Scrambled gRNA control (black) following transduction of dCas9^{VPR} in NPCs. (B) Hierarchical clustering of RNA-seq reads for all DEGs (vertical dimension) and CRISPR samples (horizontal dimension). N=5–6/group. (C) Ontological categories affected in gene-gene relationships observed in our different datasets. (D) Altered pairwise correlations on the mRNA and H3K4me3 levels in the prenatally THC-exposed NAc. Colors correspond to Pearson R values for each gene pair.
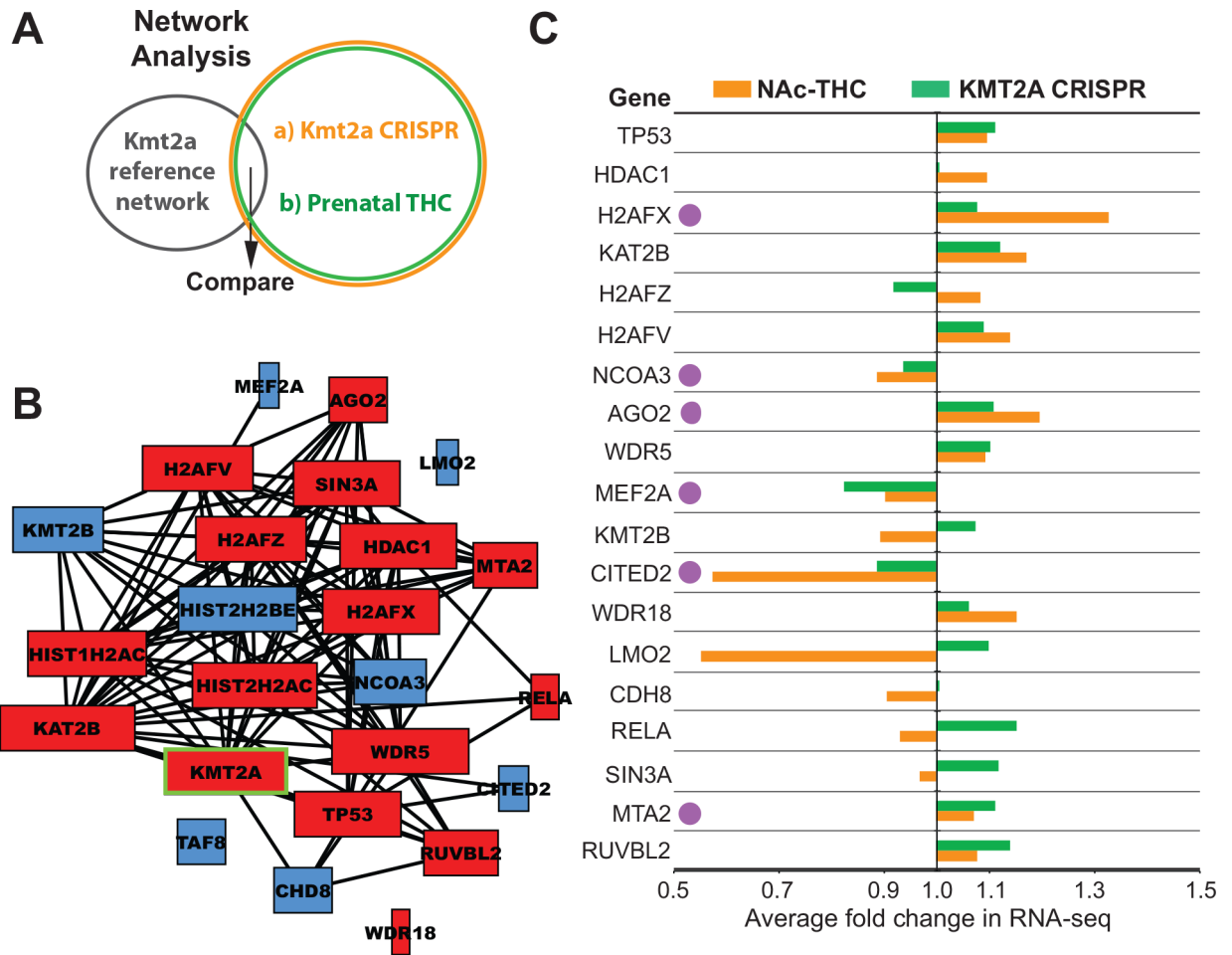
Figure 6. Affected *Kmt2a* gene network based on specific *Kmt2a* dysregulation and PTE.
(A) Network analysis approach. (B) *Kmt2a* network identified. Colors indicate genes dysregulated either in our prenatal THC or CRISPR *Kmt2a* overexpression studies. Red: upregulation, blue: downregulation. (C) mRNA level changes of genes within the affected *Kmt2a* network due to prenatal THC (green bars) and specific *Kmt2a* upregulation by CRISPR (orange bars). Purple dots indicate potentially interesting repressors.

KEY RESOURCES TABLE

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Add additional rows as needed for each resource type	Include species and sex when applicable.	Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use "this paper" if new.	Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources.	Include any additional information or notes if necessary.
Antibody	anti-Kmt2a primary antibody	Bethyl	374A	
Antibody	anti-Cas9	Millipore	7A9	
Antibody	β-Actin	Ambion	1406030	
Cell line	human neural progenitor cells	Donor from male control patient	NSB2607	HiPSC-derived cell line. Collected as fibroblasts, reprogrammed into NPCs. More information here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM2843354
Chemical Compound or Drug	Trizol	ThermoFisher	15596026	
Chemical Compound or Drug	200 mg/ml THC stock	NIDA	N/A	
Commercial Assay Or Kit	RNAqueous-micro kit	Ambion	AM1931	
Commercial Assay Or Kit	TaqMan assay	ThermoFisher	Rn01444748_m1	
Commercial Assay Or Kit	nCounter array	Nanostring	N/A	
Commercial Assay Or Kit	DNAse treatment	ThermoFisher	EN0525	
Commercial Assay Or Kit	Bioanalyzer	Agilent	G2939BA	
Commercial Assay Or Kit	uQuant plate reader	Biotek Instruments	UNK446	
Commercial Assay Or Kit	NovaSeq 6000 S1 flowcell PE50	Illumina	N/A	
Commercial Assay Or Kit	Qubit instrument	ThermoFisher	Q33239	
Deposited Data; Public Database	Gene Expression Omnibus	NCBI	GSE87610, GSE102556	
Genetic reagent	ON-TARGETplus SMART pool, rat *Kmt2a*,	Horizon Discovery	L-100995-02-0005	

Resource Type	Specific Reagent or Resource	Source or Reference	Identifiers	Additional Information
Genetic reagent	ON-TARGET nontargeting pool	Horizon Discovery	D-001810–10-05	
Genetic reagent	lentiCRISPR v2	Addgene	plasmid # 52961	
Genetic reagent	Guide RNAs	Benchling	N/A	
Genetic reagent	AAV8-hSyn-Cre-GFP	https://www.med.unc.edu/genetherapy/vectorcore/in-stock-aav--vectors/reporter-vectors/	N/A	
Transfection Reagent	JetSI transfection reagent	Polyplus Transfection	N/A	
Organism/Strain	Long-Evans female dams	Charles River Laboratories	N/A	
Organism/Strain	Mice (Kmt2a2lox/2lox)	Bred in-house		
Software; Algorithm	Gen5	Biotek Instruments	RRID:SCR_017317	
Software; Algorithm	Subread	PMID:23558742	RRID:SCR_009803	
Software; Algorithm	DESeq2	https://bioconductor.org/packages/release/bioc/html/DESeq2.html	RRID:SCR_015687	
Software; Algorithm	Voom-limma	http://bioinf.wehi.edu.au/limma/	RRID:SCR_010943	
Software; Algorithm	Bowtie	PMID:19261174	RRID:SCR_005476	
Software; Algorithm	SAMtools	PMID:21903627	RRID:SCR_002105	
Software; Algorithm	MACS	PMID:18798982	RRID:SCR_013291	
Software; Algorithm	FastQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	RRID:SCR_014583	
Software; Algorithm	diffReps	https://doi.org/10.1371/journal.pone.0065598	RRID:SCR_010873	
Software; Algorithm	ChIPseeker	http://bioconductor.org/packages/devel/bioc/vignettes/ChIPseeker/inst/doc/ChIPseeker.html#:~:text=ChIPseeker%20is%20an%20R%20package,peaks%20binding%20to%20TSS%20regions,&text=Currently%2C%20ChIPseeker%20contains%202017%2C0000%20bed%20file%20information%20from%20GEO%20database.	N/A	
Software; Algorithm	HISAT2	PMID:25751142	RRID:SCR_015530	
Software; Algorithm	STRING	PMID:21045058	RRID:SCR_005223	