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Developmental cannabidiol exposure increases anxiety and modifies genome-wide brain DNA methylation in adult female mice

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Abstract

Background: Use of cannabidiol (CBD), the primary non-psychoactive compound found in cannabis, has recently risen dramatically, while relatively little is known about the underlying molecular mechanisms of its effects. Previous work indicates that direct CBD exposure strongly impacts the brain, with anxiolytic, antidepressant, antipsychotic, and other effects being observed in animal and human studies. The epigenome, particularly DNA methylation, is responsive to environmental input and can direct persistent patterns of gene regulation impacting phenotype. Epigenetic perturbation is particularly impactful during embryogenesis, when exogenous exposures can disrupt critical resetting of epigenetic marks and impart phenotypic effects lasting into adulthood. The impact of prenatal CBD exposure has not been evaluated; however, studies using the psychomimetic cannabinoid Δ^9 -tetrahydrocannabinol (THC) have identified detrimental effects on psychological outcomes in developmentally exposed adult offspring. We hypothesized that developmental CBD exposure would have similar negative effects on behavior mediated in part by the epigenome. Nulliparous female wild-type Agouti viable yellow (A^{vy}) mice were exposed to 20 mg/kg CBD or vehicle daily from two weeks prior to mating through gestation and lactation. Coat color shifts, a readout of DNA methylation at the Agouti locus in this strain, were measured in F1 A^{vy}/a offspring. Young adult F1 a/a offspring were then subjected to tests of working spatial memory and anxiety/compulsive behavior. Reduced-representation bisulfite sequencing was performed on both F0 and F1 cerebral cortex and F1 hippocampus to identify genome-wide changes in DNA methylation for direct and developmental exposure, respectively.

Results: F1 offspring exposed to CBD during development exhibited increased anxiety and improved memory behavior in a sex-specific manner. Further, while no significant coat color shift was observed in A^{vy}/a offspring, thousands of differentially methylated loci (DMLs) were identified in both brain regions with functional enrichment for neurogenesis, substance use phenotypes, and other psychologically relevant terms.

Conclusions: These findings demonstrate for the first time that despite positive effects of direct exposure, developmental CBD is associated with mixed behavioral outcomes and perturbation of the brain epigenome.

Keywords: Cannabis, Anxiety, Memory, Prenatal, Epigenetics, Addiction, DOHaD

Background

Cannabidiol (CBD) is the primary non-psychomimetic compound found in cannabis (*Cannabis sativa*) and an FDA-approved treatment for childhood epilepsy that also shows therapeutic potential for several neuropsychiatric disorders. Use of both cannabis and CBD is rising in the USA with CBD sales expected to reach 1.8 billion

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dollars by 2022 [1], due in part to reports of their positive effects on psychological phenotypes. In rodent studies, exposure to CBD in adulthood has been shown to reduce immobility and increase swimming time in the forced swim test, a measure of depression [2, 3], increase time spent in the open arm of the elevated plus maze, a measure of anxiety [4–7], and reduce responsiveness to drugs of addiction such as morphine and cocaine [4, 8]. In human trials, CBD additionally reduces psychotic symptoms in schizophrenia [9, 10] and lowers subjective measures of anxiety [11, 12]. These findings contrast with previous research on the psychoactive cannabinoid Δ^9 -tetrahydrocannabinol (THC), which report anxiogenic and other adverse psychological effects that concurrent CBD administration may counteract [13–15]. In vitro and in vivo studies suggest that the underlying mechanisms behind CBD's actions in the brain are complex. CBD acts on a large number of targets including serotonin 1a (*5HT-1a*), peroxisome proliferator-activated receptor gamma (*PPAR γ*), and transient receptor potential cation channel subfamily V (*TRPV*) receptors, antioxidant mechanisms, and modulation of endocannabinoid levels [5, 7, 16–19]. CBD also appears to impact neurogenesis in the hippocampal dentate gyrus, one of only two sites of ongoing neurogenesis in the adult brain. Luján, Cantacorps, and Valverde demonstrated that the protective effects of CBD on voluntary cocaine intake were ameliorated by pharmacological inhibition of hippocampal neurogenesis, and Campos et al. determined that CBD's anxiolytic effects were driven by facilitating endocannabinoid-mediated neurogenesis using a mouse model of chronic unpredictable stress [16, 20].

Despite the potential for therapeutic applications of direct CBD exposure, its widespread actions in the brain and increasing use of cannabinoids during pregnancy raise concern for potential impacts on the developing fetus and subsequent adult. Administration of THC during pregnancy has been associated with negative cognitive outcomes in rodent models [21]. Notable examples include a study by de Salas-Quiroga et al., who identified sex-specific deficits in spatial memory in mice prenatally exposed to THC [22, 23], and work by Trezza et al. associating perinatal THC with altered vocalization and social and play behavior in rats [24]. Mereu et al. similarly found that the synthetic cannabinoid CB1 receptor agonist WIN 55212-2, which mimics the actions of THC, disrupted memory retention and led to hyperactive behavior in prenatally exposed adult rats [25]. Effects on addiction-related behavior have also been an area of interest for cannabinoid exposure during pregnancy, with reports of increased heroin seeking [26] and morphine self-administration [27] and modified dopamine [28] and enkephalin [29] signaling being reported. Perturbed

glutamatergic [30], GABAergic [31], and serotonergic signaling [32] have also been observed, reflecting the widespread impact of exogenous cannabinoids in the brain.

The developmental origins of health and disease (DOHaD) hypothesis state that early-life environmental exposures can mediate later life phenotypes via epigenomic perturbation [33–36]. DNA methylation is the most commonly studied epigenetic mark and occurs when a methyl group is added to the fifth carbon of cytosine in a cytosine-guanine dinucleotide (CpG) context in mammals. Differential methylation is highly relevant for neuropsychiatric diseases and has been identified in association with schizophrenia [37–42], depression [43–46], anxiety [47–50], and autism spectrum disorder [51–55]. Several studies have identified differential methylation patterns in the sperm of humans and rats directly exposed to THC, particularly at the autism candidate locus *DLGAP2*, and a study by Watson et al. demonstrated that parental THC shifts DNA methylation of genes relevant for glutamatergic signaling in the rat nucleus accumbens [56–58]. Importantly, the effects of parental exposure to CBD have not yet been explored. The presence of cannabinoids during critical windows of methylation pattern setting in development has the potential to persistently alter patterns of gene regulation in the brain; these changes are likely to contribute to adverse neuropsychiatric phenotypes in adulthood.

In this context, the aim of the present study was to investigate the effects of developmental exposure to CBD on adult behavior and the brain methylome. To this end, we subjected pregnant mice to a subchronic CBD exposure paradigm and tested their abstinent adult offspring for abnormalities in memory and anxiety behavior. Regarding DNA methylation, we utilized the Agouti viable yellow (*A^{vy}*) environmental biosensor model [59], which provides a readout of methylation changes at the *Agouti* locus via offspring coat color and has been successfully used to identify epigenomic perturbations associated with prenatal exposure to bisphenol A [60], lead [61], and other compounds. For a genome-wide perspective on a target tissue for neuropsychiatric phenotypes, we performed reduced-representation bisulfite sequencing (RRBS) in the cerebral cortex and hippocampus of adult F1 offspring. We found that developmental CBD exposure resulted in a sex-specific increase in anxiety behavior affecting female offspring and identified over 2000 differentially methylated loci in each brain region. Thousands of differentially methylated loci were additionally identified in the cortex of directly exposed F0 females in the absence of anxiety or memory changes, echoing recent studies showing modified methylation with CBD exposure and recapitulating behavior studies

demonstrating a lack of effect in the absence of a stressor. Overall, these findings provide an initial investigation into the effects of prenatal exposure to CBD, identify behavior deficits and functionally relevant methylation changes in the brain, and support the importance of neuroepigenetics in the etiology of psychiatric phenotypes.

Methods

Animals

Animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals and were treated humanely and with regard for alleviation of suffering. The study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Mice were obtained from an Agouti viable yellow (A^{vy}) colony maintained for over 220 generations with the A^{vy} allele passed through the male line, resulting in forced heterozygosity on a genetically invariant background with 93% identity to C57BL/6 [62, 63]. All animals were maintained on a standard chow diet (Envigo Teklad 19% protein 2019 breeder diet for dams and 18% protein 2018 maintenance diet for offspring) and housed in cages of 3–4 individuals on corn cob bedding with a 12-h light/dark cycle.

Exposure paradigm

Pharmaceutical-grade CBD (Epidiolex, GW Pharmaceuticals, Cambridge, UK) was purchased at the University of Minnesota Boynton Health Pharmacy (Minneapolis, MN). CBD was diluted to 10 mg/mL concentration in honey (Nice! Organic Honey, Walgreens) due to its high lipophilicity and stability at room temperature. Honey has been used successfully as a drug delivery vehicle by others [64]. Twenty-two six- to ten-week-old, sexually mature nulliparous wild-type a/a females were randomized into two groups and received either 20 mg/kg Epidiolex™ (GW Pharmaceuticals, Cambridge, UK) emulsified in honey or vehicle only daily via oral administration using the tip of a 14-gauge gavage needle for 14 days prior to mating. This dose was chosen based on previous CBD studies and approximates casual human use (~1.5 mg/kg) due to scaling factors for body surface area [3, 4, 8, 65, 66]. On day 14, F0 females were harem-mated with A^{vy}/a males (8–12 weeks of age) and daily dosing continued through gestation, lactation, and behavior testing for a total exposure time of approximately 9 weeks. All animals had access to food and drinking water ad libitum throughout the experiment in accordance with the Institute of Laboratory Animal Resources guidelines [67]. F1 animals were drug-abstinent following weaning.

Behavior procedures

F0 exposed and control dams were subjected to the Y-maze spontaneous alternation test (spatial working memory) and the marble burying task (anxiety and compulsive behavior) between 4–7 days following the weaning of pups with CBD exposure ongoing. Tests were conducted in the home mouse room during the light phase of the light–dark cycle. Each F0 female was tested twice in order to differentiate acute from cumulative CBD effects with consecutive tests being at least 24 h apart. For acute testing, dams were dosed with either 20 mg/kg CBD or vehicle between 0.5 and 1.5 h prior to testing to ensure CBD plasma levels were near C_{max} [68]. For cumulative effect testing, dams were tested approximately 24 h after the last dose to ensure CBD plasma levels were low. Adult a/a F1 offspring, which were drug-abstinent after weaning, were subjected to the same behavior tests once per animal at 12 weeks of age with at least 24 h between tests. Same sizes were as follows: F0, $n=9$ control, 7 exposed; F1, $n=17$ control, 16 exposed. F0 behavior testing was conducted on females only as males were not exposed to CBD, while F1 behavior testing was conducted on both males and females.

Y-maze spontaneous alternation

The Y-maze spontaneous alternation task is a measure of spatial working memory and exploits rodents' natural tendency to explore novel areas [69, 70]. Mice were placed at the end of one arm facing the center in a standard Y-maze (MazeEngineers, Boston, MA) consisting of a high-walled chamber with three arms connected at 120°. Investigators vacated the room, and the animal's movement was recorded on video using a tripod and digital camera for ten minutes. After ten minutes, the animal was returned to the home cage and the apparatus was sanitized with 70% alcohol to prevent scent trails from confounding subsequent runs. The sequence of entries (all four feet within the arm) was recorded from the video by an investigator blinded to treatment group, and the spontaneous alternation percentage was calculated as the number of spontaneous alternations \div (number of entries $- 2$) $\times 100$. One spontaneous alternation was counted when three consecutive entries into unique arms (e.g., A, B, C) were recorded. The total number of arm entries was also recorded as a measure of exploration.

Marble burying

The marble burying task is a measure of anxiety and compulsive behavior in mice and takes advantage of rodents' natural tendency to bury objects [71–73]. Mice were individually placed into the corner of a rat cage filled with 10 cm (height) of corn cob bedding and 20 evenly

placed marbles (4 × 5 layout) distributed on its surface. Testing was conducted with the investigator absent from the room for 30 min. After 30 min, mice were carefully removed from the test cage to avoid disturbing the bedding and the number of marbles buried was counted, with marbles at least 2/3 covered being counted as buried. The bedding was thoroughly mixed, and marbles were washed with dish soap and rinsed with 70% ethanol following each trial to prevent scent from affecting subsequent runs.

***A^{vy}/a* coat color**

The *A^{vy}* strain was used to determine whether CBD exposure imparted large-scale changes in DNA methylation in developmentally exposed offspring. Briefly, the *Agouti* gene produces a paracrine signaling molecule that determines coat color, among other functions. The upstream region of the *Agouti* gene of *A^{vy}/a* mice contains an intracisternal A particle (IAP) retrotransposon insertion that leads to constitutive, metastable expression of the gene with the magnitude of expression dependent upon stochastic DNA methylation within the insertion [74]. Shifts in methylation of these metastable loci can be triggered by in utero environmental exposures such as bisphenol A, resulting in a shifted distribution of coat colors in the affected offspring via variable production of pheomelanin [60]. Constitutive expression of the *Agouti* gene is not limited to hair follicles and thus leads to obesity, liver tumors, diabetes, and other phenotypes in *A^{vy}/a* animals. Therefore, to eliminate confounding effects, only wild-type *a/a* animals produced by *A^{vy}/a* × *a/a* breedings were used for molecular and behavioral analyses. *A^{vy}/a* F1 offspring were photographed from above at 5–7 weeks of age in order to measure coat color, a readout of DNA methylation at the metastable *Agouti* epiallele in this strain. Coat color photographs were scored on a three-category scale (low brown mottling/low methylation, medium mottling/medium methylation, high mottling or pseudoagouti/high methylation) by two investigators blinded to treatment group. Discrepant scores were settled by a third blinded investigator to identify overall and sex-specific differences in methylation between CBD and control groups.

Statistical analysis

Normality for behavior scores and coat color data were assessed using density plots and QQ plots. Between-group differences in F0 and F1 behavior scores (performed separately for both F0 dosage timing windows) were assessed using Wilcoxon rank-sum tests in RStudio. Two F1 female outliers (one control, one exposed) lying more than two standard deviations above the group mean were identified for Y-maze spontaneous alternation

and removed from the analysis. Sex:group interactions for behavior tests were assessed using a one-way analysis of variance (ANOVA). Between-group differences for F0 acute and cumulative behavior scores were assessed using Wilcoxon rank-sum tests, and paired within-group scores (acute vs. cumulative) were assessed using Wilcoxon signed-rank tests. Chi-square tests for trend were used to evaluate overall and sex-specific differences in F1 *A^{vy}* coat color between groups. Wild-type F1 weights were measured from weaning through behavior testing, and differences between groups were assessed using ANOVA, while differences in litter size were determined using Wilcoxon rank-sum tests.

DNA isolation and bisulfite sequencing

Samples were prepared, and sequencing was performed as described previously [75]. Briefly, animals were euthanized via isoflurane inhalation followed immediately by internal decapitation. The brain was removed and dissected fresh using a stereoscope to obtain cortical (F0 *n* = 6, F1 *n* = 6) and hippocampal (F1 *n* = 4) regions [76]. Tissue samples were placed directly in RNAlater (Sigma-Aldrich) and stored at 4 °C overnight, then transferred to -80 °C for long-term storage. Total genomic DNA (gDNA) was isolated from each animal using the DNeasy Blood and Tissue kit following the manufacturer's protocol (Qiagen, Hilden, Germany). A NanoPhotometer N50 system was used to check DNA yield with three biological replicates per group being chosen for further processing based on concentration and quality. Methylation analyses were performed on F0 dams and F1 females only. gDNA was bisulfite-converted following isolation using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA). Bisulfite conversion allows detection of methylated cytosines via treatment of DNA with sodium bisulfite, which causes unmethylated cytosines to be deaminated to uracils. These loci are read as thymidine by polymerases during sequencing. Genome-wide DNA methylation levels were measured using reduced-representation bisulfite sequencing (RRBS) at Diagenode, S.A. (Belgium). Briefly, DNA concentration of samples was measured using the Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific), and DNA quality was assessed using the Fragment Analyzer™ and DNF-488 High Sensitivity genomic DNA Analysis Kit (Agilent). RRBS libraries were prepared using the Premium Reduced Representation Bisulfite Sequencing Kit (Diagenode Cat# C02030033), and 100 ng of genomic DNA was used to start library preparation for each sample. Bisulfite sequencing was performed in single-end mode 50 bp (SE50) on an Illumina HiSeq 3000/4000. Quality control of reads was performed using FastQC version

0.11.8 [77], and adapter removal was performed using Trim Galore! Version 0.4.1 [78]. Bismark, a specialized tool that utilizes an in silico bisulfite-converted reference genome, was used for mapping bisulfite-treated reads [79]. The cytosine2coverage module of Bismark was used to determine the methylation state of all cytosines for every uniquely mappable read, determine their sequence context, and compute the percentage methylation. Spike-in control sequences were used to check the bisulfite conversion rates and to validate the efficiency of bisulfite treatment. The resulting cytosine loci were filtered to exclude non-CG context cytosines, loci with less than 10 reads, and loci with less than two biological replicates per group using R version 3.6.1.

DML and DMR calling, annotation, and functional enrichment

RStudio open-source software (version 3.6.1) tools were used for RRBS analysis as described previously [75]. Briefly, the *DSS* R package (version 2.32.0) was used to test RRBS data for differential methylation between CBD-exposed and control animals [80]. The *DMLtest*, *callDML*, and *callDMR* functions in *DSS* were used to identify differentially methylated CpG loci (DMLs) and regions (DMRs) with $\Delta > 0.1$ and local FDR < 0.001 . The *annotatr* R package (version 1.10.0) was used to annotate DMLs and DMRs to the mm10 genome [81]. Predicted genes and three large erroneous gene transcripts (ENSMUST00000127664.1, ENSMUST00000124096.7, and ENSMUST00000154148.7), which were present in *annotatr*'s GENCODE-based intervals but not in RefSeq when assessed using UCSC Genome Browser, were manually removed from the annotation. The *randomize_regions* function and Chi-square tests were used to compare the observed genic distribution of DMLs to the expected distribution, and the *plot_annotatr* function was used to generate figures. Functional enrichment of DML-containing genes was performed using the ToppGene suite tool ToppFun [82]. ToppFun utilizes hypergeometric distributions with Bonferroni correction to determine statistically significant enrichment in up to fourteen functional categories including Gene Ontology (GO) terms, human and mouse phenotypes, protein–protein interactions, diseases, and others [82]. Disease annotations are drawn from DisGeNET, Online Mendelian Inheritance in Man (OMIM) MedGen, and other sources. Lists of unique DML-containing genes for each assayed tissue (F1 cortex, F1 hippocampus, F0 cortex) were used as input selecting the “HGNC Symbol and Synonyms” entry type and run on default settings; terms with a Bonferroni-corrected *p*-value less than 0.05 were deemed significant.

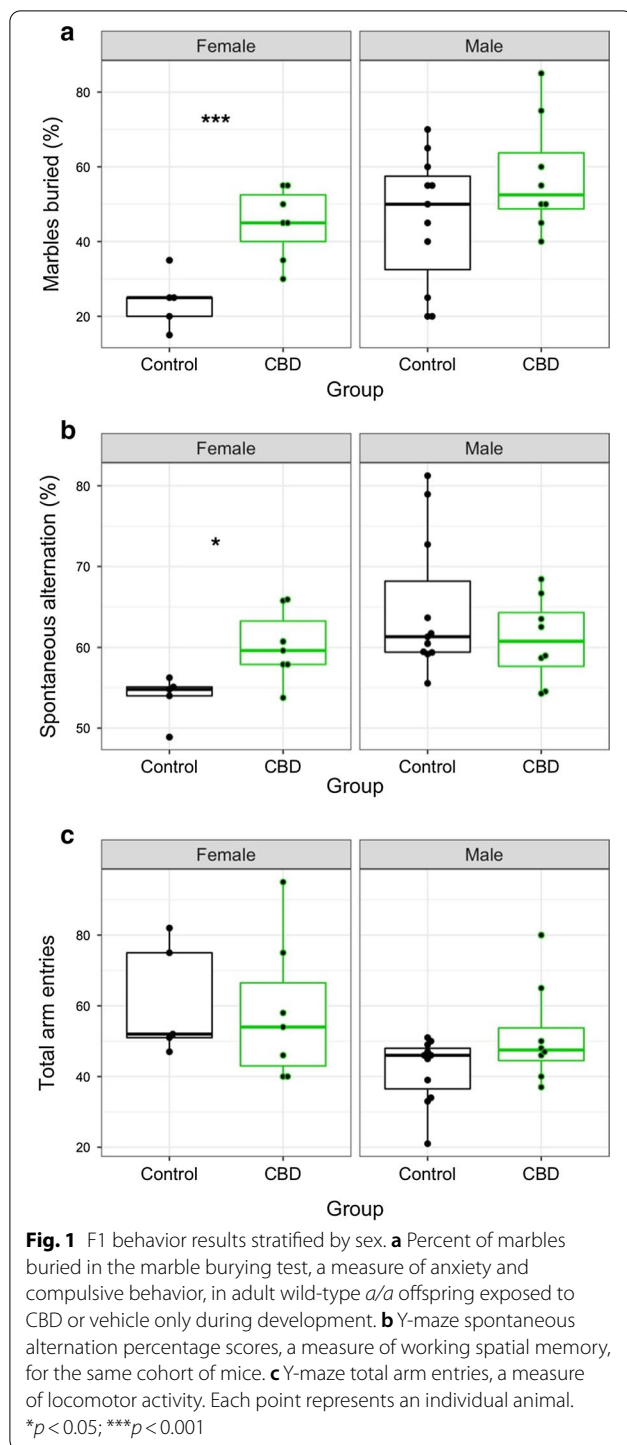
Pyrosequencing

Pyrosequencing was performed as described previously [83]. Briefly, LINE1 and intracisternal A particle (IAP) retrotransposon pyrosequencing primers were designed using Qiagen Pyromark Assay Design software version 2.0.2 and sequences from the mm10 genome. The parameters for each reaction included a thermocycler protocol of 95 °C for 30 s, an optimized temperature for 30 s, and 72 °C for 30 s repeated for 35–40 cycles. Primer sequences and conditions are presented in Additional file 1. DNA methylation level was quantitated from PCR products on a Qiagen Pyromark Q96 ID instrument. Controls consisted of a “no template control” and two wells of bisulfite converted 100% or 0% methylated control mouse DNA from EpiGentek. Methylation results were valued under criteria that the Pyromark software defined as ‘check’ or ‘passing’, with these values retained for analysis, and discarded if ‘failed’.

Results

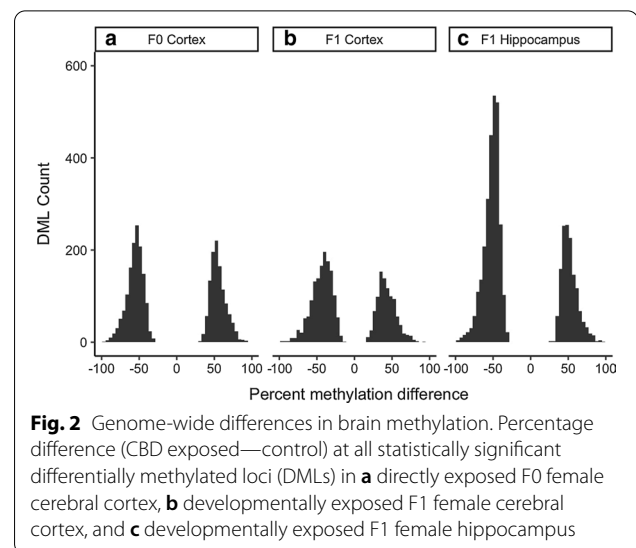
Effects of developmental and direct CBD exposure on memory and anxiety

No significant differences in F1 weight from weaning through study conclusion (12 weeks) were identified by ANOVA (Additional file 2). CBD-exposed litters contained 1.25 more pups on average when compared to control litters ($p = 0.0134$; Additional file 3). To evaluate behavioral effects associated with developmental CBD exposure, F1 offspring of both sexes were subjected to the marble burying test, a measure of anxiety and compulsive behavior, and the Y-maze spontaneous alternation test, a measure of working spatial memory. No significant differences in marble burying or Y-maze spontaneous alternation were identified between the full control and CBD-exposed F1 groups (Additional file 4); however, significant sex interactions were identified by ANOVA for both behavior tests warranting stratification by sex. A sex effect regardless of treatment group was identified by ANOVA for marble burying ($p = 0.00139$), and both a sex effect ($p = 0.0239$) and a sex:treatment interaction ($p = 0.0385$) were identified for Y-maze spontaneous alternation. Stratifying results by sex revealed that young adult female F1 offspring exposed to CBD during gestation and preweaning buried nearly twice as many marbles as unexposed female controls (Fig. 1; $p = 0.000328$) indicating an increase in anxiety behavior, while differences between control and CBD-exposed F1 males were not significant ($p = 0.156$). Y-maze spontaneous alternation percentage, a measure of working spatial memory, was also increased in exposed adult female offspring ($p = 0.0344$). The total number of Y-maze arm entries, a



measure of locomotor activity, was not significantly different between F1 groups of either sex.

Based on previous studies demonstrating anxiolytic effects for CBD and memory deficits for THC, F0 female behavior scores were also assessed during continuing CBD exposure following weaning of pups, representing



approximately nine weeks of daily exposure. F0 behavior tests were conducted twice, once during CBD's C_{max} approximately one hour after dosing and again at least 24 h after dosing to delineate acute and cumulative effects. F0 Y-maze spontaneous alternation percentages were not significantly different between control and exposed groups at either timing of dosage (Additional file 5; acute $p = 0.114$; cumulative $p = 0.791$). Similarly, differences in the total number of arm entries between F0 groups were not significantly different (acute $p = 0.7449$; cumulative $p = 0.1004$). F0 marble burying scores were also not significantly different between groups for either dosage window (acute $p = 0.524$; cumulative $p = 0.421$) or between dosage windows.

Effects of CBD on DNA methylation

In order to assess the effects of developmental CBD exposure on DNA methylation, we used the *Agouti* viable yellow (A^{vy}) environmental biosensor model. Coat color was visually assessed on a three-category scale in A^{vy}/a F1 offspring of both sexes ($n = 76$). Changes in coat color distribution in this strain represent shifts in DNA methylation at the *Agouti* locus. Chi-square tests for trend revealed that differences in coat color distribution between control and exposed F1 A^{vy}/a animals did not reach significance overall ($p = 0.204$) or within males ($p = 0.874$) or females ($p = 0.0924$) (Additional file 6).

In order to identify genome-wide DNA methylation effects at the single nucleotide level in both directly and developmentally exposed animals, reduced representation bisulfite sequencing (RRBS) was applied to female F0 and F1 wild-type *a/a* cerebral cortex and F1 wild-type hippocampus. Differential methylation comparisons revealed 4190 differentially methylated

loci (DMLs) in F1 hippocampus and 2234 DMLs in F1 cerebral cortex. Both F1 tissues exhibited a bias toward hypomethylation overall with 66.6% of DMLs being hypomethylated in hippocampus and 60.6% being hypomethylated in cortex (Fig. 2). In order to determine direct effects of CBD on the epigenome, genome-wide DNA methylation was also assessed in the cerebral cortex of chronically exposed F0 females. 2523 DMLs were identified in F0 cortex with 55% of DMLs being hypomethylated. Randomization of F1 and F0 DMLs using the R package *annotatr* revealed significant enrichment in genic regions including promoters, exons, and 5' and 3' untranslated regions for all three tissues ($p < 0.01$; Fig. 3).

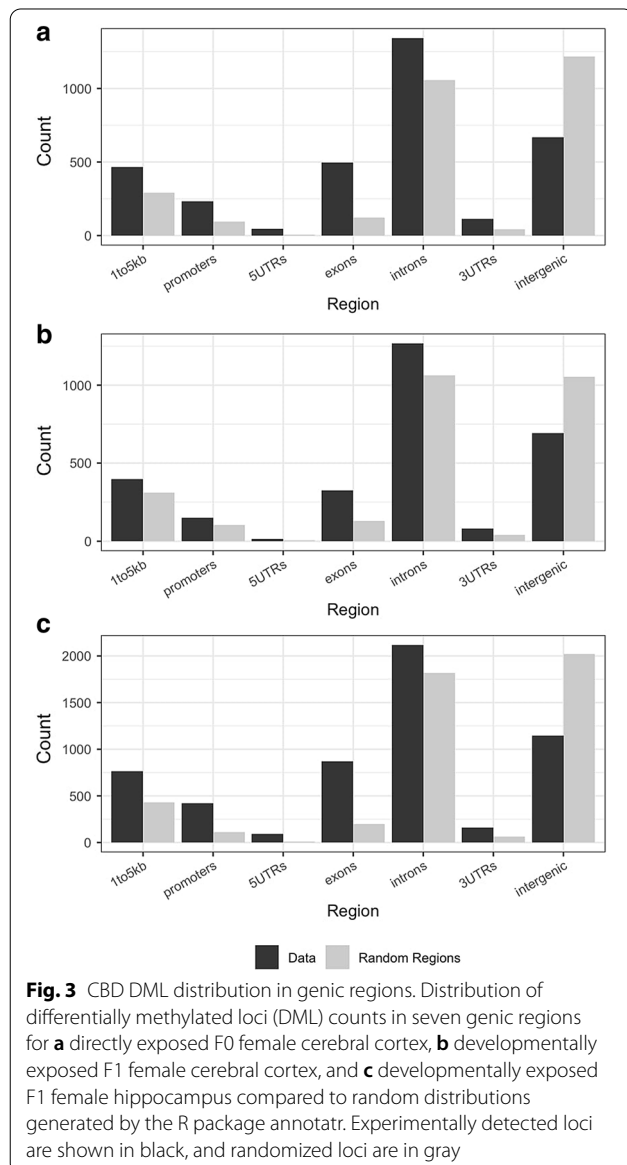


Table 1 Top DML-containing genes and mean methylation change values

Tissue	Gene	DMLs	Mean methylation (%)	SD
F1 Hippocampus	<i>Tnxb</i>	9	28.6	43.6
	<i>Ncor2</i>	8	-35.4	51.1
	<i>Prdm16</i>	8	-5.21	61.1
	<i>Zfx3</i>	8	-19.2	47.9
	<i>Gse1</i>	7	-59.7	13.9
F1 Cortex	<i>Tmem151b</i>	8	55.1	8.5
	<i>Prdm16</i>	7	-65.4	13.2
	<i>Epas1</i>	6	68.7	11.7
	<i>Fhl1</i>	6	-51.6	5.9
	<i>Gse1</i>	6	-40.0	54.6
F0 Cortex	<i>Camta1</i>	9	-13.8	59.7
	<i>Grip1</i>	7	-43.2	39.8
	<i>Cask</i>	5	-42.4	54.1
	<i>Gdf1</i>	5	-53.4	6.0
	<i>Mfsd12</i>	5	60.2	11.3

Mean methylation values (percent, exposed—control) for F1 and F0 brain regions. The mean methylation column represents the average change across all called DMLs for a given gene. Standard deviation represents the variability between DMLs within a gene

To evaluate potential functional molecular consequences of CBD exposure, genes containing the largest number of DMLs in each tissue were identified (Table 1).

No multi-CpG differentially methylated regions (DMRs) were identified in any tissue, likely due to stringent criteria for DMR calling. Notable genes containing a high number of DMLs in F1 hippocampus included nuclear receptor corepressor 2 (*Ncor2*), which contained eight primarily hypomethylated DMLs in introns and the 1–5 Kb upstream region (Additional file 7). Loss of *Ncor2* function has been associated with memory impairment and reduced social interactions via altered GABAergic signaling in mice [84–86]. The histone methyltransferase PR domain-containing 16 (*Prdm16*) contained seven intronic DMLs and one 32.9% hypomethylated promoter DML. Shimada et al. determined that *Prdm16* knockout is neonatally lethal and the gene is required for neural stem cell maintenance and neurogenesis in the postnatal hippocampal dentate gyrus [87]. In F1 cortex, transmembrane protein 151B (*Tmem151b*) contained the largest number of DMLs. The function of this gene is not well understood; however, one study associated *Tmem151b* knockout with lowered seizure threshold in mice [88]. Eight hypermethylated DMLs, all exonic, were identified in *Tmem151b* in the present study. *Prdm16* also contained seven hypomethylated DMLs in F1 cortex in addition to those found in F1 hippocampus. Additionally,

T-lymphoma invasion and metastasis-inducing protein 1 (*Tiam1*), a gene found to be involved in memory storage in a knockout mouse model [89], contained three hypermethylated and three hypomethylated intronic DMLs. Notably, autism susceptibility candidate 2 (*Auts2*) contained five hypomethylated intronic DMLs in F1 cortex; exonic mutations in this gene cause a syndromic form of intellectual disability through its involvement in neuronal migration, neuritogenesis, and transcriptional regulation [90–93]. In F1 hippocampus, DNA methyltransferase 1 (*Dnmt1*) contained one intronic DML that was 57% hypomethylated in exposed animals. DMLs were not identified in *Dnmt1* or any other DNA methyltransferases in F1 cortex or F0 cortex.

In F0 cortex, top DML-containing genes were also identified. Genes containing the largest number of DMLs included calmodulin-binding transcription activator 1 (*Camta1*), which is involved in long-term and episodic memory and has been correlated with intellectual disability when mutated [94–97]. In the present study, *Camta1* contained eight intronic DMLs and one promoter DML, which were largely hypomethylated. Additionally, glutamate receptor-interacting protein 1 (*Grip1*) contained four hypomethylated DMLs in the 1–5 Kb upstream region and three intronic DMLs in directly exposed F0 mice. In previous studies, *Grip1* knockout mice exhibited increased sociability and human *GRIP1* gain of function mutations have been correlated with more severe social deficits in autism spectrum disorder [98, 99]. Interestingly, direct CBD exposure also resulted in both hypermethylation and hypomethylation of CpGs in long interspersed nuclear element (LINE1) retrotransposons in F0 females, while intracisternal A particle (IAP) retrotransposons were unaffected (Additional file 8). Multiple genes known to interact either directly or indirectly with exogenous cannabinoids that are hypothesized to mediate some of CBD's effects (endocannabinoid, PPAR γ , and TRPV receptors) did not contain RRBS DMLs in either generation.

Gene Ontology, phenotype, and disease terms were analyzed for overrepresentation in the list of DML-containing genes for each F1 and F0 tissue. The top significantly enriched terms for each tissue are presented in Table 2, and the full results are available in Additional file 9. Top-enriched Gene Ontology terms for F0 and F1 tissues included terms related to neurogenesis, neuron differentiation and projection, ion transport, and synaptic and postsynaptic cellular components. CBD's positive effects on neurogenesis are well established [4, 7, 16, 20, 100, 101], and THC has been shown to modify neuron morphology in multiple brain regions including the nucleus accumbens, an area commonly associated with drug-related reward [102]. Prominent overrepresented

mouse phenotypes for F0 and F1 DML-containing genes included abnormal synaptic transmission and neuron morphology. Lastly, neurodevelopmental disorders, intellectual disability, epilepsy, and autistic disorders were among the most enriched disease terms. In F1 hippocampus drug abuse terms were the most numerous despite not appearing in the top five terms, with terms related to addiction and substance use occupying eight of the top twenty disease term enrichment positions (Additional file 9).

Discussion

There is increasing interest in the beneficial effects of cannabinoids for psychological phenotypes as well as for pregnancy-related concerns such as hyperemesis. This combination warrants careful investigation regarding the potential impacts of exposure to CBD during development, especially given the known negative effects of prenatal THC. The main finding of the present study is that developmental CBD exposure in mice is associated with widespread changes in the brain methylome and sex-specific effects on anxiety and memory behavior. Direct exposure during pregnancy and lactation also modifies DNA methylation with a similar magnitude but does not impart changes in memory or anxiety. Together, these data suggest that despite its previously studied benefits in direct exposure CBD use during pregnancy may have negative consequences for adult offspring, though the observed effects were mixed. Additionally, based on functional enrichment of DML-containing genes it is possible that neuroepigenetic mechanisms are mechanistically involved in developmental and direct CBD exposure's behavioral effects; additional studies will be required to more directly link these phenomena.

The present study represents the first interrogation of developmental CBD's impact on offspring psychological phenotypes. Developmental exposure to CBD as seen in F1 behavior results in sex-specific increases in anxiety and memory performance. These results parallel some findings for prenatal exposure to the psychoactive cannabinoid THC and contrast others. Trezza et al. found that prenatal THC resulted in an anxiogenic profile in adult offspring as measured by the elevated plus maze; however, Manduca et al. did not find anxiety or other behavioral abnormalities in adulthood despite neonatal differences in vocalization behavior with prenatal exposure to the synthetic cannabinoid WIN55212-2 [24, 103]. Silva et al. and de Salas-Quiroga et al. identified memory deficits with prenatal THC exposure [22, 104], which contrast with improved memory function observed in the present study; however, small sample size and the removal of outliers are important considerations and warrant further investigation into the effect. Other

Table 2 ToppFun top 5 enriched functional terms by tissue, significant with Bonferroni-corrected p values < 0.05

Tissue	GO: Molecular function	GO: Biological process	GO: Cellular component	Mouse phenotype	Disease
F1 Hippocampus	<ol style="list-style-type: none"> 1. Metal ion transmembrane transporter activity (GO:0046873) 2. Cation channel activity (GO:0005261) 3. Ion channel activity (GO:0005216) 4. Cation transmembrane transporter activity (GO:0008324) 5. Inorganic cation transmembrane transporter activity (GO:0022890) 	<ol style="list-style-type: none"> 1. Neurogenesis (GO:0022008) 2. Generation of neuron (GO:0048699) 3. Neuron differentiation (GO:0030182) 4. Neuron development (GO:0048666) 5. Cell–cell signaling (GO:0007267) 	<ol style="list-style-type: none"> 1. Synapse (GO:0045202) 2. Neuron projection (GO:0043005) 3. Cell junction (GO:0030054) 4. Somatodendritic compartment (GO:0036477) 5. Synaptic membrane (GO:0097060) 	<ol style="list-style-type: none"> 1. Abnormal synaptic transmission (MP:0003635) 2. Abnormal brain morphology (MP:0002152) 3. Abnormal CNS synaptic transmission (MP:0002206) 4. Abnormal neuron morphology (MP:0002882) 5. Abnormal lymphatic vessel endothelium morphology (MP:0010195) 	<ol style="list-style-type: none"> 1. Neurodevelopmental disorders (C1535926) 2. Intellectual disability (C3714756) 3. Epilepsy (C0014544) 4. Autistic disorder (C0004352) 5. Global developmental delay (C0557874)
F1 Cortex	<ol style="list-style-type: none"> 1. Actin binding (GO:0003779) 2. Cytoskeletal protein binding (GO:0008092) 3. Regulatory region nucleic acid binding (GO:0001067) 4. Channel activity (GO:0015267) 5. Passive transmembrane transporter activity (GO:0022803) 	<ol style="list-style-type: none"> 1. Cell morphogenesis (GO:0000902) 2. Neurogenesis (GO:0022008) 3. Cellular component morphogenesis (GO:0032989) 4. Neuron differentiation (GO:0030182) 5. Generation of neurons (GO:0048699) 	<ol style="list-style-type: none"> 1. Synapse (GO:0045202) 2. Synaptic membrane (GO:0097060) 3. Neuron projection (GO:0043005) 4. Postsynapse (GO:0098794) 5. Postsynaptic density (GO:0014069) 	<ol style="list-style-type: none"> 1. Abnormal CNS synaptic transmission (MP:0002206) 2. Abnormal synaptic transmission (MP:0003635) 	<ol style="list-style-type: none"> 1. Intellectual disability (C3714756) 2. Neurodevelopmental disorders (C1535926) 3. Global developmental delay (C0557874) 4. Developmental delay (disorder) (C0424605) 5. Congenital abnormality (C0000768)
F0 Cortex	<ol style="list-style-type: none"> 1. Channel activity (GO:0015267) 2. Passive transmembrane transporter activity (GO:002280) 3. Ion channel activity (GO:0005216) 4. Ion transmembrane transporter activity (GO:0015075) 5. Cation channel activity (GO:0005261) 	<ol style="list-style-type: none"> 1. Neurogenesis (GO:0022008) 2. Generation of neurons (GO:0048699) 3. Cellular component morphogenesis (GO:0032989) 4. Cell morphogenesis (GO:0000902) 5. Neuron differentiation (GO:0030182) 	<ol style="list-style-type: none"> 1. Neuron projection (GO:0043005) 2. Synapse (GO:0045202) 3. Postsynapse (GO:0098794) 4. Somatodendritic compartment (GO:0036477) 5. Cell junction (GO:0030054) 	<ol style="list-style-type: none"> 1. Abnormal CNS synaptic transmission (MP:0002206) 2. Abnormal synaptic transmission (MP:0003635) 3. Abnormal locomotor behavior (MP:0001392) 4. Abnormal brain morphology (MP:0002152) 5. Abnormal locomotor activation (MP:0003313) 	<ol style="list-style-type: none"> 1. Intellectual disability (C3714756) 2. Epilepsy (C0014544) 3. Autistic disorder (C0004352) 4. Neurodevelopmental disorders (C1535926) 5. Bipolar disorder (C0005586)

behavioral phenotypes for prenatal THC exposure have also been observed in animals, including altered cognitive function, emotional reactivity, and responses to drugs of abuse such as methamphetamine and opioids [24, 26, 28, 104], warranting expansion of prenatal CBD studies to these areas. Importantly, the marble-burying assay employed in the current study has significant limitations, specifically that it remains unclear whether the task measures novelty-induced anxiety or compulsive/repetitive behavior [72, 105]. Subsequent studies of prenatal CBD exposure should employ additional measures of anxiety such as the elevated plus maze or open-field tests as well as measures of compulsivity such as nestlet shredded to provide greater phenotypic accuracy. The observed sex-specific nature of developmental CBD's behavioral effects remains notable; however, investigation of differential methylation in both males and females following prenatal exposure (as opposed to assaying females only as in the current study) may help identify vulnerabilities or protective effects in each sex. Interestingly, effects on wean weight or postweaning weight for CBD were not observed in the present study in contrast to THC, which has been shown to reduce wean weight in rats [106] and reduce birth weight in rats [107] and humans [108] despite evidence for hyperphagia with direct exposure [109–111]. A lack of significant behavioral effects in the directly exposed F0 generation corresponds with previous studies such as Fogaça et al. and Gáll et al. which identified anxiolytic effects for CBD in the presence of chronic unpredictable stress but not in its absence [101, 112]. In contrast to these studies, the extended exposure window from prior to breeding through lactation employed here prevents identification of the discrete window of exposure mediating the observed changes and will require additional study. Regarding routes of prenatal exposure, cannabinoids are known to rapidly cross the placenta and are found in breast milk due to their high lipophilicity [113–117]; a study by Feinshtein et al. additionally found that CBD increased the permeability of the placenta to other xenobiotics [118].

Genome-wide DNA methylation results identified thousands of DMLs enriched in genic regions in each generation and brain region. These findings expand upon findings by Pucci et al. and Paradisi et al. who initially identified DNA methylation changes with exposure to CBD and the endocannabinoid anandamide, respectively, in keratinocytes *in vitro* [119, 120]. These studies found hypermethylation associated with CBD exposure in contrast to the current study, where hypomethylation was more predominant in both directly and developmentally exposed animals. Inclusion of only females in the F1 RRBS analysis prevents sex comparisons on the epigenetic level, and future studies will be needed

to determine whether behavior differences are reflected in differential methylation between males and females. A recent study by Sales et al., who identified changes in global DNA methylation levels in the mouse prefrontal cortex and hippocampus following direct CBD exposure, also associated CBD with hypermethylation by finding that the compound restored hypomethylation triggered by unpredictable stress [2]. While a mixture of hyper- and hypomethylation was identified in the present study, the slightly greater prominence of hypomethylation (particularly in the F1 hippocampus) agrees with our previous study of brain methylation with direct CBD exposure in male mice [75]. While site-specific methylation changes may have positive or negative effects on gene expression depending on genic region, binding of methylation readers and other factors, hypomethylation is generally associated with increased chromosomal instability, activation of transposons, and reduced cell survival in CNS neurons [121–124]. Further study is needed to determine whether transposable element methylation is reduced by prenatal CBD; however, our finding that direct CBD results in mixed differential methylation within LINE1 retrotransposons indicates that transposon methylation patterns may not correspond with genic trends. One limitation of the current study that is highly relevant to both DNA methylation and behavior outcomes is that the effects of CBD on maternal care are unknown. Differences in maternal care have been shown to impact both offspring behavior and brain DNA methylation in the hippocampus and other brain regions [125–131]. Continuous infusion of the THC-like cannabinoid receptor agonist WIN-55212-2 has been associated with reduced maternal care during lactation [132]; further study of CBD in the context of maternal care will be required to delineate its effects from those of developmental exposure.

Functional enrichment of F1 DML-containing genes revealed overrepresentation of neurogenesis, neuron morphology, and metal ion channel terms, while top disease terms included autism spectrum disorder, schizophrenia, and intellectual disability. Direct exposure to CBD has been shown to improve anxiety and memory behavior in rodents [12, 133–137] and reduce psychotic symptoms in rodent models and humans [9, 10, 138, 139], and it appears that similar pathways are affected on the epigenetic level with developmental exposure. Based on behavior results, it can be hypothesized that prenatal exposure affects these pathways in a disruptive manner; however, further characterization of prenatal CBD's behavioral effects will be required to validate the observed results. Neurogenesis was a particularly enriched term in hippocampus, and while it is not possible from these data to determine which window of neurogenesis (embryonic, postnatal, or adult) was affected

by exposure, it is important to note that CBD has been shown to stimulate adult neurogenesis in the hippocampal dentate gyrus [7, 16, 20, 100, 140]. Whether altered DNA methylation in neurogenesis pathways in the adult F1 hippocampus represents changes to ongoing adult neurogenesis or signatures of perturbation earlier in development will be a highly relevant distinction based on the differential consequences of increased neurogenesis during these windows. Enhanced adult neurogenesis has been associated with exercise, environmental enrichment, and reduced depressive symptoms [141–145], while regional increases during the developmental and postnatal windows are linked to diseases such as autism spectrum disorder [146–148].

Conclusions

Overall, the current study identified sex-specific changes in working spatial memory and anxiety behavior as well as genome-wide changes in brain DNA methylation in adult mouse offspring developmentally exposed to human-relevant doses of CBD. The collected data represent an initial inquiry into the effects of prenatal CBD exposure on the adult brain and behavioral phenotypes, indicating that significant efforts are needed to fully characterize the impacts of this compound during development.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13148-020-00993-4>.

Additional file 1: Primer information for pyrosequencing of LINE1 and IAP transposons.

Additional file 2: Body weight trends for wild-type a/a F1 offspring of both sexes from weaning through study completion (12 weeks). Shaded areas represent 95% confidence intervals. Weights did not differ significantly between groups at any time point by ANOVA ($p = 0.995$).

Additional file 3: Litter size for F1 pups differed significantly between groups with CBD-exposed litters containing 1.25 more pups on average in comparison to control litters ($p = 0.0134$).

Additional file 4: Scores for wild-type a/a F1 young adult mice in (a) marble burying, a measure of anxiety and (b) Y-maze spontaneous alternation and (c) Y-maze arm entries, measures of spatial memory, did not differ significantly between CBD-exposed and control groups when results from both sexes were combined.

Additional file 5: Scores for wild-type a/a F0 adult female mice in (a) marble burying, a measure of anxiety and (b) Y-maze spontaneous alternation and (c) Y-maze arm entries, measures of spatial memory and locomotion, did not differ significantly between animals receiving 20 mg/kg CBD daily for 9 weeks and controls for either acute (test performed near CBD Cmax) or cumulative (test performed 24 hours after last dose) runs. Likewise, paired comparisons for acute vs. cumulative scores were not statistically significant.

Additional file 6: Measurements of F1 Avy/a offspring coat color on a three category scale (L: low methylation/yellow; M: medium methylation/mottled; H: high methylation/pseudoagouti) revealed a lack of statistically

significant differences between CBD-exposed and control groups when assessed (a) as a whole and (b) stratified by sex.

Additional file 7: Table of all unique, statistically significant differentially methylated loci (DMLs) identified between CBD-exposed and control tissues consisting of F0 cerebral cortex, F1 cerebral cortex, and F1 hippocampus.

Additional file 8: Methylation values (four CpG positions and mean) for CpGs lying in (a) mLINE1 and (b) IAP retrotransposons in F0 female cerebral cortex and hippocampus. Significant hypermethylation was identified at mLINE1 position 1 and hypomethylation at mLINE1 position 2 in CBD-exposed tissues, both in cerebral cortex only. Other mLINE1 positions, mean methylation, and IAP positions and mean were not found to be significantly different between groups. Each point represents an individual animal. * = $p < 0.05$, ** = $p < 0.01$.

Additional file 9: Complete results returned by the functional enrichment tool ToppFun for lists of DML-containing genes in F0 cerebral cortex, F1 cerebral cortex, and F1 hippocampus.

Abbreviations

CBD: Cannabidiol; THC: Δ^9 -Tetrahydrocannabinol; 5HT-1a: 5-Hydroxytryptamine (serotonin) 1a; PPARy: Peroxisome proliferator-activated receptor gamma; TRPV: Transient receptor potential cation channel subfamily V; GABA: Gamma aminobutyric acid; DOHaD: Developmental Origins of Health and Disease; A^y: Agouti viable yellow; RRBS: Reduced-representation bisulfite sequencing; IAP: Intracisternal A particle; ANOVA: Analysis of variance; gDNA: Genomic deoxyribonucleic acid; LINE1: Murine long interspersed nuclear element 1; DML: Differentially methylated locus; DMR: Differentially methylated region; F0: Filial generation 0; F1: Filial generation 1; Ncor2: Nuclear receptor corepressor 2; Prdm16: PR domain-containing 16; Tmem151b: Transmembrane protein 151b; Tiam1: T-lymphoma invasion and metastasis-inducing protein 1; Aut2: Autism susceptibility candidate 2; Dnmt1: DNA methyltransferase 1; Camta1: Calmodulin-binding transcription activator 1; Grip1: Glutamate receptor-interacting protein 1.

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Authors' contributions

CF and NW conceived the study. NW, CF, MC, and CD performed the animal experiments. NW, MC, and CD performed the molecular experiments. NW performed the bioinformatics. NW and MC wrote the manuscript. CF edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The raw datasets generated during the current study are available in the Sequence Read Archive repository at Accession No. PRJNA655745; <https://www.ncbi.nlm.nih.gov/sra/PRJNA655745>. Processed datasets generated during the current study are available as Additional files.

Ethics approval and consent to participate

All animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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