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Subacute cannabidiol alters genome-wide DNA methylation in adult mouse hippocampus

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Abstract

Use of cannabidiol (CBD), the most abundant non-psychoactive compound found in cannabis (*Cannabis sativa*), has recently increased as a result of widespread availability of CBD-containing products. CBD is FDA-approved for the treatment of epilepsy and exhibits anxiolytic, antipsychotic, prosocial, and other behavioral effects in animal studies and clinical trials, however, the underlying mechanisms governing these phenotypes are still being elucidated. The epigenome, particularly DNA methylation, is responsive to environmental input and can govern persistent patterns of gene regulation affecting phenotype across the life course. In order to understand the epigenomic activity of cannabidiol exposure in the adult brain, 12-week-old male wild-type *a/a* Agouti viable yellow (*A^{vy}*) mice were exposed to either 20 mg/kg CBD or vehicle daily by oral administration for fourteen days. Hippocampal tissue was collected and reduced-representation bisulfite sequencing (RRBS) was performed. Analyses revealed 3,323 differentially methylated loci (DMLs) in CBD-exposed animals with a small skew toward global hypomethylation. Genes for cell adhesion and migration, dendritic spine development, and excitatory postsynaptic potential were found to be enriched in a gene ontology term analysis of DML-containing genes, and disease ontology enrichment revealed an overrepresentation of DMLs in gene sets associated with autism spectrum disorder, schizophrenia, and other phenotypes. These results suggest that the epigenome may be a key substrate for CBD's behavioral effects and provides a wealth of gene regulatory information for further study.

Keywords

Epigenetics; Cannabis; Autism; Schizophrenia

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Statement of author 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 approved of the manuscript.

The authors have no conflicts of interest.

Introduction

Cannabidiol (CBD) is the primary non-psychoactive derivative of the cannabis plant (*Cannabis sativa*) and an increasingly popular nutraceutical, with CBD sales in the US predicted to reach 1.8 billion dollars by 2022 [Total CBD consumer sales U.S. 2014–2022 | Statista, March 18 2020]. Discovered in 1940 and its structure determined in 1963, CBD acts on multiple pathways in the brain and modulates the psychoactive effects of Δ^9 -tetrahydrocannabinol (THC) [Crippa et al. 2018; Mannucci et al. 2017; Mokrysz et al. 2020; Murphy et al. 2017; Zuardi et al. 1982; Malone et al. 2009]. CBD alone has recently been investigated for its therapeutic potential in psychiatric phenotypes including anxiety, depression, sociality, drug craving, and others in animal studies and a limited number of clinical trials (Supplemental Table I) [de Faria et al. 2020; Skelley et al. 2020; Masataka 2019; de Mello Schier et al. 2014; Schubart et al. 2011; Osborne et al. 2017; Ren et al. 2009; de Carvalho and Takahashi 2017; Mandolini et al. 2018; Campos et al. 2012; Leweke et al. 2012; McGuire et al. 2018; Crippa et al. 2011; Bergamaschi et al. 2011; Zuardi et al. 1993; Bhattacharyya et al. 2018; Sales et al. 2020; Réus et al. 2011; El-Alfy et al. 2010]. Previously studied mechanisms of CBD action include indirect stimulation of endocannabinoid receptor CB1 via delayed hydrolysis and reuptake of the endocannabinoid anandamide (AEA), action at the serotonin 1a (*5HT-1a*), peroxisome proliferator-activated receptor gamma (*PPAR γ*), and transient receptor potential cation channel subfamily V (*TRPV*) receptors, neuroprotection, stimulation of neurogenesis, and antioxidant activity [Alline C. Campos et al., 2013; Alline Cristina Campos & Guimarães, 2008; Hampson et al., 1998; Pumroy et al., 2019; Schiavon et al., 2016; Vallée et al., 2017]. Despite a large number of purported targets, a significant gap regarding the impacts and molecular mechanisms of CBD exposure in psychiatric disease exists in the literature, which has previously focused on acute and single-bolus exposures.

Modulation of the brain epigenome is an emerging mechanism of importance in the pathophysiology of psychiatric disease [Wanner et al. 2019; Wockner et al. 2014; Nardone et al. 2014; Swartz et al. 2017; Nestler et al. 2016; Ladd-Acosta et al. 2014]. DNA methylation, defined by the addition of a methyl group to the fifth carbon of cytosine in a cytosine-guanine dinucleotide (CpG) context, is the most frequently studied epigenetic mark and is strongly mediated by environmental input [Bernal and Jirtle 2010; Hollander et al. 2020; Plusquin et al. 2019; Wilson and Sengoku 2013]. Maintenance of DNA methylation patterns is critical for normative brain development and affects gene expression via interactions with transcription factors, splicing machinery, chromatin features, and other mechanisms [Watson et al. 2015; Spiers et al. 2015; Jaffe et al. 2016; Takizawa et al. 2001; Lister et al. 2013; Fagiolini et al. 2009; Nugent et al. 2015]. Aberrant methylation patterns have been associated with psychiatric phenotypes including autism spectrum disorder (ASD), major depressive disorder (MDD), and anxiety, [Fuchikami et al., 2011; Ladd-Acosta et al., 2014; McCoy et al., 2019; Stefano Nardone et al., 2017; Sabunciyan et al., 2012; Simmons et al., 2012] indicating a critical need to determine how environmental exposures shift disease-relevant DNA methylation patterns in the brain. Exposure to whole-plant cannabis, THC, and synthetic cannabinoids has been associated with methylation changes in the rodent hippocampus and nucleus accumbens (NAc), two brain regions broadly regulating memory

and reward, respectively [DiNieri et al., 2011; Murphy et al., 2018; Spano et al., 2007; Tomas-Roig et al., 2017]. Abnormalities in the hippocampus have also been identified in association with autism spectrum disorder, major depressive disorder, and schizophrenia (SZ) [Ito et al., 2017; Kundakovic et al., 2015; Lieberman et al., 2018; Rivero et al., 2015; Snyder et al., 2011; Thongkorn et al., 2019].

We hypothesized that changes in DNA methylation are involved in CBD's mechanism of action with respect to psychiatric phenotypes, and that these changes affect gene pathways related to previously characterized mechanisms of action and behavioral outcomes. To explore this hypothesis, we performed genome-wide DNA methylation analysis via reduced representation bisulfite sequencing (RRBS) in the adult mouse hippocampus. These data provide novel insight into epigenetic mechanisms contributing to CBD's psychological effects.

Materials and Methods

Animals

Mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) 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). Animals were obtained from an agouti viable yellow (Avy) colony maintained for over 200 generations with 93% genetic similarity to C57BL/6 [Weinhouse et al., 2014]. Seven animals per group were maintained on a standard chow diet and housed in cages of 3–4 individuals on corn cob bedding with a 12-hour light/dark cycle.

Cannabidiol 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 palatability and stored at room temperature. Based on the maximum recommended dose of Epidiolex for treatment of Dravet Syndrome, mice were administered 20 mg/kg CBD or vehicle *per os* (PO) using the tip of an 18-gauge gavage needle once daily for fourteen days [Küster et al., 2012]. This dose is within range of previous animal studies and approximates a human dose much lower than 20 mg/kg due to scaling factors for body surface area [Nair and Jacob 2016; Klein et al. 2011; Linge et al. 2016; Luján et al. ; Stark et al. 2020]. Body weights were recorded weekly and differences between groups were assessed by two separate Student's t-Tests, one for each week in which weights were measured. At the end of the exposure period animals were euthanized and hippocampal brain tissues were snap frozen and stored at –80°C until further processing.

DNA isolation and bisulfite conversion

Total genomic DNA (gDNA) from snap frozen hippocampus was isolated from each animal (n = 14) using the DNeasy Blood and Tissue kit following the manufacturer's protocol (Qiagen, Hilden, Germany). DNA yield was checked using a NanoPhotometer N50 system

and three biological replicates per group were selected for further processing based on concentration and quality. After isolation, gDNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA). Briefly, to detect methylated cytosines in genomic DNA (gDNA), DNA is treated with sodium bisulfite and then sequenced. Unmethylated cytosines are deaminated to uracils during the bisulfite treatment, and are read as thymidine by polymerases during sequencing.

Bisulfite sequencing

Genome-wide DNA methylation levels were measured using reduced-representation bisulfite sequencing (RRBS) at Diagenode, S.A. (Belgium). DNA concentration of samples was measured using the Qubit® dsDNA BR Assay Kit (Thermo Fisher 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 [Andrews, 2010]. Adapter removal was performed using Trim Galore! Version 0.4.1 [Krueger, 2015]. Bismark, a specialized tool that utilizes an *in-silico* bisulfite converted reference genome, was used for mapping bisulfite-treated reads [Krueger & Andrews, 2011]. The cytosine2coverage module of Bismark was used to infer 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 calling and annotation

RStudio open source software (version 3.6.1) tools were used for RRBS analysis. The *DSS* R package (version 2.32.0) was used to test RRBS data for differential methylation between CBD-exposed and control animals [Park & Wu, 2016]. The *DMLtest* function in *DSS* was used to identify differentially methylated CpG loci (DMLs) and regions (DMRs). Smoothing parameters were set to false, the delta threshold was set to 0.1 and the p-value threshold was set to 0.001 so that all loci with a posterior probability greater than 1-threshold were deemed DMLs. The *annotatr* R package (version 1.10.0) was used to annotate DMLs and DMRs to the mm10 genome [Cavalcante & Sartor, 2016]. The *annotate_regions* function was used to annotate the abundance of DMLs within CpG and genic regions. The *randomize_regions* function was used to create randomized regions for DMLs to compare the CpG and genic distribution of the data to values based on expected frequency in the genome and chi-square goodness-of-fit tests were performed in R to evaluate the distributions statistically. The *plot_annotatr* function was used to generate figures.

Gene Ontology and Disease Ontology enrichment

The *topGOR* package (version 2.37.0) was used to annotate DML-containing genes with corresponding Gene Ontology (GO) terms while accounting for the topology of the GO

graph [Alexa & Rahnenfuhrer, 2010]. Reference gene and GO term keys were obtained from the org.Mm.eg.db genome-wide annotation for mouse [Carlson, 2019]. The *topGO* function new was used to generate test statistics using the Fisher test option. The *go_table* function was used to display the most significant nodes and corresponding p-values.

The Human-Mouse Disease Connection (HMDC) “Associations of Human and Mouse Genes with DO Diseases” (Mouse Genome Informatics) gene set was used to find overrepresented disease processes in the list of mouse genes containing DMLs [Eppig et al., 2015]. The HMDC association list contains Disease Ontology (DO) terms, gene symbols, and organism names for genes found to be associated with disease phenotypes through either human or mouse studies. The number of DML-containing genes annotated to each DO term in the HMDC dataset was calculated using R and the total number of DMLs per disease was divided by the number of genes per disease to yield an average ratio of DMLs per gene for each condition. Fisher’s exact tests were used to compare these values to the expected ratio, which was calculated by dividing the total number of DMLs within genes found in the HMDC dataset by the total number of unique HMDC disease genes. Benjamini-Hochberg adjusted p-values were calculated for the top ten DO terms containing the largest number of DMLs.

Results

Animal weights were not significantly different between CBD exposed and control groups at the beginning or end of the exposure (Figure S1; $p = 0.765$ and $p = 0.906$). Reduced-representation bisulfite sequencing (RRBS) is a quantitative method that was used to compare genome-wide DNA methylation profiles at single-nucleotide resolution between exposed and control groups. Data for more than 2 million CpG sites per animal were generated using this method, and differential methylation comparisons were only made for sites covered by at least 2 biological replicates per group. Loci with $\leq 10X$ coverage and $<10\%$ difference in means between groups were also eliminated.

Methylation comparisons revealed 3,323 significantly differentially methylated loci (DMLs; $p < 0.001$) and 2 differentially methylated regions (DMRs) consisting of 5 CpGs each between CBD-exposed and control animals (Supplemental Files 1–4). One hypomethylated DMR spanning 59 nucleotides was identified in Polypeptide N-Acetylgalactosaminyltransferase 2 (*Galnt2*), a gene with low regional specificity in the brain involved in glycosylation of peptides in the Golgi apparatus and thought to be involved in type 2 diabetes mellitus [Holleboom et al., 2011]. The second DMR was hypermethylated by 40% and located intergenically on chromosome 1. In contrast to one previous *in vitro* CBD study detailing region-specific hypermethylation associated with *DNMT1* activation, we find in the subacutely exposed adult mice that the *de novo* methylator *Dnmt3a* contains an intronic DML exhibiting 74% hypomethylation while no differential methylation was identified in *Dnmt1* [Pucci et al., 2013]. The flanking sequence surrounding the DML in *Dnmt3a* did not contain a known regulatory element binding site according to the ORegAnno database [Griffith et al., 2008].

Approximately 44% of DMLs exhibited hypermethylation in the exposed group while 56% of the loci were hypomethylated (Figure 1). DML distribution throughout the genome was significantly different than expected based on random sampling of the genome, with a bias toward CpG shores and shelves ($p < 0.0001$) as well as genic regions ($p < 0.0001$; Figure 2) indicative of functionally consequential differences between the two groups. Sixty-one percent of DMLs fell in genic regions (including introns and 1- to 5-kb upstream regions) and were identified in 1593 genes with a maximum of 23 being found in the gene *Galnt2* (Figure 3). *Galnt2* DMLs were localized to introns with a mixture of hypo- and hypermethylation averaging to a mean difference of -17% (Supplemental File 1). The top ten genes containing the most DMLs are listed in Figure 3. Other single genes containing a large number of DMLs included Down Syndrome Cell Adhesion Molecule Like 1 (*Dscaml1*), a gene involved in dendritic arborization containing 5 DMLs with an average of 40% hypermethylation found in the 5'UTR and introns, and Reticulon 1 (*Rtn1*), an endoplasmic reticulum peptide involved in neuroendocrine secretion containing 6 DMLs [Montesinos, 2014]. *Rtn1* DMLs were localized primarily to exons with a mean difference in methylation of -67% in exposed animals (Supplemental Data 1); this gene was identified in a genome-wide study of depression by Amare et al. as a novel locus for co-occurring MDD and SZ [Amare et al., 2019]. No DMLs were identified in genes for endocannabinoid, serotonin, *PPAR γ* , or *TRPV* receptors.

Gene ontology enrichment analysis using biological process (BP) terms revealed 184 significant terms ($p < 0.01$) consisting of 1471 significant genes with at least five genes annotated to each term. The top fifteen most significantly enriched GO terms are shown in Table II. The topmost pathways enriched for DML-containing genes were cell adhesion (GO:0007155, $p < 0.0001$), cell migration (GO:0016477, $p < 0.0001$), and cell morphogenesis involved in differentiation (GO:0000904, $p < 0.0001$). Other notable terms included dendritic spine development, behavior, and excitatory postsynaptic potential.

In order to evaluate DML enrichment in the context of disease, the list of genes containing DMLs were compared to the Human Mouse Disease Connection (HMDC) database, which aggregates genes associated with disease phenotypes in either mouse or human studies. The top ten disease ontology (DO) terms by number of DMLs are listed in Table II, with p-values representing a Fisher's exact test of enrichment in average DMLs per gene compared to expected based on the total number of DMLs within genes found in the HMDC dataset divided by the total number of unique HMDC disease genes. The list of most enriched terms included autism spectrum disorder (ASD; $p < 0.001$), type II diabetes mellitus (T2D; $p < 0.001$), and schizophrenia (SZ; $p < 0.001$).

Overall, subacute CBD administration in adult mice significantly impacted the DNA methylation of approximately 0.11% of CpG sites under investigation. The loci were not evenly distributed, with bias toward exons, promoters and 5' regions. Gene ontology and disease context analyses both contained genes involved in neuronal function and synaptic organization.

Discussion

There is increasing interest in both the role of epigenetics in psychiatric disease and the mechanisms governing CBD's behavioral effects. This study is the first interrogation of CBD's effects on genome-wide DNA methylation *in vivo* and examines the response of the hippocampal methylome to subacute exposure in adult mice. Our RRBS data identified over 3,000 loci exhibiting a biologically relevant degree of differential methylation between control and exposed groups with a geographic bias toward genic regions indicative of functionally consequential changes. Notably, identification of only two differentially methylated regions (DMRs) was likely related to stringent filtering criteria to minimize false positive results. GO analysis of DMLs revealed enrichment of terms for behavior, neuronal projection morphogenesis, and cell migration, and top DO enrichment terms included autism spectrum disorder and schizophrenia (SZ). Changes in the methylome reveal an additional layer of complexity likely involved in mediating CBD's benefits for these phenotypes, however, expression data from CBD exposed humans and mice are needed to understand the molecular effects of the identified DMLs.

Previous studies have demonstrated that CBD improves sociality in animal models of autism spectrum disorder (ASD), a neurodevelopmental disorder characterized by persistent deficits in social behavior and communication, sensory reactivity, and other features [Al-Dewik et al., 2020, Osborne et al., 2017; Kaplan et al., 2017]. Pathways for synaptic signaling and plasticity are strongly implicated in ASD, and Disks large-associated protein 2 (*Dlgap2*), containing hypomethylated intronic DMLs in our study, is an ASD risk gene involved in synapse organization that has also been shown to be responsive to the psychoactive cannabinoid THC by others [Ebrahimi-Fakhari & Sahin, 2015; Isshiki et al., 2014]. In a study by Schrott et al., *Dlgap2* exhibited differential methylation as a consequence of THC exposure in both rat and human sperm as well as the nucleus accumbens of rats whose fathers were exposed to THC [Schrott et al., 2019]. Similarly, SH3 and multiple ankyrin repeat domains 3 (*Shank3*), a scaffolding protein required for synapse formation and function, contained both hypo- and hypermethylated exonic DMLs in our study and is associated with ASD pathology [Monteiro & Feng, 2017]. Notably, while ASD is frequently heritable, relatively few ASD cases exhibit genetic variants in *Shank3* or other synaptic proteins, and it has been hypothesized that epigenetics play a larger role in the disease than previously thought [Keil & Lein, 2016; Patel et al., 2015; Persico & Napolioni, 2013]. Differential DNA methylation in human ASD patients has been identified in multiple brain regions and includes dysregulation of histone deacetylase 4 (*HDAC4*), a known downregulator of synaptic genes [Ladd-Acosta et al., 2014; S. Nardone et al., 2014]. The gene *Hdac10* contained a strongly hypomethylated promoter DML in our study (Supplemental Data 1) reflecting a possible mechanistic connection between CBD and ASD worthy of further interrogation.

CBD has also been shown to rescue social deficits produced by low dose THC in rats and improved social interaction behavior in the prenatal infection (poly I:C) rat model of schizophrenia (SZ) [Malone et al., 2009; Osborne, Solowij, Babic, et al., 2017]. SZ is a severe mental illness characterized by relapsing delusions, disorganized thinking, and auditory and visual hallucinations [Osborne, Solowij, & Weston-Green, 2017]. In human

clinical trials, CBD administration is associated with fewer positive psychotic symptoms both alone and in combination with THC with a superior side effect profile compared to traditional antipsychotics [Leweke et al., 2012; McGuire et al., 2018; Morgan & Curran, 2008]. Our findings suggest that DNA methylation may be involved in CBD's antipsychotic effects in SZ and support previous literature highlighting the significant contribution of the epigenome to this disease. Multiple SZ-associated loci not included in the HMDC DO annotation were found to contain DMLs including a hypomethylated intronic DML in the metabotropic glutamate receptor 7 (*Grm7*) gene and a hypermethylated DML upstream of catechol-O-methyltransferase domain containing 1 (*Comtd1*; Supplemental Data 1)[Ohtsuki et al., 2008; Wockner et al., 2014]. While the functional consequences of these changes are unclear, gene expression, binding of regulatory proteins, and expression of small RNAs are all plausible consequences of differential methylation that may be relevant to CBD's impact on psychotic symptoms.

While specific loci for MDD, anxiety, and other psychiatric phenotypes did not appear in the GO or DO results, GO terms for Wnt signaling and cell projection, morphogenesis, and differentiation in the hippocampus draw attention to the positive effects of CBD on adult neurogenesis. Adult neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus and deficits in this process have been associated with psychiatric disorders including major depressive disorder, autism spectrum disorder, and anxiety [Hsieh & Eisch, 2010; Sahay & Hen, 2007; Wegiel et al., 2010]. CBD promotes hippocampal neurogenesis *in vivo*, and differential methylation of the *de novo* methyltransferase *Dnmt3a* in our study may have implications for the compound's mechanism of action [Esposito et al., 2011]. Wu et al. found that *Dnmt3a* is required for neurogenesis and its methylation of nonpromoter regions in neurogenic genes increases expression by antagonizing Polycomb repression [H. Wu et al., 2010; Z. Wu et al., 2012]. Further study is needed to determine whether *Dnmt3a* expression is affected by CBD-mediated differential methylation.

In conclusion, the present study shows evidence that subacute CBD administration modifies DNA methylation in the adult mouse hippocampus in a manner that is relevant for human psychiatric disease. These findings provide an initial survey of the effects of CBD on the methylome and include both established and novel loci for further interrogation of CBD's mechanisms of action relevant to neuropsychiatric phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:
Density plot of degree of differential methylation (exposed - control) for RRBS loci passing filtering criteria. The plot indicates that most detected DMLs have over 50% difference in methylation between groups.

Figure 2: Differentially methylated loci counts by (top) CpG region and (bottom) genic region in comparison to expected random distributions generated by the R package *annotatr*. Experimentally detected loci are in black, randomized loci are in gray for comparison.

Gene Symbol	DMLs	Mean	Minimum	Maximum
Galnt2	23	-16.9	-94.6	77.9
Rtn1	6	-66.6	-84.9	-53.9
Spock1	6	-16.6	-72.5	94.9
Dscaml1	5	40.0	-75.3	84.2
Gpc6	5	-39.6	-74.7	88.0
Lrmda	5	15.8	-66.0	73.0
Ptpn3	5	-66.7	-89.3	-56.6
Rnf112	5	-77.0	-78.5	-76.0
Tenm2	5	25.9	-76.1	95.6
Tmem267	5	-76.4	-82.3	-62.2

Figure 3:

Top ten genes by number of DMLs (left) and density plot of DMLs per gene (right).

Table I:

Top fifteen enriched biological process Gene Ontology (GO) terms with the annotated, significant, and expected number of genes organized by p-value.

GO ID	Term	Annotated	Significant	Expected	P-value
GO:0007155	Cell adhesion	1298	176	82.26	8.4e-20
GO:0016477	Cell migration	1448	180	91.77	6.2e-14
GO:0000904	Cell morphogenesis involved in differentiation	804	124	50.96	1.6e-10
GO:0006355	Regulation of transcription, DNA-templated	2784	258	176.44	4.8e-10
GO:0060070	Canonical Wnt signaling pathway	279	47	17.68	7.5e-10
GO:1903508	Positive regulation of nucleic acid-templated transcription	1540	156	97.60	2.3e-09
GO:0120039	Plasma membrane bounded cell projection morphogenesis	691	110	43.79	2.1e-08
GO:0060996	Dendritic spine development	122	26	7.73	3.8e-08
GO:0007610	Behavior	728	91	46.14	7.8e-08
GO:0045597	Positive regulation of cell differentiation	1097	141	69.53	2.3e-07
GO:0060079	Excitatory postsynaptic potential	86	20	5.45	9.6e-07
GO:0050890	Cognition	326	45	20.66	9.8e-07
GO:0051271	Negative regulation of cellular component movement	316	50	20.03	1.5e-06
GO:0043087	Regulation of GTPase activity	295	41	18.70	1.9e-06
GO:0030029	Actin filament-based process	750	103	47.53	2.2e-06

Table II:

Top ten Disease Ontology (DO) terms organized by number of DMLs and enrichment (average DMLs/gene). The specific number of DMLs for each gene is indicated in parenthesis.

DO ID	Disease Name	Genes	Gene Symbols	DMLs	DMLs per gene	P-value
DOID:006004 1	Autism spectrum disorder	10	<i>Dlgap4</i> (3), <i>Shank3</i> (3), <i>Cadps2</i> (2), <i>Arid1b</i> (1), <i>Camk2a</i> (1), <i>Lrfd2</i> (1), <i>Prex1</i> (1), <i>Shank2</i> (1), <i>Tsc1</i> (1), <i>Wdfr3</i> (1)	15	1.50	<0.0001
DOID:9532	Type 2 diabetes mellitus	5	<i>Tcf7l2</i> (4), <i>Cdkal1</i> (3), <i>Irs1</i> (2), <i>Abcc8</i> (1), <i>Ptpn1</i> (1)	11	2.20	<0.0001
DOID:5419	Schizophrenia	5	<i>Nr4a2</i> (3), <i>Shank3</i> (3), <i>Srgap3</i> (2), <i>Magi2</i> (1), <i>Tcf4</i> (1)	10	2.00	<0.0001
DOID:12930	Dilated cardiomyopathy	6	<i>Esrrb</i> (2), <i>Prox1</i> (2), <i>Gnaq</i> (1), <i>Ppp3ca</i> (1), <i>Rxra</i> (1), <i>Srf</i> (1)	8	1.33	<0.0001
DOID:10283	Prostate cancer	5	<i>Zfhx3</i> (3), <i>Cdh1</i> (1), <i>Ehbp1</i> (1), <i>Mxi1</i> (1), <i>Rnasel</i> (1)	7	1.40	<0.0001
DOID:9256	Colorectal cancer	4	<i>Bub1b</i> (2), <i>Dlc1</i> (2), <i>Smad7</i> (2), <i>Axin2</i> (1)	7	1.75	<0.0001
DOID:3827	Congenital diaphragmatic hernia	3	<i>Gata4</i> (3), <i>Slit3</i> (2), <i>Sox7</i> (1)	6	2.00	<0.0001
DOID:9074	Systemic lupus erythematosus	4	<i>Def6</i> (2), <i>Inpp5d</i> (1), <i>Rxra</i> (1), <i>Thr5</i> (1)	5	1.25	0.0005
DOID:6419	Tetralogy of Fallot	3	<i>Gata4</i> (3), <i>Dock1</i> (1), <i>Gata6</i> (1)	5	1.67	0.0002
DOID:9744	Type 1 diabetes mellitus	3	<i>Ptpn2</i> (3), <i>Cd38</i> (1), <i>Nos2</i> (1)	5	1.67	0.0002