Environmental Epigenetics of Diesel Particulate Matter Toxicogenomics

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Received: 28 August 2020; Accepted: 8 October 2020; Published: 10 October 2020

Abstract: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by disruptions in social communication and behavioral flexibility. Both genetic and environmental factors contribute to ASD risk. Epidemiologic studies indicate that roadway vehicle exhaust and in utero exposure to diesel particulate matter (DPM) are associated with ASD. Using the Comparative Toxicogenomics Database (CTD), we identified genes connected to DPM exposure and ASD, extracted the known enhancers/promoters of the identified genes, and integrated this with Assay for Transposase Accessible Chromatin (ATAC-seq) data from DPM-exposed human neural progenitor cells. Enhancer/promoter elements with significantly different chromosome accessibility revealed enriched DNA sequence motifs with transcription factor binding sites for EGR1. Variant extraction for linkage disequilibrium blocks of these regions followed by analysis through Genome Wide Association Studies (GWAS) revealed multiple neurological trait associations including exploratory eye movement and brain volume measurement. This approach highlights the effects of pollution on the regulatory regions of genes implicated in ASD by genetic studies, indicating convergence of genetic and environmental factors on molecular networks that contribute to ASD. Integration of publicly available data from the CTD, cell culture exposure studies, and phenotypic genetics synergize extensive evidence of chemical exposures on gene regulation for altered brain development.

Keywords: autism spectrum disorder; diesel particulate matter; epigenetics; toxicogenomics

1. Introduction

There has long been a known relationship between the environment and human health. Exposures to environmental contaminants have been linked to diseases including cancer [1], respiratory illnesses [2], and neurodevelopmental disorders [3]. A growing body of research has supported the hypothesis that environmental conditions, like malnutrition, chemical, and viral exposures during in utero development can have negative effects on both adult health and early childhood health [4]. Air pollution is a ubiquitous environmental contaminant that impacts human health. Air pollution from vehicle exhaust is a complex mixture of organic chemicals, particulate matter, and volatile gases. Diesel particulate matter (DPM) is a specific component of air pollution that originates from truck exhaust and has been linked to adverse health consequences including lung cancers, asthma, cardiovascular disease [5], low birth weight [6], and autism spectrum disorders (ASD) [7]. The exact mechanisms by which DPM impacts...
human health is likely a complex mechanism, with growing evidence that DPM exposure contributes to oxidative stress [8] and inflammation [9].

Given the evidence environmental toxins impact human health, it is vital that a public database curates the experimental data reporting these effects. The Comparative Toxicogenomics Database (CTD) is a public database that aims to advance understanding of environmental exposure impact on human health [10]. The CTD contains information on the interactions between genes, diseases, and chemicals [10]. The CTD is a useful resource for examining previously published evidence on the effects of environmental toxins, providing insights into the chemical, gene, and disease interactions. Resources such as the CTD provide a database for the development of hypothesis generation and testing using extensive genomic technologies, from gene regulation to genomic variant extraction. Here, we generated hypotheses using publicly curated data in the CTD on the role of air pollution on the expression of genes involved in autistic disorder and examined these alterations to gene expression with ATAC-seq data we generated in human neural progenitor cells exposed to an increasing dose of diesel particulate matter (DPM). ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) is used to identify chromatin accessibility genome-wide [11]. The technique involves the use of transposon insertions in regions where the chromatin is open and accessible, allowing for subsequent sequencing of the region. Any regions in the DNA where the chromatin is closed will prevent sequencing. The ability of ATAC-seq to identify regions of open chromatin makes it an ideal technique for examining epigenetic mechanisms of gene expression. ATAC-seq has been widely used to probe biological questions of gene regulation including normal human tissues [12], differences between cancerous and healthy cells [13], effects of therapeutics on cells [14], nucleosome mapping [15], enhancer identification [16], and transcription factor binding sites [17]. This technique was used here to examine the effects of DPM on chromatin accessibility at the enhancers for genes identified by the CTD as related to both ASD and susceptible to vehicle exhaust particulate matter.

2. Materials and Methods

2.1. Cell Culture

ReNcell CX human neural progenitor cells (Sigma Millepore #SC007) were maintained in tissue culture flasks at 37 °C and 5% CO₂ in ReNcell NSC Maintenance Medium supplemented with FGF and EGF in laminin-coated flasks. Diesel particulate matter was purchased from Sigma, serially diluted in a small volume of dimethyl sulfoxide (Sigma-Aldrich #NIST2975) and added to the ReNcell CX medium for 24 hours prior to harvest. A total of 50,000 cells from each dose (n = 2) were used for ATAC-seq protocol as described previously [11]. Fragmented DNA libraries were prepared using the Illumina Nextera Index Kit and adaptors (cat no. # FC-121-1030, FC-121-1011). Sequencing was performed by the Van Andel Institute Genomics Core, using Illumina NextSeq 500, paired end sequencing with all samples run over two flow cells.

2.2. ATAC-Seq Analysis

ATAC-seq analysis was performed using the ENCODE ATAC-seq pipeline [18] where same sample replicates from the two flow cells were concatenated and aligned against the hg38 index. Pipeline default parameters were used with the exception of bowtie2, set for very sensitive and maximum insert size at 1000 bp [19]. MACS2 output files were filtered of mitochondrial reads, PCR duplicates and blacklisted regions.

2.3. Database Analysis

The genes associated with both vehicle exhaust and particulate matter were downloaded from the CTDbase (data accessed July 2020). Data was filtered for genes that occur in both datasets and then further filtered to extract out the genes that are curated into the database as being associated with autism spectrum disorder (ASD). The UCSC genome browser [20], using the GRCh38/hg38 genome
build, was used with GeneHancer [21] regulatory elements to find the enhancers associated with the ASD associated genes identified in the CTDbase.

2.4. ATAC-Seq Differential Accessibility Analysis and Visualization

The sequences from these 772 enhancer regions of 78 ASD DPM genes from the CTD were extended 300 bp upstream and downstream of the start and end positions using the GenomicRanges R package. Differential Accessibility (DA) analysis of just these regions was performed using CSAW following recommended methodology by the developers [22] using the loess normalization and MACS2 peaks [23]. Batch effects were accounted for using the edgeR package in the design matrix using model.matrix according to methodology recommended [24]. Pairwise DA was used for each concentration of DPM using control DPM0 as baseline. DPM10 had 60 regions of significant DA (Pval < 0.05), DPM20 had 64 significant DA regions, DPM50 had 76 significant DA regions and DPM100 had 156 significant DA regions. Annotation of regions was performed by the ChIPseeker and bioMaRt R packages to the nearest promoter region. Peaks were visualized using Integrative Genomics Viewer [23] using generated bigwig fold change signal files from MACS2 [23]. The gene enhancer regions that had an FDR < 0.05 and a log2FC compared to the control cells of <−1.0 or >1 were split to the negative and positive genes and the sequences were submitted to the MEME [25] database and the identified motifs were submitted to TOMTOM [26] to identify the transcription factors that bind to those motifs, with the JASPAR [27] redundant vertebrates 2018 option. STRING [28] analysis and an interactome of the genome-wide ASD associated genes [29] were performed on the identified genes with significant enhancer regions and transcription factors.

2.5. Genomic Variant Extraction

The 80 significant regulation regions identified were parsed through gnomADv3 [30] for any genomic variants in a diverse population of 71,702 whole genomes. Variants were filtered through total allele frequency greater than 0.0002. Those variants with an rsID were parsed through linkage disequilibrium (LD) using SNiPA [31] within any of the 1000 genome phase 3 v5 populations, yielding a total list of variants connected in inheritance with >0.8R² to those variants within the enhancers. The genome-wide association studies GWAS catalog represents a highly curated list of variants to traits that reach genome wide significance, as reviewed by an expert panel of geneticists. The GWAS catalog [32] was obtained on 8/23/2020 and parsed for any SNPs from the LD list, searching rsIDs in LD to enhancers against the rsIDs within the GWAS catalog. From this, the connected traits to matched rsIDs from the GWAS catalog were reviewed for any that have neurological function.

3. Results

To examine the role of diesel particulate matter (DPM) on gene expression, we used a multifaced approach by combining gene expression data about air pollution and DPM from the CTD, linking of enhancers to those differentially expressed genes using GeneHancer, analysis of the identified enhancers by ATAC-seq of DPM-exposed neural progenitor cells, and genomic variant phenotype associations from GWAS (Figure 1). We used two search terms in CTD to identify the genes that are affected by two forms of pollution, vehicle emissions and particulate matter. For both vehicle emissions and particulate matter, of the top 20 biological pathways affected by the pollutant, three were relevant to ASD molecular biology: gene expression (#6), developmental biology (#9), and axon guidance (#17). Both pollutants are correlated with ASD on CTD, ranking 2nd for vehicle emissions and 28th for particulate matter. For both vehicle emissions and particulate matter, of the top 20 biological pathways affected by the pollutant, three were relevant to ASD molecular biology: gene expression (#6), developmental biology (#9), and axon guidance (#17). Both pollutants are correlated with ASD on CTD, ranking 2nd for vehicle emissions and 28th for particulate matter. There are a total of 9150 genes associated with vehicle exhaust and 10,373 genes associated with particulate matter. We took the overlapping 4530 genes and then extracted the genes associated with ASD [33,34], resulting in a total of 78 genes. There ASD genes in the CTD are curated from the literature and the Online Mendelian Inheritance in Man (OMIM) database. Of these genes labeled as associated with ASD in the CTD, 42 are considered ASD risk genes in the Simons Foundation Autism Research Initiative (SFARI) database [35] and eight of the genes were found to be significant in a recent exome sequencing of ASD [36].
A total of 772 regulatory regions were examined in ATAC-seq data on ReNcell CX neural progenitor cells exposed to five concentrations of DPM (0, 10, 20, 50, 100 µg/mL). Of these 772 regulatory regions, 641 were found in the ATAC-seq dataset, with 80 of the elements having an FDR less than 0.05 at the 100 µg/ml DPM dose relative to the control. Further parsing of the data using a cut off less than −1.0 log2 fold change (FC) or greater than +1.0 log2FC resulted in 22 negative regions of interest and seven positive regions (Table S1). Out of the 29 combined regions, 22 are uniquely associated to genes, with only PRKCB having multiple regulatory regions (both negative and positive). The genes with the highest positive log2FC regulatory regions include PTGS2, PTEN, and PRKCB (Figure 2). The genes with the biggest negative log2FC regulatory regions were APC, SHANK3, and IGF1 (Figure 2). A STRING plot of the 22 genes shows that many of the genes are associated with each other and have overlapping biological functions (Figure 3). There biological processes include nervous system development and response to stress. This overlap of the genes with altered enhancer regions shows the DPM exposure is likely affecting these regulatory regions of the genes which are enriched in response to stress (i.e., hypoxia and toxic substances exposures) and developmental processes.

Since these genes themselves are involved in similar biological processes during development and response to stress, it is possible that DPM alters transcription through overlapping intracellular mechanisms. Similar recognition sequences within the elements regulated by the same transcription factors may be activated by DPM. Using the MEME-suite [25], we identified redundant repetitive sequences (DNA motifs) in the 80 regulatory regions (FDR < 0.05), resulting in motifs that are repeatedly found in the regulatory regions. The three motifs (Figure 4A) all have significant e-values of less than 1 × 10−14. The motifs were assessed through TomTom using the JASPER database to match motifs to transcription factor recognition, identifying 29 unique transcription factors that map to the top MEME motif result. The top transcription factor results for the 1st MEME motif is EGR1 (Figure 4B). Egr1, or the early growth response protein, is regulated in oxidative stress [37], and the EGR1 number 1 curated chemical interaction is with particulate matter in the CTD [38]. When we compared the 29 unique transcription factors using a STRING plot, we found an enrichment in genes related to the biological functions: gene expression, cell population proliferation, response to chemicals and stress, and regulation of DNA binding (Figure 4C). As both the 22 genes with altered enhancer regions and the transcription factors that target these enhancer rights have shared biological processes of responses to stress and general cellular processes like cell proliferation and developmental process, it shows that DPM exposure is targeting specific biological functions. It has previously been shown that DPM

![Flow chart of analysis. Data from the publicly available comparative toxicogenomic database (CTD) identified 4539 genes that were differentially expressed following exposure to both vehicle exhaust and particulate matter. Genetic studies have implicated 78 of those genes in autism spectrum disorder (ASD). Enhancer regions for these 78 genes were identified and analyzed for differential accessibility by ATAC-seq on DPM-exposed human neural progenitor cells. Motif analyses of the open chromatin identified transcription factor binding sequences.](image)
exposure causes oxidative stress [39,40]. This gives possible insight into how DPM exposure affects
gene regulation by targeting the enhancer regions of genes and their transcription factors, which can
affect expression of the genes that are critical for development.

![Image](image_url)

**Figure 2.** Top gene enhancers that are differentially accessible when exposed to increasing doses of
diesel particulate matter (DPM). (A)–(C): three enhancer regions that have negative log2FC in chromatin
accessibility compared to the control following increasing DPM exposure, and are associated with genes
*APC* (A), *SHANK3* (B), and *IGF1* (C). (D)–(F): the enhancer regions with positive log2FC compared
to the control following increasing DPM exposure, and are associated with the genes *PTGS2* (D),
*PTEN* (E), and *PRKCB* (F). Both the enhancers for *PTGS2* (D) and *PTEN* (E) are located in the distal
intergenic region.

To test the impact on neuronal development for these identified regulatory regions, we extracted
genomic variants linked to any region with FDR significance. This analysis is an independent observation
of the identified regulatory regions connected to neurological phenotypes, with no gene level ontology
biases for connection of neurological function. Using the gnomADv3 database of 71,702 whole genomes,
we identify 60,186 unique variants within the regulatory regions, with 3250 populations, imputing all SNPs that are coinherited with SNPs within the regulatory regions identified.

Categorizing the identified variants as either 0.1 allele frequency within the population. Normalizing the number of variants per number
of bases within each regulatory region, we identified elements associated with *LOC730100* and *SHANK3*
for all variants to have high SNP density, with marked density of high allele frequency variants within
elements for *GADD45B*, *IGF1*, *MET*, *ELF5*, and *GABBR2* (Figure 3). Of these variants, 1207 have
rsID numbers and are connected to 10,659 SNPs based on 0.8 R^2^ linkage disequilibrium from all
populations, imputing all SNPs that are coinherited with SNPs within the regulatory regions identified.
Assessing all of the SNPs through the GWAS catalog [32] (196,813 associations from 4671 publications)
identified 120 associations, with 12 (10%) of them linked to neurological phenotypes (Table 1) including
self-reported educational attainment, multiple sclerosis, exploratory eye movement measurement,
schizophrenia, risk-taking behavior, and brain volume measurement. The most interesting observation
was rs703545, which is associated with brain volume measurement ($p = 1.00 \times 10^{-8}$). rs703545 has a
0.9 R^2^ to rs2114912, 0.86 to rs2607988, and 0.85 to rs2245763, all of which are found within enhancers
for *IGF1*. There SNPs are present within all populations based on the single nucleotide variant
12-102943000-A-G(CRCh37) in v2.1.1 in gnomAD [30]. IGF1 is a high-confidence ASD gene [35], which has significant decreasing accessibility following DPM treatment (Figure 2C). Overall, this suggests that regulatory region integrated mapping for DPM based on the CTD and ATAC-seq of exposure has neurological phenotype connections.

**Figure 3.** STRING analysis of the genes associated with the enhancers of interest. (A) The 4 genes on the far left are the genes with enhancer regions that are more accessible following DPM exposure and the 17 on the right enhancer regions that are less accessible following DPM exposure. The single gene that has both negative and positive enhancer regions, PRKCB, is highlighted in the red box in the middle of the plot. The gene SHANK3 is not included on this chart because it is not in the STRING human protein database. Gene ontology enrichment terms are highlighted by color. There is an enrichment for the following biological processes based on gene ontology: response to stress (FDR 0.00041), nervous system development (FDR 0.00046), developmental process (FDR 0.00046), response to hypoxia (FDR 0.00021), and response to toxic substance (FDR 1.84 × 10^{-5}). (B) Interaction map of the 22 genes with enhancers that are differentially accessible visualized using an interaction network of genome wide ASD genes.

**Figure 4.** MEME motif analysis of the regulatory regions. (A) shows the 3 MEME motifs found for the 80 GeneHancer regions (FDR < 0.05) associated with the ASD curated genes in the CTD. (B) EGR1 is the top transcription factor that recognizes the top identified motif based on TomTom and JASPAR database search. (C) STRING plot of the transcription factors that recognize the top MEME motif. Colors correspond to the biological processes’ enriched terms based on gene ontology in the network. The biological processes found to be enriched include: negative regulation of gene expression (FDR 4.30 × 10^{-15}), regulation of cell population proliferation (FDR 1.17 × 10^{-7}), response to chemical (FDR 0.0289), regulation of DNA binding (FDR 0.0404), response to endogenous stimulus (FDR 0.0015), and cellular response to stress (0.0031).
Figure 5. Genomic variants found within top regions. The first four box and whisker plots are based on gnomAD v3 whole genome extraction and the last based on all rsID listed variants. All values are the number of variants mapped per base pairs (bp) of element. Outliers are labeled based on GeneHancer connected gene. The four gnomAD v3 plots show all variants (far left) followed by cutoffs for allele frequency within all populations (cutoffs for 0.001, 0.01, and 0.1).

Table 1. Neurological linked GWAS to regulated enhancers. All SNPs found within identified enhancers in addition to all population linked 0.8R2 SNPs were queried within the GWAS catalog obtained on 8/23/2020. In red text is a SNP linked to the highlighted region in Figure 2C. CHR = chromosome, POS = position.

| Linked Regulatory Region | PubMed_ID | MAPPED_TRAIT | RISK ALLELE | CHR_ID | CHR_POS | p-Value |
|--------------------------|-----------|--------------|-------------|--------|---------|---------|
| chr5:51031736-51032889   | 30038396  | self-reported educational attainment nicotine dependence symptom count, depressive symptom measurement exploratory eye movement measurement | rs12620796-A | 2      | 51060711 | 9.00×10^{-11} |
| chr5:148730602-148971200 | 3028706  | unipolar depression, alcohol dependence multiple sclerosis mathematical ability schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment self-reported educational attainment | rs57108954-T | 5      | 148857822 | 3.00×10^{-6} |
| chr5:148730602-148971200 | 26242244 | unipolar depression, alcohol dependence multiple sclerosis mathematical ability schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment self-reported educational attainment | rs17108911-7 | 5      | 148903759 | 6.00×10^{-6} |
| chr7:22600600-22602886   | 29071344  | unipolar depression, alcohol dependence multiple sclerosis mathematical ability schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment self-reported educational attainment | rs2905347-G | 7      | 22580700 | 6.00×10^{-6} |
| chr7:11686839-116776343 | 1910793  | multiple sclerosis mathematical ability schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment self-reported educational attainment | rs10243024-7 | 7      | 116706549 | 6.00×10^{-6} |
| chr7:11784647-117850990 | 30038396  | multiple sclerosis self-reported educational attainment | rs17430287-A | 8      | 11784408 | 4.00×10^{-8} |
| chr9:4506589-4512250     | 29503163  | schizophrenia, response to risperidone multiple sclerosis | rs16921385-A | 9      | 4507513 | 4.00×10^{-8} |
| chr10:88060002-88390829  | 31604244  | schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment | rs1819577-A | 10     | 88067364 | 1.00×10^{-7} |
| chr10:88060002-88390829  | 30038396  | schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment | rs1426619-T | 10     | 88331783 | 1.00×10^{-11} |
| chr10:88060002-88390829  | 30038396  | schizophrenia, response to risperidone multiple sclerosis self-reported educational attainment | rs1426619-T | 10     | 88331783 | 1.00×10^{-10} |
| chr11:27720334-28567694  | 30643258  | risk-taking behavior brain volume measurement | rs16918024-T | 11     | 28566879 | 8.00×10^{-6} |
| chr12:102474603-102536836 | 31678660 | risk-taking behavior brain volume measurement | rs703545-7 | 12     | 102549222 | 1.00×10^{-6} |

4. Discussion

The epigenetic effects of environmental exposures impact human development and health. Publicly available databases like the CTD are important tools in evaluating the effects of chemicals on not only the genome but also the epigenome. The curated CTD contains information on 2,159,899 chemical–gene interactions (13,618 unique chemicals) and 27,723,024 gene–disease associations as of July 2020 [10]. As presented here, this data can be a strong tool in evaluating the role of the environment on health. By building onto the knowledge of previous experiments using ATAC-seq data from DPM-exposed progenitor cells, new insights into how environmental chemical exposures affect human health can be identified.

A closer examination of the top three genes with enhancers that are less accessible (APC, SHANK3, and IGF1) shows that genes associated with DPM response cause changes to gene expression or
methylation states. SHANK3 was found to have a reduced mRNA expression levels in early postnatal rats exposed to DPM [41]. The APC gene is hypermethylated in cells following DPM exposure [42].

In addition to the less accessible enhancers, there are also alterations to PTGS2 [43], PTEN [9,44], PRKCB [45] expression upon exposure to DPM. We have not only found enhancers for genes that are related to processing this stress, but also those regions of the genome that have similar recognition sequences that also attract transcription factors related to the processing of toxic substances. Some of these transcription factors are known to have shared biological functions. In the case of PTEN regulation, the transcription factor found to target the motif in the PTEN enhancer region is a target for the EGR1 has been shown to control expression [46].

EGR1 (NGFI-A, zif268) is an immediate early gene and inducible transcription factor. Within the CTD, 1236 gene–chemical interactions are identified [38]. The top chemical interactor for EGR1 is particulate matter with 43 curated interactions. EGR1 has been shown to have altered gene expression in the response to environmental pollutants like arsenic [47], bisphenol A, and lead [48]. Exposure of bronchial epithelial cells to diesel particulate matter has been shown to decrease expression of EGR1 [49].

It has been shown to be involved in pulmonary inflammation following particulate matter exposure [50], increased expression by polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposures [51] and increased mRNA levels have been found in microvascular endothelial cells following ultrafine particle and cigarette smoke extract exposures [52]. Additionally, Egr-1 expression is disrupted by neonatal ethanol exposure [53], is differentially impacted by age and experience [54], and can be induced by both anxiogenic and anxiolytic drugs, indicating a sensitivity of Egr-1 to physiological homeostasis [55]. The function of EGR1 in neurodevelopment includes regulation of synaptic plasticity [56] and maturation of neurons in the dentate gyrus [57]. For an extensive review of established function of EGR-1 in neurodevelopmental disorders and upstream and downstream targets, see Duclot and Kabbaj [58].

Of the 78 genes that were curated as having an association with ASD in the CTD, 41 are also in the SFARI database. While most of these genes curated to be associated with ASD in the CTD are the results of rare single gene mutations, many also are the result of associations of common genetic variants to ASD. Of the 29 enhancers identified here as affected by DPM exposure, they are associated with 22 specific genes, 11 of which are considered ASD risk genes [35] (three high confidence, two strong candidate, and six suggestive evidence genes). These three high confidence genes are SHANK3, UBE3A, and PTEN, with both UBE3A and PTEN having more than one enhancer affected by DPM exposure. GWAS studies demonstrated that exposures to air pollution alter gene expression of ASD genes SHANK3 [41], GADD45B [59], and IGF1 [60].

Many ASD risk genes are the result of genetic mutation. But sequencing studies have shown that there are loss-of-function variants for genes in unaffected individuals. There mutations may be exacerbated by environmental factors and this interaction contributes to disease risk. One key example of this is the MET gene. Studies have shown that individuals with risk variants in the MET promoter combined with high exposures to traffic related pollution have an increased risk of ASD [61]. Additionally, PRKCB gene variants have decreased gene expression in individuals with a diagnosis of ASD [62], and also show decreased expression following exposures to particulate matter [63].

There two examples of genetic variants that can also be altered by an environmental agent indicates that there are likely epigenetic mechanisms that contribute to disease state when combined. Additionally, there is a well-established role of the paternal age to incidence of ASD in offspring, where rare genetic changes due to age and environmental exposures of sperm increase genes identified in ASD and other neurodevelopmental disorders [64]. Many studies have thus established that not only rare variants contribute to ASD, but that there is a complex interplay of environmental exposures with genetics to contribute to elevated risk of ASD [65–67]. The role of environmental exposures on neurodevelopment in ASD can be extended to other neurodevelopmental and psychiatric disorders. The ASD risk genes are commonly key components of basic neurodevelopment and are also frequently implicated in other neurodevelopmental and psychiatric disorders such as schizophrenia [68], intellectual disability [69], and epilepsy [70].
There are some limitations to the study. The CTD can help to give insights into the relationship between genes and toxins, but care should be taken to examine the gene–disease relationship critically. The CTD is a useful tool for hypothesis generation, but it is susceptible to the same candidate gene biases as other databases. Of the 78 genes related to ASD based on their curation in the CTD, there was little overlap with databases like SFARI [35] and other publications [36], indicating discrepancies between the list of ASD risk genes. The CTD genes associated with ASD and particulate matter were used for consistency throughout the analysis, which influences the enhancer regions used. It is possible that using ASD associated genes from other sources may yield different enhancers. In addition to a bias from the database, it is possible that the enhancer regions identified could be modulating gene expression for multiple genes [71].

While this work was focused on vehicle exhaust/particulate, this workflow can be applied to studying the toxicogenomic mechanisms of other diseases and pollutants. Our results clearly build onto previously curated data that indicates vehicle exhaust/particulate matter can have wide ranging effects to gene expression and regulation. By examining chromatin accessibility, we gained insights into possible mechanisms of DPM exposure on development. DPM exposure has been previously linked to alterations in oxidative stress [72], brain development [73], and inflammation [74]. We have shown here that gene enhancers that regulate ASD related genes are also altered following DPM exposure. There alterations to the enhancers could provide much needed insights into how DPM affects gene expression, and how these alterations in gene expression could drive altered neurobiology during fetal brain development.

5. Conclusions

Exposure to diesel particulate matter affected the chromatin accessibility of autism risk genes. Transcription factor binding sites in these regulatory regions are affected by DPM exposures likely affecting gene expression.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-4601/17/20/7386/s1, Table S1: List of enhancer regions.

Author Contributions: Conceptualization, S.M.B., K.L., J.W.P., and D.B.C.; methodology, S.M.B., K.L., J.W.P.; formal analysis, S.M.B., K.L., and J.W.P.; writing—original draft preparation, S.M.B., K.L., and J.W.P.; writing—review and editing, S.M.B., K.L., B.L.T., J.W.P., and D.B.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by National Institutes of Health (NIH) R56ES029064 (to D.B.C.), NIH K01ES025435 (to J.W.P.), the Spectrum–MSU Alliance Fund, and Michigan State University.

Acknowledgments: The authors thank the Van Andel Genomics Core for providing facilities and ATAC sequencing services.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Moorthy, B.; Chu, C.; Carlin, D.J. Polycyclic Aromatic Hydrocarbons: From Metabolism to Lung Cancer. Toxicol. Sci. 2015, 145, 5–15. [CrossRef] [PubMed]
2. Sciara, R.; Borghini, A.; Montuschi, P.; Gerosa, G.A.; Ricciardi, W.; Moscato, U. Impact of air pollution on respiratory diseases in urban areas: A systematic review. Daniele Ignazio La Milia. Eur. J. Public Health 2017, 27. [CrossRef]
3. Rock, K.D.; Patisaul, H.B. Environmental Mechanisms of Neurodevelopmental Toxicity. Curr. Environ. Health Rep. 2018, 5, 145–157. [CrossRef] [PubMed]
4. Gluckman, P.D.; Hanson, M.A.; Cooper, C.; Thornburg, K.L. Effect of in utero and early-life conditions on adult health and disease. N. Engl. J. Med. 2008, 359, 61–73. [CrossRef]
5. Lee, B.J.; Kim, B.; Lee, K. Air Pollution Exposure and Cardiovascular Disease. Toxicol. Res. 2014, 30, 71–75. [CrossRef]
6. Bell, M.L.; Belanger, K.; Ebisu, K.; Gent, J.F.; Lee, H.J.; Koutrakis, P.; Leaderer, B.P. Prenatal Exposure to Fine Particulate Matter and Birth Weight. Epidemiol. Camb. Mass 2010, 21, 884–891. [CrossRef]
20. Kent, W.J.; Sugnet, C.W.; Furey, T.S.; Roskin, K.M.; Pringle, T.H.; Zahler, A.M.; Haussler, D. The Human Genome Browser. Nucleic Acids Res. 2002, 30, 277–280. [CrossRef] [PubMed]
21. Fishilevich, S.; Nudel, R.; Rappaport, N.; Hadar, R.; Plaschkes, I.; Iny Stein, T.; Rosen, N.; Kohn, A.; Twik, M.; Mott, J.; Ehrlich, M.E.; Hurd, Y.L.; Roussos, P. An atlas of chromatin accessibility in the adult human brain. Genome Res. 2018, 28, 1243–1252. [CrossRef] [PubMed]
22. Rendeiro, A.F.; Schmidt, C.; Strefford, J.C.; Walewska, R.; Davis, Z.; Farlik, M.; Oscier, D.; Bock, C. Chromatin accessibility maps of chronic lymphocytic leukemia identify subtype-specific epigenome signatures and transcription regulatory networks. Nat. Commun. 2016, 7, 11938. [CrossRef] [PubMed]
23. Kagohara, L.T.; Zamuner, F.; Davis-Marcisak, E.F.; Sharma, G.; Considine, M.; Allen, J.; Yegnasubramanian, S.; Gaykalova, D.A.; Fertig, E.J. Integrated single-cell and bulk gene expression and ATAC-seq reveals heterogeneity and early changes in pathways associated with resistance to cetuximab in HNSCC-sensitive cell lines. Br. J. Cancer 2020, 123, 101–113. [CrossRef] [PubMed]
24. Schep, A.N.; Buenrostro, J.D.; Denny, S.K.; Schwartz, K.; Sherlock, G.; Greenleaf, W.J. Structured nucleosome fingerprints enable high-resolution mapping of chromatin architecture within regulatory regions. Genome Res. 2015, 25, 1757–1770. [CrossRef] [PubMed]
25. Daugherty, A.C.; Yeo, R.W.; Buenrostro, J.D.; Davis, Z.; Greenleaf, W.J.; Kundaje, A.; Brunet, A. Chromatin accessibility dynamics reveal novel functional enhancers in C. elegans. Genome Res. 2017, 27, 2096–2107. [CrossRef] [PubMed]
26. Li, Z.; Schulz, M.H.; Look, T.; Begemann, M.; Zenke, M.; Costa, L.G. Identification of transcription factor binding sites using ATAC-seq. Genome Biol. 2019, 20, 45. [CrossRef] [PubMed]
27. Nair, S.; Kim, D.S.; Perricone, J.; Kundaje, A. Integrating regulatory DNA sequence and gene expression to predict genome-wide chromatin accessibility across cellular contexts. Bioinformatics 2019, 35, i108–i116. [CrossRef] [PubMed]
28. Wilson, M.R.; Reske, J.J.; Holladay, J.; Wilber, G.E.; Rhodes, M.; Koeman, J.; Adams, M.; Johnson, B.; Su, R-W; Joshi, N.R.; et al. ARID1 and PI3-kinase pathway mutations in the endometrium drive epithelial transdifferentiation and collective invasion. Nat. Commun. 2019, 10, 3554. [CrossRef]
29. Kent, W.J.; Sugnet, C.W.; Furey, T.S.; Roskin, K.M.; Pringle, T.H.; Zahler, A.M.; Haussler, D. The Human Genome Browser at UCSC. Genome Res. 2002, 12, 996–1006. [CrossRef] [PubMed]
30. Fishilevich, S.; Nudel, R.; Rappaport, N.; Hadar, R.; Plaschkes, I.; Iny Stein, T.; Rosen, N.; Kohn, A.; Twik, M.; Safran, M.; et al. GeneHancer: Genome-wide integration of enhancers and target genes in GeneCards. Database 2017, 2017, [CrossRef] [PubMed]
31. Lun, A.T.L.; Smyth, G.K. csaw: A Bioconductor package for differential binding analysis of ChIP-seq data using sliding windows. Nucleic Acids Res. 2016, 44, e45. [CrossRef] [PubMed]
32. Robinson, J.T.; Thorvaldsdottir, H.; Winckler, W.; Guttman, M.; Lander, E.S.; Getz, G.; Mesirov, J.P. Integrative Genomics Viewer. Nat. Biotechnol. 2011, 29, 24–26. [CrossRef] [PubMed]
33. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010, 26, 139–140. [CrossRef] [PubMed]
34. Machanick, P.; Bailey, T.L. MEME-ChIP: Motif analysis of large DNA datasets. Bioinformatics 2011, 27, 1696–1697. [CrossRef] [PubMed]
35. Gupta, S.; Stamatoyannopoulos, J.A.; Bailey, T.L.; Noble, W.S. Quantifying similarity between motifs. Genome Biol. 2007, 8, R24. [CrossRef]
36. Fornes, O.; Castro-Mondragon, J.A.; Khan, A.; van der Lee, R.; Zhang, X.; Richmond, P.A.; Modi, B.P.; Correard, S.; Gheorghe, M.; Baranasi, D.; et al. JASPAR 2020. Update of the open-access database of transcription factor binding profiles. Nucleic Acids Res. 2020, 48, D87–D92. [CrossRef]

References

[1] Volk, H.E.; Lumrnan, F.; Penfold, B.; Hertz-Picciotto, I.; McConnell, R. Traffic-Related Air Pollution, Particulate Matter, and Autism. JAMA Psychiatry 2013, 70, 71–77. [CrossRef] [PubMed]
[2] Mohan Kumar, S.M.J.; Campbell, A.; Block, M.; Veronese, B. Particulate matter, oxidative stress and neurotoxicity. Neurotoxicology. 2008, 29, 479–488. [CrossRef]
[3] Motta, V.; Angelici, L.; Nordio, F.; Bollati, V.; Fossati, S.; Frascati, F.; Tinaglia, V.; Bertazzi, P.A.; Battaglia, C.; Baccarelli, A.A. Integrative Analysis of miRNA and Inflammatory Gene Expression After Acute Particulate Matter Exposure. Toxicol. Sci. 2013, 132, 307–316. [CrossRef] [PubMed]
[4] Davis, A.P.; Grondin, C.J.; Johnson, R.J.; Scially, D.; McMorran, R.; Wiegers, J.; Wiegers, T.C.; Mattingly, C.J. The Comparative Toxicogenomics Database: Update 2019. Nucleic Acids Res. 2019, 47, D948–D954. [CrossRef] [PubMed]
[5] Buenrostro, J.; Wu, B.; Chang, H.; Greenleaf, W.; ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. Curr. Protoc. Mol. Biol. Ed. Frederick M Ausubel Al 2015, 109, 21–29. [CrossRef] [PubMed]
28. Franceschini, A.; Szklarczyk, D.; Frankild, S.; Kuhn, M.; Simonovic, M.; Roth, A.; Lin, J.; Minguiz, P.; Bork, P.; von Mering, C.; et al. STRING v9.1: Protein–protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* 2013, 41, D808–D815. [CrossRef]
29. Krishnan, A.; Zhang, R.; Yao, V.; Theesfeld, C.L.; Wong, A.K.; Tadych, A.; Volfovsky, N.; Packer, A.; Lash, A.; Troyanskaya, O.G. Genome-wide prediction and functional characterization of the genetic basis of autism spectrum disorder. *Nat. Neurosci.* 2016, 19, 1454–1462. [CrossRef]
30. Karczewski, K.J.; Francioli, L.C.; Tiao, G.; Cummings, B.B.; Alföldi, J.; Wang, Q.; Collins, R.L.; Laricchia, K.M.; Ganna, A.; Birnbaum, D.P.; et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 2020, 581, 434–443. [CrossRef]
31. Arnold, M.; Raffier, J.; Pfeuffer, A.; Suhre, K.; Kastenmüller, G. SNIpA: An interactive, genetic variant-centered annotation browser. *Bioinformatics* 2015, 31, 1334–1336. [CrossRef] [PubMed]
32. Buniello, A.; MacArthur, J.A.L.; Cerezo, M.; Harris, L.W.; Hayhurst, J.; Malangone, C.; McMahon, A.; Morales, J.; Mountjoy, E.; Sollis, E.; et al. The NHGRI-EBI GWAS Catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. *Nucleic Acids Res.* 2019, 47, D1005–D1012. [CrossRef] [PubMed]
33. Curated [Vehicle Exhaust-Autism Spectrum Disorders] Data Were Retrieved from the Comparative Toxicogenomics Database (CTD), MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina. World Wide Web. Available online: http://ctdbase.org/ (accessed on 1 July 2020).
34. Curated [Particulate Matter-Autism Spectrum Disorders] Data Were Retrieved from the Comparative Toxicogenomics Database (CTD), MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina. World Wide Web. Available online: http://ctdbase.org/ (accessed on 1 July 2020).
35. Abraham, B.S.; Arking, D.E.; Campbell, D.B.; Mefford, H.C.; Morrow, E.M.; Weiss, L.A.; Menashe, I.; Wadkins, T.; Banerjee-Basu, S.; Packer, A. SFARI Gene 2.0: A community-driven knowledgebase for the autism spectrum disorders (ASDs). *Mol. Autism* 2013, 4, 36. [CrossRef] [PubMed]
36. Satterstrom, F.K.; Kosmicki, J.A.; Wang, J.; Breen, M.S.; De Rubeis, S.; An, J.-Y.; Peng, M.; Collins, R.; Grove, J.; Klei, L.; et al. Large-Scale Exome Sequencing Study Implies Both Developmental and Functional Changes in the Neurobiology of Autism. *Cell* 2020, 180, 568–584.e23. [CrossRef]
37. Stuart, J.R.; Kawai, H.; Tsai, K.K.C.; Chuang, E.Y.; Yuan, Z.-M. c-Abl regulates Early Growth Response Protein (EGR1) in response to oxidative stress. *Oncogene* 2005, 24, 8085–8092. [CrossRef]
38. Curated [EGR1-Chemical] Data Were Retrieved from the Comparative Toxicogenomics Database (CTD), MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina. Available online: http://ctdbase.org/ (accessed on 10 October 2020).
39. Ehnsifaf, M.; Tameh, A.A.; Farzadkia, M.; Kalantari, R.R.; Zavareh, M.S.; Nikzaad, H.; Safari, A.J. Exposure to nanoscale diesel exhaust particles: Oxidative stress, neuroinflammation, anxiety and depression on adult male mice. *Ecotoxicol. Environ. Saf.* 2019, 168, 338–347. [CrossRef]
40. Kim, Y.-D.; Lantz-McPeak, S.M.; Ali, S.F.; Kleinman, M.T.; Choi, Y.-S.; Kim, H. Effects of ultrafine diesel exhaust particles on oxidative stress generation and dopamine metabolism in PC-12 cells. *Environ. Toxicol. Pharmacol.* 2014, 37, 954–959. [CrossRef]
41. Li, K.; Li, L.; Cui, B.; Gai, Z.; Li, Q.; Wang, S.; Yan, J.; Lin, B.; Tian, L.; Liu, H.; et al. Early Postnatal Exposure to Airborne Fine Particulate Matter Induces Autism-like Phenotypes in Male Rats. *Toxicol. Sci.* 2018, 162, 189–199. [CrossRef]
42. Hou, L.; Zhang, X.; Tarantini, L.; Nordio, F.; Bonzini, M.; Angelici, L.; Marinelli, B.; Rizzo, G.; Cantone, L.; Apostoli, P.; et al. Ambient PM exposure and DNA methylation in tumor suppressor genes: A cross-sectional study. *Part. Fibre Toxicol.* 2011, 8, 25. [CrossRef]
43. Bos, I.; Boever, P.D.; Emmerichs, J.; Buekers, J.; Vanoirbeek, J.; Meeusen, R.; Poppel, M.V.; Nemery, B.; Nawrot, T.; Panis, L.I. Changed gene expression in brains of mice exposed to traffic in a highway tunnel. *Inhal. Toxicol.* 2012, 24, 676–686. [CrossRef]
44. Tsamou, M.; Vrijens, K.; Madhlooum, N.; Lefevre, W.; Vanpoucke, C.; Nawrot, T.S. Air pollution-induced placental epigenetic alterations in early life: A candidate miRNA approach. *Epigenetics* 2018, 13, 135–146. [CrossRef] [PubMed]
45. Cui, L.; Shi, L.; Li, D.; Li, X.; Su, X.; Chen, L.; Jiang, Q.; Jiang, M.; Luo, J.; Ji, A.; et al. Real-Ambient Particulate Matter Exposure-Induced Cardiotoxicity in C57/B6 Mice. *Front. Pharmacol.* 2020, 11. [CrossRef] [PubMed]
64. Kong, A.; Frigge, M.L.; Masson, G.; Besenbacher, S.; Sulem, P.; Magnusson, G.; Gudjonsson, S.A.; Sigurdsson, A.; Jonasdottir, A.; Jonasdottir, A.; et al. Rate of de novo mutations and the importance of father’s age to disease risk. *Nature* 2012, 488, 471–475. [CrossRef] [PubMed]
65. Janecka, M.; Mill, J.; Basson, M.A.; Goriely, A.; Spiers, H.; Reichenberg, A.; Schalkwyk, L.; Fernandes, C. Advanced paternal age effects in neurodevelopmental disorders—Review of potential underlying mechanisms. *Transl. Psychiatry* **2017**, *7*, e1019. [CrossRef] [PubMed]

66. Grabrucker, A.M. Environmental Factors in Autism. *Front. Psychiatry* **2013**, *3*. [CrossRef] [PubMed]

67. Chaste, P.; Leboyer, M. Autism risk factors: Genes, environment, and gene-environment interactions. *Dialogues Clin. Neurosci.* **2012**, *14*, 281–292.

68. Guan, J.; Cai, J.J.; Ji, G.; Sham, P.C. Commonality in dysregulated expression of gene sets in cortical brains of individuals with autism, schizophrenia, and bipolar disorder. *Transl. Psychiatry* **2019**, *9*, 1–15. [CrossRef]

69. Srivastava, A.K.; Schwartz, C.E. Intellectual disability and autism spectrum disorders: Causal genes and molecular mechanisms. *Neurosci. Biobehav. Rev.* **2014**, *46*, 161–174. [CrossRef]

70. Srivastava, S.; Sahin, M. Autism spectrum disorder and epileptic encephalopathy: Common causes, many questions. *J. Neurodev. Disord.* **2017**, *9*. [CrossRef]

71. Karnuta, J.M.; Scacheri, P.C. Enhancers: Bridging the gap between gene control and human disease. *Hum. Mol. Genet.* **2018**, *27*, R219–R227. [CrossRef]

72. Gangwar, R.S.; Bevan, G.H.; Palanivel, R.; Das, L.; Rajagopalan, S. Oxidative stress pathways of air pollution mediated toxicity: Recent insights. *Redox Biol.* **2020**, *34*, 101545. [CrossRef]

73. Haghani, A.; Johnson, R.; Safi, N.; Zhang, H.; Thorwald, M.; Mousavi, A.; Woodward, N.C.; Shir Mohammadi, F.; Coussa, V.; Wise, J.P.; et al. Toxicity of urban air pollution particulate matter in developing and adult mouse brain: Comparison of total and filter-eluted nanoparticles. *Environ. Int.* **2020**, *136*, 105510. [CrossRef]

74. Campbell, A.; Oldham, M.; Becaria, A.; Bondy, S.C.; Meacher, D.; Sioutas, C.; Misra, C.; Mendez, L.B.; Kleinman, M. Particulate Matter in Polluted Air May Increase Biomarkers of Inflammation in Mouse Brain. *Neuro Toxicol.* **2005**, *26*, 133–140. [CrossRef] [PubMed]

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