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Main Manuscript for

Developmental pyrethroid exposure disrupts folate metabolism in mouse brain

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Abstract:

Environmental and genetic risk factors, and their interactions, contribute significantly to the etiology of neurodevelopmental disorders (NDDs). Recent epidemiology studies have implicated pyrethroid pesticides as an environmental risk factor for autism and developmental delay. Our previous research showed that low-dose developmental exposure to the pyrethroid pesticide deltamethrin in mice caused male-biased changes in the brain and in NDD-relevant behaviors that persisted into adulthood. Here, we used a metabolomics approach to determine the broadest possible set of metabolic changes in the adult male mouse brain caused by low-dose developmental pyrethroid exposure. Using a litter-based design, we exposed mouse dams during pregnancy and lactation to deltamethrin (3 mg/kg or vehicle every 3 days) at a concentration well below the EPA-determined benchmark dose used for regulatory guidance. We raised male offspring to adulthood and collected whole brain samples for untargeted high-resolution metabolomics analysis. Developmentally exposed mice had disruptions in 116 metabolites which clustered into pathways for folate biosynthesis, retinol metabolism, and tryptophan metabolism. Perturbagen analysis on split-sample transcriptomics data from the same mice identified a folate inhibitor as the drug causing the most similar effect to DPE, thus confirming that developmental pyrethroid exposure disrupted folate metabolism. These results suggest that DPE directly disrupts folate metabolism in the brain, which may inform both prevention and therapeutic strategies.

Introduction:

Neurodevelopmental disorders (NDDs) are a cluster of lifelong brain disorders including autism spectrum disorders, attention deficit hyperactivity disorder (ADHD), learning disabilities, sensory impairments, schizophrenia, and intellectual disabilities[1,2]. These are often incurable disorders with limited biomarkers and treatment options[3]. The prevalence of NDDs is rapidly rising, affecting as many as 17% of children in the United States[4]. Research has historically focused on genetic factors in the etiology of NDDs, however, recent studies have shown that genetic anomalies can only account for 30-40% of all cases, and that environmental risk and gene-environment interactions may contribute substantially the risk for autism[5-9]. Environmental toxicants such as air pollution[10], heavy metals (lead and mercury)[11], persistent organic pollutants (DDT and polychlorinated biphenyls)[12,13], flame retardants (polybrominated diphenyl ethers and organophosphate esters)[14,15], and pesticides (organophosphates and pyrethroids)[16] have been implicated in risk for NDDs in humans[17,18]. Understanding the risk factors associated with NDDs and their underlying molecular mechanisms is critical for prevention and the development of therapeutic strategies.

One potential environmental factor of concern is developmental exposure to pyrethroid pesticides. Pyrethroids are neurotoxic chemicals commonly used for agriculture and mosquito fogging, and in household applications such as insect sprays, flea collars, and head lice shampoos[19]. Pyrethroids are thought to be safe for adults and have low environmental impact, making them one of the most widely used pesticide classes in the US, with 70-80% of the general US population having detectable pyrethroid metabolites in their blood[20,21]. Nonetheless, epidemiology studies have shown that prenatal exposure to pyrethroids is a risk factor for NDDs[16,22,23] and that pyrethroid metabolite levels in the blood of pregnant women is associated

with poor brain development in the child[24]. Moreover, pyrethroid metabolites in blood and urine are associated with the risk for ADHD in children[20,21].

Mouse models have been developed to study comparable effects of developmental exposure to deltamethrin, a type II pyrethroid, in humans[25]. Deltamethrin exposure in mice during pregnancy and lactation at doses well below the Environmental Protection Agency (EPA) benchmark dose of 14.5 mg/kg is sufficient to cause a broad range of changes in the brain and behavior of the offspring. Behaviorally, deltamethrin exposure during development results in male-biased hyperactivity, repetitive behaviors, reduced vocalizations, impulsivity, and learning deficits[20,25]. In the brain, developmental deltamethrin exposure leads to broad changes in striatal dopamine signaling, altered levels of voltage-gated sodium channels, elevated endoplasmic reticulum stress, altered brain-derived neurotrophic factor (BDNF) levels, and long-term changes in molecular pathways for circadian rhythms and synaptic plasticity[26-32]. However, the underlying processes altered by deltamethrin exposure to drive these broad changes are unknown, hindering the identification of effective therapeutic targets or the identification of biomarkers.

Metabolomics – the quantitative measure of all low-molecular-weight biological molecules detectable in a sample using advanced analytical chemistry techniques[33] – is beginning to be recognized as an invaluable tool for translational research related to biomarker discovery, drug target discovery, drug responses, and disease mechanisms[34]. Mass spectrometry-based metabolomics detects and quantifies thousands of molecular features simultaneously, which are identified by a combination of retention time and signature[35]. These molecular features are then mapped onto specific metabolites using annotated reference databases. Metabolomics has the potential to identify metabolic processes that are disrupted by developmental exposure to pesticides in an unbiased manner, providing the foundation for testable hypotheses identifying biomarkers or drug targets and describing mechanisms driving the broad changes in the brain observed with developmental pesticide exposure.

In this study, we exposed pregnant mouse dams to a low level of deltamethrin (3 mg/kg every 3 days) throughout pregnancy and lactation. This dose was chosen based on prior studies showing behavioral and neurological effects in mice at this dose[20,25,32] and because it is substantially lower than the EPA-determined benchmark dose[19]. Because developmental pyrethroid exposure (DPE) is a whole-body manipulation, we chose the whole brain as our level of investigation, and we performed untargeted high-resolution metabolomics on whole-brain tissue collected from the resulting adult male offspring. We hypothesized that low-dose developmental pyrethroid exposure would lead to long-lasting changes in brain metabolism that would be detectable in the adult brain.

Materials and Methods:

Animals. Experimental subjects were the adult (P56+) male offspring of female C57BL6/J mouse dams. Virgin dams were kept on a 12/12 hour light cycle and given access to water and high-fat breeder food ad libitum. Dams were housed in female pairs, then in trios with a male sire until 3 days prior to birth, and finally singly housed through birth and weaning. Offspring were weaned at 20-22 days of age into single-sex cages of 3-5 mice and were socially housed continuously into adulthood. All procedures were approved by the Emory University IACUC and performed in accordance with the US Animal Welfare Act.

Chemicals. Deltamethrin (Sigma, St. Louis, MO) is the reference pyrethroid selected by the EPA (EPA, 2011). Aliquots were prepared by dissolving deltamethrin (Sigma, St. Louis, MO) in acetone (Sigma), mixing with corn oil (Mazola, Memphis, TN), allowing the acetone to evaporate overnight, and then storing at -80° C in capped borosilicate glass vials (Fisher Scientific, Hampton, NH) until the day of use. Aliquots of vehicle were prepared by allowing a similar volume of acetone to evaporate in corn oil.

Study design. The study design consisted of two independent groups: pesticide exposed and vehicle control. Mouse dams were exposed to deltamethrin (or vehicle) during preconception, pregnancy, and lactation as described below. The experimental groups for these experiments were composed of a single adult male offspring from each of the resulting litters.

Experimental units. Since experimental exposure was given to the dam, the experimental unit was the dam/litter, and each dam/litter was counted as N=1. A single male offspring was chosen at random to represent the litter.

Elimination criteria. Dams and their litters were eliminated from the study if they did not consume 90% of the peanut butter containing the pesticide or vehicle during 90% of the feedings (see below), if they did not give birth within 28 days of being paired with a sire, or if their litter did not contain at least one male and one female pup.

Sample size. A total of 40 female mice, pre-screened for peanut butter preference, (N=20 per group) were offered experimental or control treatment in this study. Three dams were eliminated due to inconsistent consumption, and two dams failed to produce a litter within 28 days of pairing. Four whole-brain samples were eliminated by the HERCULES High Resolution Metabolomics Core for failing quality control. This left N=18 exposed and N=13 control samples.

Developmental pyrethroid exposure (DPE). Mice were developmentally exposed to the pyrethroid pesticide deltamethrin as previously described[25]. Starting two weeks prior to being paired with a male, dams were fed deltamethrin (3 mg/kg by voluntary oral consumption in peanut butter/corn oil) or vehicle once every 3 days, continuing throughout pre-conception, pregnancy, and weaning. Dams were weighed immediately prior to each feeding, and the appropriate amount of corn oil was mixed with JIF peanut butter (J.M. Smucker, Orville, OH) to achieve the desired dose. To ensure that cagemates and offspring did not consume the peanut butter, adult cagemates were temporarily separated and peanut butter was provided in an elevated position. Dams that failed to consume 90% of the peanut butter during 90% of the feedings were eliminated from the study. Offspring were only exposed to deltamethrin indirectly through the dam. Offspring were weaned from the dam as above and received no additional treatment until experimental testing.

Tissue. Adult male offspring were sacrificed by rapid decapitation and dissected. The brains were flash-frozen using liquid nitrogen and pulverized. The resulting powder was aliquoted for use in split-sample multiomics[32].

Untargeted high-resolution metabolomics. Pulverized brain tissue aliquots were shipped on dry ice to the HERCULES High Resolution Metabolomics Core at Emory University. Sample preparation and high-resolution tandem liquid chromatography-mass spectrometry (LC-MS) were performed by the Core. The Metabolomics Core sent the results of LC-MS metabolomics in the form of raw feature tables for both c18neg and hilicpos modes, which were generated by the Core using xMSanalyzer and xMSannotator[36,37].

Preprocessing. The R package xmsPANDA was used for data filtering of features with missing values, log2 transformation, and quantile normalization[38]. xmsPANDA was then used to identify features of interest based on two criteria: (1) the feature is significantly different between the two groups ($p<0.05$ uncorrected, Limma t-test); and (2) the feature is important in discerning between the two groups, as determined by a variable importance in the projection (VIP) score >2 (partial least-squares discriminant analysis). VIP scores, p-values and log fold change were used to generate volcano and Manhattan plots and to conduct unsupervised two-dimensional hierarchical clustering analysis.

Annotation. Features of interest were mapped to the KEGG and HMDB databases using the web-based tool, MetaboAnalyst 5.0[39,40]. Feature tables from xmsPANDA were used as input into the tool's modules for data filtering, normalization, feature selection, and statistical analysis. Mummichog pathway analysis was used with default parameters.

Transcriptomics data analysis. Split-sample transcriptomics was performed on the same samples as previously described[41].

Perturbagen Analysis. Post-processed differential gene expression data were compared to the iLINCS online database using concordant and discordant perturbagen analysis[42,43]. The R packages drugfindR and biomaRt were used. The following parameters were used: Output_lib ="CP", filter threshold =0.075, similarity threshold = 0.321.

Results:

Feature analysis. We performed untargeted high-resolution metabolomics on whole-brain tissue from adult male DPE mice. Mass spectrometry detected 120,416 unique molecular features in the positive and negative ion modes (Supplemental File 1A-1B). Preprocessing of the raw data identified 731 molecular features of interest that were significantly different between DPE mice and controls ($p<0.05$ uncorrected) and were important in identifying differences between the groups (VIP > 2) (Figure 1; Supplemental Figures S1-S3). Hierarchical clustering analysis using these 731 features was able to confidently separate the two groups (adjusted $r=0.37$; $p=0.040$; sample average silhouette width=0.12; Figure 2). These 731 features mapped on to 116 annotated metabolites of interest (Supplemental File 1C).

Metabolite Pathway Analysis. The 116 annotated metabolites of interest were used to identify metabolic pathways that were significantly altered in DPE mice. Metabolites for folate biosynthesis, retinol metabolism, and tryptophan metabolism were significantly different between DPE mice and controls (hypergeometric test, $p<0.02$; Figure 3, Supplemental Figures S4-S6, Supplemental File 1D).

Perturbagen Analysis. We compared the split-sample transcriptome data from DPE mice to the iLINCS online database of compound-induced transcriptional changes using perturbagen analysis. The primary "concordant" perturbagens (having a similar transcriptional signature as DPE) were trimethoprim (similarity = 0.511), a folate synthesis inhibitor[44]; and AG14361 (similarity = 0.497), an inhibitor of mammalian target of rapamycin (mTOR) signaling and apoptosis[45,46] (Table 1). The primary "discordant" perturbagens (having the opposite transcriptional signature as DPE) were pelitinib (similarity = -0.498), an epidermal growth factor receptor (EGFR) inhibitor[47]; and mebendazole (similarity = -0.489), an inhibitor of microtubule synthesis[48] (Table 2).

Discussion:

Adult mice exposed during development to low levels of deltamethrin showed a pattern of altered small molecule metabolites in three key metabolic pathways in whole brain. Compared to vehicle-exposed controls, DPE mice showed altered folate biosynthesis, a process that is particularly critical for preventing neural tube defects during neurodevelopment. Additionally, the whole-brain transcriptional profile of adult DPE mice showed a high similarity to the transcriptional profile associated with trimethoprim, a drug that inhibits folate synthesis. These results suggest a long-term effect of DPE on folate metabolism at the whole-brain level. DPE mice also had altered retinol and tryptophan metabolism, two processes related to circadian rhythms. These findings are in line with prior research that showed reduced whole-brain gene transcription of two clock genes, Per2 and Ciart, in DPE mice[32]. This is the first study using an unbiased metabolomics approach to identify long-term effects of DPE on the brain in need of further investigation in future hypothesis-driven studies.

Folate is critical for early neural development and is universally recognized as a key prenatal vitamin for the prevention of neural tube defects. Prenatal folate supplementation in humans decreases the risk for autism, developmental delay, and other NDDs[49,50], and mouse models of maternal folate deficiency consistently show increases in neural tube defects and tumor risk[51,52]. In our study, male mice exposed to deltamethrin during development showed altered folate metabolism in the brain in adulthood. The long-term effect of DPE on folate metabolism in the mouse brain may represent a primary cause of the male-biased effects of DPE on NDD-relevant behaviors[25] and on NDD-relevant molecular pathways for synaptic plasticity[32]. Folate is also a precursor in dopamine biosynthesis, which may explain changes in the dopamine system in DPE mice[25]. These results support the hypothesis that maternal folate supplementation in mice may help prevent the NDD-relevant phenotypes induced by DPE, which is congruent with the prediction made by human epidemiology showing a protective effect of prenatal folate against the increased risk for NDDs contributed by pyrethroid pesticides[53].

Trimethoprim, a folate synthesis inhibitor, is the most significant hit in our concordant perturbagen analysis. This analysis compared transcriptomic profiles of gene expression between the iLINCS database of drug effects on cell culture, and split samples of whole brain from our mouse subjects. The consistency of these findings across these levels of analysis suggests a multi-modal effect of DPE on folate-related molecular processes. Trimethoprim acts to inhibit dihydrofolate reductase, which is part of an enzymatic pathway that converts dietary folate into more water soluble, bioactive forms[54,55]. Genetic polymorphisms in dihydrofolate reductase and other enzymes in this pathway have been implicated in autism risk, hence stressing the importance of a functional folate biosynthesis pathway in neurodevelopment[56-58]. Direct measures of cerebral and serum folate, as well as proteins in the folate biosynthesis pathway, are an important follow-up to this study.

Perturbagen analysis also identified two discordant perturbagen drugs, an EGFR inhibitor and microtubule synthesis inhibitor, as having highly dissimilar transcriptome profiles to those of the DPE mouse brain. These dissimilarities may suggest a relative increase in growth factor signaling and microtubule synthesis in DPE mouse brains. Multiomic integration of transcriptome and proteome data from DPE mouse brains also identified increases in the activity of kinases responsible for synaptic plasticity and an overall alteration in molecular pathways for synapse function[32]. These molecular changes in the DPE mouse brain could reflect an increase in dendritic spine growth and synaptogenesis. Brain samples from autistic

patients show an increase in the number of dendritic spines, owing primarily to the proliferation of short, immature spines[59]; a phenotype that is commonly observed in mouse models of autism risk genes[60]. Direct assessment of dendritic spines in DPE mice would provide more insight into these findings.

Tryptophan metabolism was also altered in the brains of adult DPE mice. Tryptophan is an essential amino acid and is metabolized along two metabolic pathways: a small amount of tryptophan is metabolized via the methoxyindole pathway, which produces serotonin and melatonin; while the majority of tryptophan is metabolized via the kynurenine pathway, which regulates immune response and is considered neuroprotective[61,62]. Placental tryptophan metabolism is an essential source of serotonin in fetal neurodevelopment[63-65], and disruptions in serotonin signaling during fetal brain development cause a wide range of neural defects[66]. The kynurenine pathway generates two compounds, kynurenic acid and quinolinic acid, and disruptions in both of these compounds have been associated with NDDs and autism[67]. Furthermore, acute deltamethrin exposure in rats decreases kynurenic acid production by 31%, suggesting that deltamethrin may have a direct effect on this pathway[68].

Both altered retinol metabolism and altered melatonin (via tryptophan metabolism) in DPE mouse brains may provide links between DPE and altered circadian rhythm. DPE mice show reduced transcription of critical clock genes at the whole-brain level[32]. During development, retinoids play a key role in neuronal differentiation, neural tube development, and neurite outgrowth[69]. In adulthood, retinol metabolism and retinoic acid are critical for the regulation of both circadian and seasonal rhythms[70]. Melatonin also plays an essential role in circadian rhythms in the brain and is one of the most reliable markers of the sleep-wake cycle[71]. Disruption of circadian rhythms and sleep are among the most common comorbidities in developmental disorders[72,73]. Finally, retinoic acid is highly expressed in the dopamine system and is a key regulator of dopamine synthesis and signaling[74,75], both of which are disrupted in DPE mice[20,25]. Thus, our non-biased metabolomics study adds to the literature highlighting retinol and tryptophan metabolism as worthy targets for future investigations into the long-term effects of developmental pyrethroid exposure.

There are key strengths and limitations to this study that must be considered. We chose the whole brain as the most appropriate level of analysis because DPE is a whole-body manipulation. Examining whole-brain tissue strengthens our ability to see metabolic effects permeating every brain region and dominant cell type, which are the most likely to be good targets for whole-body pharmacological treatment. However, more specific effects on individual brain regions or minority cell types are unlikely to be represented in our data. The use of a single dose of deltamethrin is also a key limitation. The 3 mg/kg dose was chosen because it is below the EPA-determined benchmark dose of 14.5 mg/kg for humans and is sufficient to cause a wide range of behavioral, transcriptional, and proteomic changes in mice[20,25,29,32]. Nonetheless, a careful look at dose-dependent effects of DPE is a critical next step. Finally, our study looked only at the effects of DPE on male mouse brains. This was primarily due to the male-biased effects of DPE in prior studies in mice. However, including female subjects in future studies would allow us to see sex-dependent changes in brain metabolism and further identify the root causes of the male bias in the changes in NDD-relevant behaviors in DPE mice.

Pyrethroid pesticides are among the most commonly used in the US, and the exposure level of the general public is high. If developmental exposure to pyrethroid pesticides increases risk for autism and other developmental disorders, this represents a critical public health challenge.

Here, we used a non-biased approach to show that developmental exposure to a low level of deltamethrin in mice caused changes in brain metabolism related to folate, tryptophan, and retinol metabolism, which are strongly related to developmental disorders in humans. Much work remains to verify these findings using specific targeted methods, which may point to potential avenues for prevention and treatment.

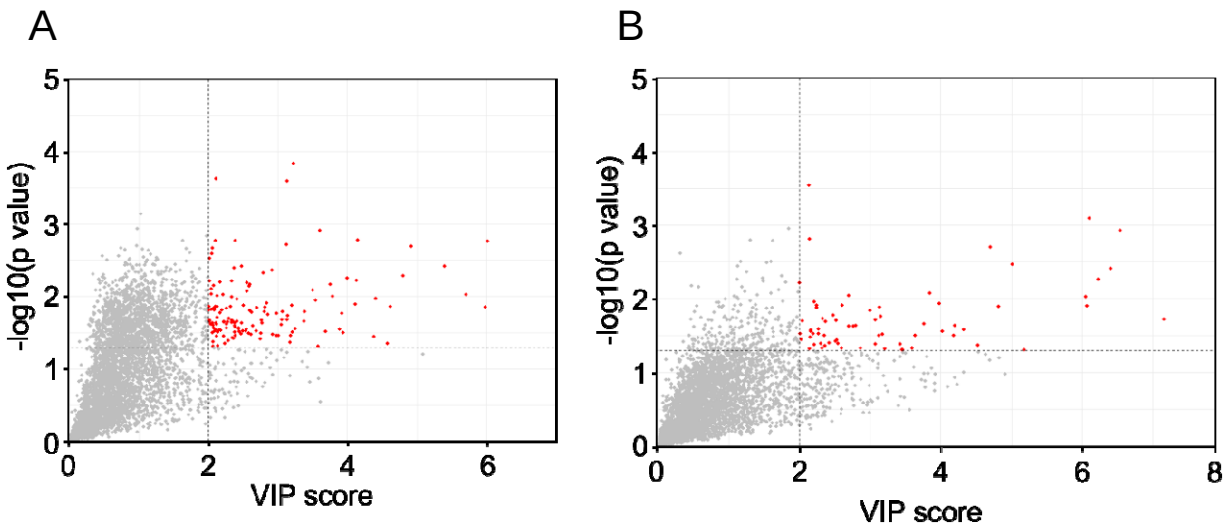

Figure 1. Molecular features of interest. Preprocessing of raw feature tables identified (A) positively charged and (B) negatively charged molecular features that were significantly different between groups (by p-value) and important in modeling differences between the groups (by VIP score).

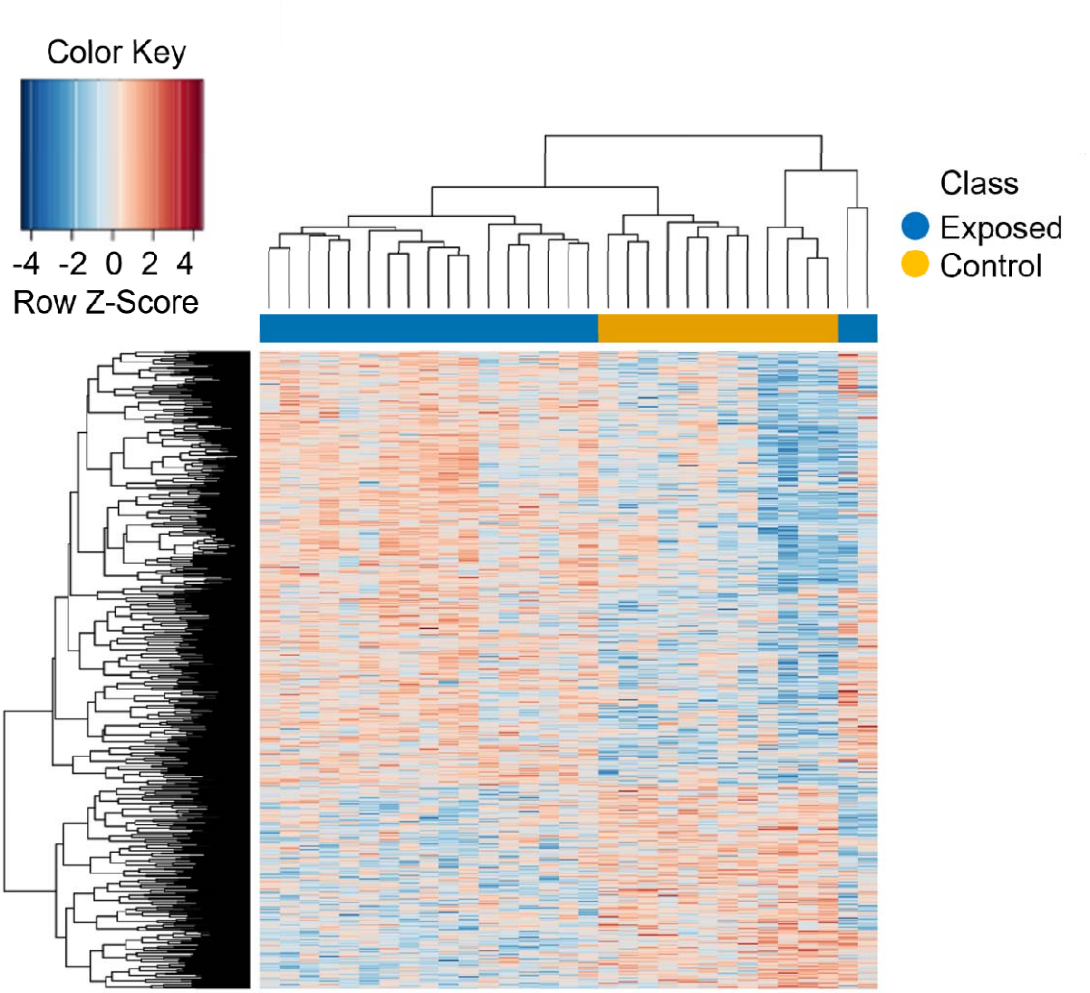

Figure 2. Hierarchical clustering heatmap. Hierarchical clustering analysis was performed using features of interest and was able to confidently separate the two groups (adjusted r=0.37; p=0.040). Group subjects are clustered along the x-axis, with features of interest (mz/rt) on the y-axis.

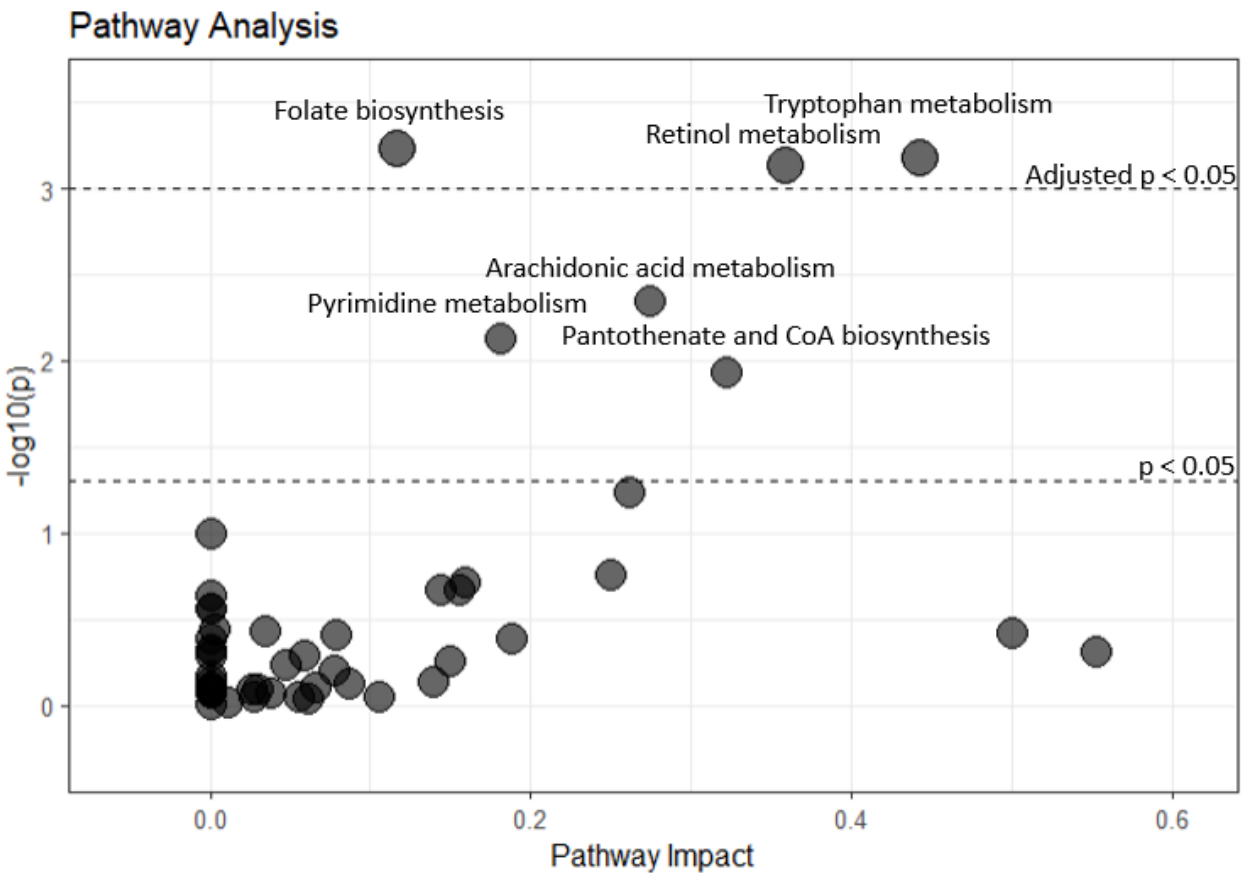

Figure 3. Metabolite pathway analysis. Pathway analysis on the 116 annotated metabolites of interest was used to map the metabolites to metabolic pathways. Pathways were evaluated by statistical significance (p-value) and the enrichment of metabolites in the pathway (pathway impact). Dashed horizontal lines show the cutoff values for raw $p < 0.05$, and $p < 0.05$ after adjustment for false discovery rate.

A

Perturbagen	**Similarity**
Trimethoprim	0.5112
AG 14361	0.4967
Erlotinib	0.4894
13-Hydroxy carbamate	0.4691
BCB03_000082	0.4646

B

Perturbagen	**Similarity**
Pelitinib	-0.4981
Mebendazole	-0.4890
CHEMBL2143628	-0.4632
AZD-1152-HQPA	-0.4540
Rifampicin	-0.4461

Table 1. Concordant and discordant perturbagens. Perturbagen analysis showed the similarity between the transcriptome profiles of DPE mouse brain tissue (compared to control) and "perturbagen" drugs applied to cell culture (compared to control) from the iLINCS database. Similarity scores are Pearson r values, with larger positive or negative values indicating more correlation between data sets. (A) Concordant perturbagens with a positively correlated transcriptome profile. (B) Discordant perturbagens with a negatively correlated transcriptome profile.

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Author Contributions:

JPB and GWM designed the exposure study, and JPB designed the analysis strategy. All samples were collected in the lab of GWM by JPB and transferred to the lab of JPB. MAC processed all samples, distributed them to the core facilities, and coordinated the initial analysis of all data. JHN and ASI participated in the final analysis of all data and assembly of figures under the guidance of JPB. JPB, MAC, and JHN interpreted all results. NS performed background research and prepared the manuscript. KLN provided experimental, administrative, and managerial support. All authors participated in writing the manuscript, approved the final version, and share responsibility for the content.

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Conflict of Interest Statement:

The authors declare no competing interests.

Data Availability Statement:

All raw data for metabolomics, including all metadata, are available in the supplementary materials.

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