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Complex epigenetic patterns in cerebellum generated after developmental exposure to trichloroethylene and/or high fat diet in autoimmune-prone mice

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Abstract

Trichloroethylene (TCE) is an environmental contaminant associated with immune-mediated inflammatory disorders and neurotoxicity. Based on known negative effects of developmental overnutrition on neurodevelopment, we hypothesized that developmental exposure to high fat diet (HFD) consisting of 40% kcal fat would enhance neurotoxicity of low-level (6 µg/kg/day) TCE exposure in offspring over either stressor alone. Male offspring were evaluated at ~6 weeks of age after exposure beginning 4 weeks pre-conception in the dams until weaning. TCE, whether used as a single exposure or together with HFD, appeared to be more robust than HFD alone in altering one-carbon metabolites involved in glutathione redox homeostasis and methylation capacity. In contrast, opposing effects of expression of key enzymes related to DNA methylation related to HFD and TCE exposure were observed. The mice generated unique patterns of anti-brain antibodies detected by western blotting attributable to both TCE and HFD. Taken together, developmental exposure to TCE and/or HFD appear to act in complex ways to alter brain biomarkers in offspring.

Introduction

Impaired glutathione redox regulation, increased oxidative stress, and mitochondrial dysfunction are common features of several neurological disorders including autism spectrum disorder or ASD,^{1–3} psychiatric disorders including schizophrenia^{4–6} bipolar disorder,^{7, 8} Alzheimer's disease,^{9, 10} and Parkinson's disease.^{11–13} Since glutathione is the major intracellular anti-oxidant in the brain, disruption of glutathione homeostasis by a decrease in the active reduced form of glutathione (GSH) together with an increase in the inactive oxidized disulfide (GSSG) may promote cell damage and neurotoxicity with potential to enhance susceptibility to neurologic disorders.¹⁴ The glutathione pathway also

Conflicts of interest

There are no conflicts to declare

intersects with the methionine cycle which is the critical supplier of cellular methyl groups through the formation of S-adenosylmethionine (SAM). Transfer of methyl groups from SAM yields S-adenosylhomocysteine (SAH), which in turn is hydrolyzed to adenosine and homocysteine. Homocysteine is then able to either regenerate methionine, or form cysteine; the rate limiting precursor for glutathione synthesis. Functionally, deficits in any of these key pathway metabolites could lead to neurotoxicity by altering epigenetic maintenance, redox defense, mitochondrial reactions, oxidative stress, and gene expression.¹⁵

Studies have shown that disruption of these responses to external factors during critical windows of development can be quite subtle and sufficient enough to predict later life onset of certain neurologic disorders.¹⁶ One potential environmental factor is the solvent trichloroethylene (TCE). TCE is an industrial pollutant that has been used for decades as a degreasing agent. Its widespread use and inappropriate disposal over the years has resulted in its prevalence as a common environmental contaminant detected in surface and groundwater. Based on the likelihood of exposure together with negative health impacts, TCE is consistently ranked 16th out of 275 list of hazardous chemicals by the Centers of Disease Control/Agency for Toxic Substances and Disease Registry.

The human health effects of TCE have been extensively studied.¹⁷ TCE has been identified as a carcinogen and is linked to adverse birth outcomes including congenital heart defects.¹⁸ Both occupational and environmental exposure to TCE has been associated with a variety of autoimmune and immune-mediated inflammatory diseases.^{19, 20} Increasing evidence has suggested that enhanced inflammation is linked to neurological disorders. Evidence has linked TCE exposure to Parkinson's disease.^{21, 22} Human exposure to occupational and in some cases environmental levels of TCE has been associated with behavioral alterations in children when exposure occurred during development.^{23, 24} A recent *in vitro* study showed that the primary TCE metabolite, trichloroacetaldehyde hydrate (TCAH), impaired mitochondrial function in lymphoblastoid cell lines derived from subjects with ASD.²⁵ This finding provided further mechanistic evidence and important implications for the role of TCE in neurologic disorders.²⁶

Rodent studies by Blossom *et al.* documented that both pre- and postnatal exposure to TCE promoted oxidative stress and glutathione redox imbalance commensurate with alterations in methyl metabolites in male mouse cerebellum.^{27, 28} Some of the associated alterations included decreased neurotrophin levels (e.g., BDNF), DNA hypomethylation. Interestingly, these seemingly modest yet statistically significant differences translated into real functional behavioral deficits including increased locomotor activity.

Studies in human populations focusing solely on single chemical exposures are limited by the potential multiplicity of risk factors and their capability to generate complex effects. Co-exposure studies that mimic the complexity of the human condition are needed to insure that the impact of a risk factor is not under-estimated. One increasingly common factor is obesity and associated metabolic dysfunction. More than 50% of American women of childbearing age are overweight/obese,²⁹ and ~ 75% of women will gain excessive weight during pregnancy.³⁰ Maternal obesity and associated metabolic disorders are risk factors for neurodevelopmental issues including impaired cognition, motor skills, inattention, anxiety,

and ASD.^{31–34} The underlying mechanism of this effect is not completely understood, however; redox-related metabolic mechanisms that may alter cellular methylation pathways may play a role. In a recent study, maternal methyl donor supplementation altered cognitive performance in offspring from dams fed HFD.³⁵

Now that some baseline neurologic responses to TCE alone have been established in our lab, it is important to study how they are altered by other relevant exposures. Controlled animal studies are perhaps the best way to study the contribution of potentially interacting factors to an alteration in phenotype. Due to known adverse neurologic effects in animal models of single exposure to either HFD or TCE, it was hypothesized that co-exposure, over either stressor alone, would augment endpoints associated with neurotoxicity in our model.

Experimental

Mice.

Male MRL^{+/+} mice (also known as MRL/MpJ mice) have been used to evaluate TCE-induced neurotoxicity as described previously.^{27, 28} MRL^{+/+} mice are autoimmune-prone and spontaneously develop a relatively mild lupus-like disease late in life (50% mortality at 17 months). These mice are also used as a model of neuropsychiatric lupus^{36–38} and to study pharmacologically-induced neurotoxicity.^{39, 40} Because many immunologic and neurologic disorders such as ASD are accompanied by immune dysfunction and autoimmunity,⁴¹ MRL^{+/+} mice were used in the current study. Since our previous studies used males, we confined this investigation to male mice. The female offspring were not discarded but utilized in a separate study with different endpoints.⁴²

Exposure strategy.

As shown in Figure 1, beginning at 4 weeks of age, female MRL^{+/+} mice (Jackson Laboratories; Bar Harbor, ME) to be used as breeding dams were randomly assigned to one of four exposure groups as described.⁴² The four experimental groups consisted of: 1) mice receiving vehicle control drinking water and 10% kcal fat diet or “control” mice (TCE⁻/HFD⁻); 2) mice receiving vehicle control drinking water and 40% kcal fat diet or “HFD” (TCE⁻/HFD⁺); 3) mice receiving TCE in the drinking water and 10% kcal fat diet or “TCE” (TCE⁺/HFD⁻); 4) mice receiving TCE in the drinking water and 40% kcal fat diet or “co-exposed” (TCE⁺/HFD⁺). The female dams were mated with previously untreated male MRL^{+/+} mice. The TCE was administered (0 or 0.05 µg/ml) in drinking water as previously described.⁴³ At weaning [postnatal day (PND) 21] the male pups were separated from females and group housed according to their exposure for the duration of the experiment.

Maternal TCE exposure.

The TCE-containing drinking water was changed 3 times/week to offset degradation of TCE. Non TCE groups were administered water containing only vehicle (1% Alkamuls EL-620), the reagent used to solubilize TCE. All drinking water was Ultrapure and unchlorinated (Milli-Q) to ensure that chlorination or its by-products did not confound the results. The dose of TCE in µg/kg/day, was calculated as described previously for female offspring.⁴² As in previous studies, we estimated the TCE exposure, and did not measure

TCE in blood or tissues based on its short half-life and technical difficulties with measuring low levels of TCE and metabolites in rodents. However, it is widely known that TCE is lipophilic, crosses placenta, and is detectable in human breast milk. In this study, the male offspring received TCE in the drinking water indirectly; pre conception, and by maternal exposure. Thus, the dose pups received was an estimate based on maternal water consumption. The maternal TCE dose was calculated based on average amount of TCE-containing water consumed per dam divided by the mean maternal body weight/dam (prenatal and pregnancy weights) and a previously determined 20% degradation of TCE in the water bottles.⁴⁴ The estimated maternal TCE exposure was ~6µg/kg/day. This dose is considerably lower than the current 8-h permissible exposure limit (PEL) established by the Occupational Safety and Health Administration for TCE, which is 100 ppm or approximately 76 mg/kg/day. While the dose administered in the current study (50 µg/ml or 50 ppb) was higher than the maximum contaminant level (MCL) for municipal water supplies (5 ppb), there have been instances of environmental exposures from contaminated drinking water that, when estimating cumulative impact of exposure from ingestion, inhalation, and dermal contact, far exceeded this exposure.⁴⁵

Diet.

All dams received purified diets purchased from Research Diets (New Brunswick, NJ). A subset of mice were fed a moderately high fat “western” diet consisting of 40% kcal fat (D12079B). Dams not receiving HFD were provided a protein, cholesterol, and sucrose-matched control diet consisting of 10% kcal fat (D14042701). When male pups were weaned, all groups received standard rodent chow (Harlan 7027) *ad libitum* for an additional 3 weeks until study terminus when the mice were approximately 6 weeks of age. This study was approved by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Experimental endpoints.

At 6 weeks of age, male mice were randomly selected (1 per litter) (n=8). Brains were extracted, weighed, dissected and immediately flash frozen in liquid nitrogen and stored in -80°C until use. Cerebellum was used in all assays based on alterations in this region by TCE in previous studies.^{27, 28} Biomarkers of redox homeostasis and transmethylation metabolites were assessed. mRNA was extracted and expression of several key neurotrophins and DNA methylation-related genes were evaluated by qRT-PCR. Since all MRL+/+ mice have a tendency to produce serum anti-nuclear antibodies, brain-specific antibodies were instead assessed in the serum as a measure of autoreactivity which represents a potential mechanism of neurotoxicity.⁴⁶

HPLC quantification of transsulfuration and transmethylation metabolites in cerebellum.

The methodological details for metabolite detection by HPLC have been described previously.⁴⁷ The analyses were performed using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (3 mM; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan) purchased from ESA, Inc. (Chemsford, MA). Extracts from cerebellar tissue were directly injected onto the column (Beckman Autosampler model 507 E). All metabolites were quantified using a model 5200 Coulochem II and CoulArray

electrochemical detection system equipped with a dual analytical cell (5010), a 4 channel analytical cell (6210), and a guard cell (5020). The concentrations of metabolites in the cerebellum were calculated from peak areas and standard calibration curves using HPLC software. Intracellular results are expressed as nmol/mg protein using the BCA Protein Assay Kit (Pierce, Rockford, IL). The percentage of oxidized glutathione is expressed in absolute glutathione equivalents and was calculated as $[2\text{GSSG}/(\text{GSH} + 2\text{GSSG}) \times 100]$, where 2GSSG is the oxidized glutathione concentration and GSH is the reduced glutathione concentration (nmol/mg protein).

Quantitative reverse transcriptase polymerase chain reaction.

Fluorescence-based quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was conducted in brain tissue as described.⁴⁸ For each sample of cerebellar tissue, complimentary DNA (cDNA) was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, California). Gene expression was evaluated by qRT-PCR using Bio-Rad iTaq SUBR Green Supermix Primers from Integrated DNA Technologies. Fold change in expression was determined using the delta-delta C_t method based on normalization to the reference gene *pgk1*, known to be a reliable control for mouse brain regions.⁴⁹ Samples were run in duplicate and averaged to obtain a mean fold change expression. The results were reported as fold change in cerebellum from treated mice compared with expression in cerebellum from control mice.

Western blotting to detect brain-specific antibody production.

Proteins from cerebellum isolated from untreated MRL+/+ mice prepared as described⁵⁰ and separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto nitrocellulose, and subsequently immunoblotted with pooled sera from each of the 4 treatment groups followed by secondary antibody, HRP-conjugated goat anti-mouse immunoglobulin (IgG). Mouse myeloma IgG was run in adjacent lanes and detected by the secondary antibody. The IgG was used to normalize exposure times for the western blots. Quantification of western blotting was conducted using densitometric analysis of total anti-liver antibodies normalized to Myeloma IgG (MIgG) run in adjoining lanes and expressed as optical density (OD).

Statistical analysis.

Summary statistics such as mean and standard deviation (SD) are presented for each treatment group. Data were first evaluated with a 2-way analysis of variance (ANOVA) to obtain the overall effects of the 2 main factors, TCE and HFD, and the interaction between the two. All analyses were followed by Tukey's *post hoc* tests with an overall significance level of 0.05. The 6 pre-specified pairwise comparisons included 1) (TCE⁻/HFD⁻) vs. (TCE⁻/HFD⁺) 2) (TCE⁻/HFD⁻) vs. (TCE⁺/HFD⁻) 3) (TCE⁻/HFD⁻) vs. (TCE⁺/HFD⁺) 4) (TCE⁻/HFD⁺) vs. (TCE⁺/HFD⁻) 5) (TCE⁻/HFD⁺) vs. (TCE⁺/HFD⁺) and 6) (TCE⁺/HFD⁻) vs. (TCE⁺/HFD⁺). P values indicating statistical significance from multiple comparisons are reported in the graphs and the tables. All analyses were completed using the data analysis software, GraphPad Prism 7.0 (LaJolla, CA).

Results and discussion

HFD and TCE co-exposure increased body weight in male offspring.

General characteristics of the dams were reported previously.⁴² There were no group-related differences in any of the parameters assessed in the dams including food and water consumption, body weight and maternal fat depots. As shown in Table 1, mean maternal body weights were not different among the groups when measured at three time points; two weeks prior to conception and mid-pregnancy [~gestational day (GD) 9], and at euthanasia. Thus, the relatively brief timeframe of exposure did not alter maternal body weight. Because the study focused on neurotoxic effects in offspring from maternal exposure, brain-related assessments were not conducted in the dams. One male mouse per each litter (n=8) was selected from each group and were euthanized at approximately 6 weeks of age.

Breeding was staggered, and the pups were not born on the same date. The mean age of the pups at study terminus was not statistically different among groups, and equal to an average of PND 43 (Table 1). At weaning, all pups were removed from their respective maternal exposures. Body weights were recorded at study terminus. Mean body weight in the co-exposure group was statistically different relative to controls. Two-way ANOVA revealed no interaction effects. However, there was a statistically significant main effect of HFD (p=0.009) but not for TCE.

In addition to body weights, both absolute brain weight and brain weights relative to body weight were documented at study terminus. Pairwise analysis revealed no statistically significant differences. However, based on two-way ANOVA assessment for brain weights relative to body weight, there were statistically significant main effect for HFD-only group (p=0.02) not observed with TCE exposure. Thus, the combination of treatments administered maternally appeared to have a greater impact on body weight of male offspring potentially attributable to HFD.

TCE and HFD altered redox metabolites in cerebellum.

In our previous investigations, TCE exposure impaired glutathione redox ratio and decreased methionine in cerebellum in male mice at PND 42 when the exposure occurred on a continuous basis postnatally beginning at PND 0.²⁷ In this experiment, male mice were similarly assessed at ~6 weeks of age with the exposure being strictly pre-conception and maternal. The data presented in Figure 2 depict the concentrations of GSH, GSSG, the calculated glutathione redox ratio, and the calculated percentage of oxidized glutathione equivalents. Compared to untreated control mice, the mean GSH concentration in cerebellar tissue was decreased in the TCE group by ~24%. As expected, the mean levels of GSSG were enhanced by TCE exposure, (~33%), and this effect was even more evident in the co-exposure group when compared to untreated controls (~44%) Although mean GSSG in the HFD group was not changed relative to control, the effect was different compared to the co-exposure group.

The ratio of GSH/GSSG is often used as a marker for oxidative stress. When GSH/GSSG ratios were calculated, compared to the vehicle control group, there was a decrease in the TCE-only exposure group (~49%), and this effect was even more pronounced in the co-

exposure group (~52%) compared to control). Likewise, the mean GSH/GSSG ratio was decreased in the HFD group by ~33% vs. no-treatment controls. The calculated percentage of oxidized glutathione is another sensitive measure of impaired redox balance. The results showed that the TCE exposure increased the mean percentage of oxidized GSH by ~51%, and this effect was slightly more pronounced with co-exposure (~52% increase). Interaction and main effects after two-factor ANOVA analysis are reported in Table 2. Together, TCE either alone or administered in combination with HFD was more likely than the HFD group to alter thiol levels in the cerebellum.

TCE altered transmethylation metabolites in cerebellum.

Next, we examined several transmethylation metabolites in cerebellum as an indicator of methylation potential. As shown in Figure 3, mean levels of cerebellar methionine decreased in the TCE group (~29% decrease). Mean levels of SAM were not different among the groups. However, SAH, a potent inhibitor of cellular methyltransferase enzymes including DNMTs, was increased by ~38% with TCE exposure, and this effect was even more pronounced in the co-exposure group (~46% increase). The SAM/SAH ratio is often used as a measure of cellular hypomethylation. TCE, whether alone or in combination with HFD decreased this ratio by ~22.1% and ~43.9%, respectively. Results of two-factor ANOVA interaction and main effects are reported in Table 2. Thus, similar to its effects on thiols concentrations, the presence of TCE, either with or without HFD co-exposure, appeared to be an important factor in the alteration of transmethylation metabolites indicating cellular deficits in methylation capacity.

TCE and HFD exposure modulate DNA methylation markers in cerebellum.

DNA methylation is catalyzed by specific DNA methyltransferases (DNMTs). SAM is the methyl donor in DNMT-mediated DNA methylation, and elevated SAH levels can inhibit these enzymes. To evaluate whether the low SAM/SAH ratio translated to epigenetic alterations, the expression of several genes known to be regulated by, or associated with, DNA methylation were examined. *Dnmt1* methylates the daughter strands of newly replicated DNA to promote the inheritance of methylation patterns⁵¹. As depicted in Figure 4, consistent with the reported overall low SAM/SAH ratio, mean mRNA expression of *Dnmt1* was downregulated in all exposure groups relative to control. Expression of *Dnmt3a*, which codes for an enzyme that catalyzes *de novo* DNA methylation, was decreased by 2.3 fold in the HFD group relative to control. This effect carried over to the co-exposure group where mean levels were 2.0-fold lower compared to control. Results of interaction and main effects are presented in Table 2. Collectively, these changes in mRNA expression of enzymes important in DNA methylation demonstrated the potential for maternal HFD and/or TCE to alter epigenetic processes in cerebellum.

IAP (intracisternal A particle) is a retrotransposon normally transcriptionally silent due to DNA methylation of their long terminal repeat sequences.⁵² Because DNA methylation is typically related to retrotransposon silencing we used expression of *Iap* as an indicator of global changes in DNA methylation. As such, increased expression of *Iap* is typically reflective of hypomethylation. We have examined the expression of *Iap* in CD4⁺ T cells in mice treated with TCE as a surrogate marker of global methylation.⁵³ As shown in Figure 4,

mean levels of *Iap* from the TCE-only exposure group were higher (1.9-fold) compared to the HFD-only group. Co-exposure appeared to mitigate this effect.

Next, we assessed the expression of neurotrophic factors including BDNF, which is important neurotrophin in the brain that regulates inflammation, oxidative stress, as well as neural growth. BDNF mRNA expression in the brain has been shown to be altered by TCE⁴⁸ and HFD exposure.⁵⁴ We chose to evaluate BDNF expression due to its well documented regulation by DNA methylation and altered expression in the brain or blood in neurologic disorders.⁵⁵ Interestingly, some opposing effects from the treatments were detected when BDNF levels were assessed by qRT-PCR (Figure 5). Mean BDNF expression in HFD group increased compared to controls by ~1.7-fold and by 2.0-fold when compared to the TCE group. In the co-exposure group, the mean levels of BDNF were decreased compared to the HFD-only group by 3.1-fold. Other genes that encode for neurotrophins (i.e., NGF and NT3) were assessed, and not statistically different among the groups. In addition to BDNF, several proinflammatory cytokines and chemokines were assessed including IL-1 β , TNF- α , and IL-6. However, treatment-related differences were impossible to detect based on little to no mRNA expression (data not shown). Results of two-factor ANOVA for mRNA expression are presented in Table 2.

Altered immunoreactivity of anti-brain antibodies observed in TCE and HFD exposed mice.

MRL+/+ mice are autoimmune-prone and naturally develop antibodies directed towards a variety of self-antigens, including anti-nuclear antibodies (ANAs), over time as disease progresses. Any modifying effect of an environmental stressor on ANAs are difficult to detect as the baseline levels increase spontaneously in these mice as they age in the absence of any exposure.⁵⁶ In addition to ANAs, sera reactivity to autoantigens present in organs and tissues can be used to help identify more specific targets of an autoimmune response. Consequently, our lab has developed a technique to assess the presence of autoantibodies in sera directed towards self-proteins. Our studies have shown that TCE-exposed MRL +/+ mice, but not non-autoimmune prone mice, generate anti-liver antibodies.⁴⁴ Furthermore, TCE-treated mice also produced unique immunoreactivity to liver and brain proteins after co-exposure to HFD³⁹ and mercury,⁴⁷ respectively.

Here we examined sera to test if TCE and/or HFD exposure was associated with brain-reactive antibodies. Based on the low volume of sera obtained from individual mice coupled with the relatively high volume of sera required for western blotting, sera was pooled from 2–3 mice in each group (n=8). The experiment was repeated 4 times using 4 individual pools from each group. As depicted in Figure 6A, co-exposure was associated with a more diverse staining pattern compared to other groups with more proteins detected in a fairly large range; between the molecular weight of 37–90 kDa. However, there were unique differences associated with other groups. For example, TCE-only exposure produced reactivity towards a protein of approximately 15 kDa not found in other groups. Likewise, mice fed HFD generated sera reactivity of a unique band between 50–75 kDa not detected in other groups. Control groups generated a band around 20 kDa not found in other groups. Thus, sera from MRL+/+ mice reacted to an array of brain proteins specific to their exposure.

Based on the staining patterns, co-exposure appeared to interfere with immunoreactivity of the 15 kDa band in the TCE group. Likewise, combined exposure also interfered with the 50–75 kDa band detected in the HFD group.

Quantification by densitometric analysis of mIgG run in adjoining lanes was used to normalize exposure times for the individual western blots, and demonstrated the increase in the total amount of anti-liver antibodies in TCE-treated mice (Figure 6B). Although we did not detect statistically significant differences in total protein, each group demonstrated unique bands attributable to their exposure.

Discussion

In the current study, we hypothesized that the combination of two environmental risk factors for neurotoxicity consisting of TCE and HFD administered prior to conception and prenatally would have more robust effects in offspring on several endpoints related to oxidative stress, redox status, cellular methylation, and autoreactivity over either treatment alone. TCE and HFD instead acted in a complex manner for which further study is needed to understand the extent of the effects of these exposures. The endpoints related to glutathione redox status were more pronounced with TCE exposure regardless of whether or not HFD was present. A similar pattern was observed when transmethylation metabolites were examined. For instance, methionine was only reduced in TCE- groups, and HFD, either alone or in combination with TCE, did not alter the level of this metabolite. The meaning of this result is not clear. There appeared to have been more variability in the samples from HFD group which might have precluded a statistically significant effect despite the trend observed.

The decrease in the ratio of SAM/SAH by TCE in the presence or absence of HFD as shown by our results would predict reduced methylation potential (e.g., hypomethylation) and decreased DNMTs, with TCE exposure. Methylation of repetitive sequences including retrotransposons depends on adequate expression of *Dnmt1* during DNA replication. A reduction in *Dnmt1* may contribute to hypomethylation on a global scale. Consistent with our SAM/SAH results, *Dnmt1* gene expression was decreased significantly in all groups relative to control. A similar pattern was observed with *Dnmt3*. The implication of these findings are not clear, but it has been established that expression of *de novo* DNA methyltransferases in the brain is dynamic and important early (first three weeks of life in mice) and then decreases.⁵⁷ Thus, it might have been more relevant to assess these methylation markers earlier in life. Regardless, this result implied that HFD can alter epigenetic processes in cerebellum associated with changes in DNMTs.

To further confirm this finding, a marker of global methylation, mRNA expression of retrotransposon, *Iap*, was examined. In previous studies in our lab, *Iap* was increased by chronic TCE exposure in effector/memory CD4⁺ T cells in adult mice⁵⁰ which translated into more specific DNA methylation alterations using both targeted bisulfite next-generation sequencing⁵⁸ and genome-wide methods.⁵⁹ In the current study TCE did not have this effect in cerebellum. However, *Iap* was decreased with HFD exposure indicating DNA hypomethylation consistent with the decline in SAM/SAH. Interestingly, TCE appeared to

mitigate this effect in the co-exposure group. The meaning of this effect is not clear. Although to our knowledge no reports in the literature exist concerning the effects of maternal obesity/HFD on brain *Iap* levels in offspring, environmental toxicant exposure has been found to increase *Iap* levels in the brain leading to variable methylation in response to perinatal lead exposure.⁶⁰ The expression of endogenous retroviruses including *Iap* were increased in the brain of valproic acid-exposed autism mouse models⁶¹ suggesting that endogenous retroviruses like *Iap* may also serve as biomarkers of atypical brain development. These results are in agreement with human studies showing that other similar retrotransposons such as LINE-1 are increased in schizophrenia,⁶² and autism brain.⁶³ Thus, global methylation marks may be tissue-specific, and differentially expressed depending upon timing of exposure.

In rodent models, exposure to toxicants such as ethanol⁶⁴ modulated BDNF in the brain in a DNA methylation dependent manner. Similarly, the association between BDNF, metabolic syndrome, and obesity has been proposed to play a protective role in diet-induced obesity models. Here, BDNF levels were increased with HFD exposure, and while TCE alone had little impact on BDNF relative to controls, when the two exposures were combined, TCE reversed a protective HFD effect. At this point it is difficult to predict the meaning of this result and whether or not the modulation of BDNF levels by TCE/HFD are relevant to the DNA methylation results. Future experiments to assess the effects of these exposures on DNA methylation on a larger genome-wide scale are needed to fully understand the meaning of these results. However, these data form the rationale and basis for this next important step.

Other findings included serological signs of autoimmunity in the form of anti-brain antibodies. When antibodies reactive with brain antigens were examined after normalizing to mIg (25 and 50 kDa bands), and quantified, there were no statistically significant differences in the amount of reactive proteins among each of the group. The use of pooled samples might have resulted in the sample variability from a single outlier mouse. However, in this assessment, we focused on the unique staining patterns observed. Perhaps most striking was the effect in the TCE group where a band of approximately 15kda was detected and not present in other groups. Because we have observed this band in prior studies, current studies are underway to identify this TCE-specific protein. Although there are several possibilities, it is noteworthy that antibodies to anti-synuclein, of about 14.5 kda, have been detected in individuals with Parkinson's disease.⁶³ The link between TCE exposure and Parkinson's disease in both human and experimental models has been well established. Thus, one potential mechanism involving a TCE-induced anti-brain response to proteins involved in this disease represents a pathway for future research. It is also plausible that the ~15kda band unique to TCE exposure represents reactivity to a protein indicative of autoimmunity found in the MRL mice. Older MRL mice naturally generate antibodies to nuclear constituents including DNA, chromatin, and histones. As such, subunits of histone such as H2A and H2B are approximately 14–15 kDa.

Although anti-brain antibodies have not been described in the context of HFD feeding in mouse models or in obese individuals, anti-brain antibodies have been detected with increased frequency relative to unaffected individuals with other neurologic disorders

including ASD and schizophrenia, and Parkinson's disease.^{41, 65–67} Anti-brain antibodies have similarly been detected in lupus patients.^{68, 69} How this finding correlates to the other endpoints examined in current study are not clear. One limitation of this assay is the necessity of pooling sera from individual mice in groups of 2–3 per treatment group in order to generate enough material to conduct the western blotting.

In general, body weights were similar with the exception of the co-exposure group. It is not clear why the exposure to HFD-alone did not appear to increase weight. However, the mice were only weighed at study terminus 3 weeks after removal of maternal TCE and/or HFD. Thus, it is possible that weights could have normalized to a certain degree. Other challenges that could relate to the greater impact of TCE on several endpoints relative to HFD include the baseline adiposity of the MRL+/+ mouse.⁷⁰ The MRL mouse weighs about 10 g more than conventional age-equivalent C57BL/6, and have been reported to be resistant to some metabolic alterations and hyperglycemia related to obesity observed in conventional mouse strains.⁷¹ It is possible that these issues may have limited a more robust HFD effect. In addition, this study was conducted in male offspring, and effects may have been different in females relative to males. Studies are under way in our laboratory to determine co-exposure responses in both male and female C57BL/6 mice, the standard model for diet-induced obesity.⁷² Behavioral studies are also planned to correlate changes in brain biomarkers with functional differences that will enable us to better extrapolate our findings to human disease.

Conclusions

This study has important relevance to human health and provides novel information for potential neurotoxic effects of common environmental exposures. Both TCE and HFD, both singly and together promoted alterations in adult male offspring when exposures were strictly maternal. In addition, the dams were exposed pre-conception. Studies have shown that environmental exposures such as alcohol impact the germline, and the epimutations are inherited in the offspring and alter neurologic phenotype.⁷³ Similar responses have been observed in models of HFD/obesity when the exposure occurred prior to conception underscoring the importance of including the pre-conception time of exposure in developmental studies.^{74–77} Also notable was that these changes were observed 3 weeks after exposures were removed which further underscores the importance of further study into the mechanisms behind the persistence of developmental programming effects in the brain and future impacts on behavior. Based on results reported here and by others underscores the importance of epigenetic mechanisms with these exposures. Future studies will be dedicated to understanding how long-lasting effects are maintained and how the individual environmental stressors relevant to neurotoxicity interact with one another.

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Figure 1. Experimental Design.

Starting at 4 weeks of age female MRL^{+/+} mice were randomly assigned to one of 4 treatment groups. Each group consisted of 1) Vehicle and 10% kcal fat diet (TCE⁻/HFD⁻); 2) Vehicle and 40% kcal fat diet (TCE⁻/HFD⁺); 3) TCE and 10% kcal fat diet (TCE⁺/HFD⁻), and 4) TCE and 40% kcal fat diet (TCE⁺/HFD⁺). All exposures began preconception, 4 weeks prior to breeding, and continued during gestation and lactation. Male offspring were weaned at 3 weeks after which TCE was removed from the purified drinking water and a standard diet was implemented. Mice were euthanized 3 weeks later at ~6 weeks of age and cerebellum/sera were assessed for parameters as described in detail in materials and methods section.

Figure 2. Effects of TCE and HFD co-exposure on brain thiols.

Male mice were euthanized and whole brain was harvested, weighed, and flash frozen in liquid nitrogen. Analyses of metabolites were performed using HPLC methodology as described in detail in materials and methods. The levels or concentrations of thiol metabolites were calculated from peak areas and standard calibration curves using HPLC software. Intracellular results are expressed as nmol/mg protein using BCA protein assay kit. Each symbol in the graph represents data from one randomly selected individual male mouse/litter (n=8). Data were analyzed by 2-way ANOVA to test for significant interaction and main effects between the two variables (TCE $\times$ HFD) and are presented in Table 2. Shown in the graphs are p values for the 6 pairwise Tukey's *post hoc* comparisons. Results are statistically different (*p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.00005).

Figure 3. Methyl metabolites.

Analyses of transmethylation metabolites were carried out as described. Each symbol in the graph represents data from one randomly selected individual male mouse/litter ($n=8$). Data were analyzed by 2-way ANOVA to test for significant interaction and main effects between the two variables (TCE $\times$ HFD) and are presented in Table 2. Shown in the graphs are p values for the 6 pairwise Tukey's *post hoc* comparisons. Results are statistically different (* $p < 0.05$; *** $p < 0.0005$).

Figure 4. TCE and HFD alter enzymes important in global DNA methylation.

Cerebellar tissue was dissected from whole brain that was harvested and flash frozen at study terminus. RNA was isolated and converted to cDNA for q-RT-PCR as described. Fold-change values (log2) for each gene relative to pgk1 housekeeping gene are presented in the graphs. Shown in the graphs are p values for the 6 pairwise Tukey's post hoc comparisons. Results are statistically different (*p<0.05; **p<0.005; ***p<0.0005).

Figure 5. mRNA levels of neurotrophins in the cerebellum.

qRT-PCR was performed using cerebellum from 8 mice/group collected at study terminus as described in materials and methods. Results are represented in the graphs as a mean (SD) fold change on a log2 scale. Data were analyzed by 2-way ANOVA to test for significant interaction and main effects between the two variables (TCE $\times$ HFD) and are presented in Table 2. Shown in the graphs are p values for the 6 pairwise Tukey's *post hoc* comparisons. Results are statistically significant (*p < 0.05; **p < 0.005).

Figure 6. TCE and HFD exposure promoted distinct anti-brain antibody profiles.

Sera from mice in each treatment group collected at study terminus were pooled (sera from 8 mice/group) and reacted with brain homogenate from an untreated MRL+/+ control mouse and separated by SDS-PAGE. Shown is a representative blot showing the staining pattern. The blots were repeated 4 times. Anti-brain antibodies were normalized to mouse myeloma IgG run in adjoining lanes.

Table 1.

HFD and TCE co-exposure increased body weight in male offspring

	TCE ⁻ HFD ⁻	TCE ⁻ HFD ⁺	TCE ⁺ HFD ⁻	TCE ⁺ HFD ⁺
Dams				
Pre-pregnancy BW	37.1 (3.8)	39.0 (2.7)	37.4 (3.4)	38.6 (1.2)
Mid-gestation BW	45.9 (5.2)	44.6 (4.3)	44.5 (6.3)	44.7 (5.6)
Postpartum BW	43.7 (6.1)	42.5 (4.9)	44.4 (5.0)	44.5 (3.6)
Male pups				
Age (days)	41.8 (2.7)	43.8 (3.5)	44.5 (2.8)	43.7 (4.5)
BW	29.3 (3.4)	32.8 (2.5)	31.6 (4.4)	34.3 (2.2) *
ABW	0.401 (0.04)	0.415 (0.06)	0.458 (0.03)	0.405 (0.08)
ABW/BW	0.014 (0.002)	0.012 (0.001)	0.015 (0.001)	0.011 (0.002)

Dams were weighed 2 weeks prior to breeding, once during mid-gestation (~GD 9), and again 3 weeks after pups were born. All body weights (BW) and brain weights are represented in grams. Male offspring were weighed at study terminus (PND 43). Male offspring were euthanized and whole brain was extracted and weighed and represented independently (ABW) or in BW ratios (ABW/BW). Numbers represent the mean and SD of the 4 maternal exposure groups. Data were analyzed by 2-way ANOVA to test for significant interaction and main effects of the 2 variables (TCE × HFD), and these results are reported in the text.

* Statistically significant compared to (TCE⁻/HFD⁻) group (p<0.05) determined by Tukey's *post hoc* comparisons conducted as described in the "experiment" section.

Table 2.

p values from two factor ANOVA analysis of cerebellar metabolites and gene expression analysis

	Interaction	HFD	TCE
GSH	0.03	0.53	0.05
GSSG	0.95	0.009	0.0002
GSH/GSSG	0.01	0.003	<0.0001
Percent oxidized GSH	0.08	0.06	0.002
Methionine	0.08	0.39	0.02
SAM	0.25	0.25	0.25
SAH	0.39	0.01	0.0007
SAM/SAH	0.19	0.03	<0.0001
<i>Iap</i>	0.59	0.003	0.007
<i>Dnmt1</i>	0.002	0.003	0.38
<i>Dnmt3a</i>	0.06	0.01	0.19
<i>Bdnf</i>	0.003	0.21	0.0002
<i>ntf3</i>	0.99	0.02	0.83
<i>ngf</i>	0.74	0.08	0.11

Data were analyzed by 2-way ANOVA to test for significant interaction and main effects of the 2 variables (HFD $\times$ TCE). Shown in the table are the p values from the analysis (*p<0.05 is statistically significant).