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Epigenetic effects of low perinatal doses of flame retardant BDE-47 on mitochondrial and nuclear genes in rat offspring

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Abstract

Polybrominated diphenyl ethers (PBDEs) are known endocrine disrupting chemicals used commonly as flame retardants in everything from electronics to furniture. Exposure to PBDEs during early development has been linked to neurodevelopmental delays. Despite mounting evidence of neurological harm from PBDE exposure, the molecular mechanisms underlying these effects on brain function remain unknown. We examined the effects of perinatal exposure to BDE-47, the most biologically active and prevalent BDE congener in North America, on epigenetic patterns in the frontal lobe of Wistar rats. Dams were gavaged with BDE-47 (0.002 and 0.2 mg/kg body weight) at gestation days 9 and 16, and postnatal days 1, 8, and 15. Frontal lobes from offspring at postnatal day 41 were collected to measure 5-methylcytosine (5mC) in mitochondrial cytochrome c oxidase genes (*Mt-co1*, *Mt-co2*, and *Mt-co3*), global nuclear 5-hydroxymethylcytosine (5hmC) content, 5mC in repetitive elements L1Rn, and 5mC in nuclear genes (*Bdnf*, *Crhr1*, *Mc2r*, *Nr3c1*, and *Snea*) related to behavioral and brain functions in the nuclear genome. We observed a significant decrease in %5mC in *Mt-co2* (difference from control= -0.68%, p=0.01 at the 0.2 mg/kg BDE-47). 5mC in repetitive elements L1Rn decreased at 0.002 mg/kg BDE-47 (difference= -1.23%, p=0.02). Decreased nuclear 5mC was observed in *Bdnf* and *Nr3c1* in BDE-47 exposed rats. However, we did not observe significant effects of PBDE toxicity on DNA methylation patterns for the majority of genes in the brain.

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Keywords

BDE-47; PBDE; Endocrine Disruptors; Epigenetics; DNA methylation; Brain

1.1 INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are organic chemicals used as flame retardants in a range of materials including textiles, plastics, wire insulation, and automobiles. PBDEs are toxic and accumulate in blood, breast milk, and fat tissue in humans, as well as in soil, sediment, and household dust (de Wit 2002).

PBDEs are highly lipophilic and therefore tend to accumulate in lipids or fats. They also easily cross the placenta and transfer to newborns through breastfeeding (Cui et al., 2012). Lipid-rich tissues, like the brain, have shown damage following PBDE exposure, possibly due to increased bioaccumulation (Mitchell et al., 2012). In rodents, gestational or postnatal exposure to the most prevalent PBDE congeners (i.e., PBDE 47, 99, 153, and 209) found in human blood (Fischer et al., 2006; Vizcaino et al., 2011) and in the environment (de Wit 2002) has been associated with significant postnatal hyperactivity (Branchi et al., 2002), decreased habituation capacity, and irreversible defects in the cholinergic system (e.g., marked hypoactivity after a nicotine challenge, decreased cholinergic binding sites in the hippocampus) (Eriksson et al., 2002; Goodman 2009). We previously observed substantial BDE-47-induced hyperactivity in offspring from rats exposed to low-dose BDE-47, which is considered the most biologically active PBDE congener and is found commonly in human blood samples across the U.S. and Canada (Suvorov et al., 2009). We have also reported significant changes in gene expression from the brain frontal lobe in the same animals (Suvorov and Takser 2011) occurring 3 weeks [postnatal day (PND) 40] after exposure. Consistent with animal experiments, three epidemiological studies have reported attention deficit and cognitive delay in relation to PBDE levels in blood (Gascon et al., 2012; Lengyel et al., 1985; Roze et al., 2009).

PBDE congeners have the potential to cross the cell membrane and accumulate in cells, particularly in mitochondria (Huang et al., 2010). Mitochondria produce the cellular energy that is critical to brain function. Mitochondrial cytotoxicity following PBDE exposure appears to occur by inhibition of mitochondrial respiration and dissipation of the mitochondrial membrane potential (Pereira et al., 2013). Even small amounts of PBDEs (e.g., 0.02 mM of BDE-49) inhibit electron transport at Complex V and Complex IV in neural mitochondria, as shown both in animal and *in vitro* studies (Napoli et al., 2013), which can affect the brain's energy balance. Despite these findings, the root cause of PBDE-induced mitochondrial toxicity has remained elusive.

Recently, mitochondrial DNA methylation has been identified as a novel epigenetic mechanism with specific sensitivity to environmental exposures (Byun and Baccarelli 2014; Byun et al., 2013). Altered DNA methylation in mitochondria, as in the nuclear genome, leads to dysregulated gene expression (Feng et al., 2012). Given data that suggest that PBDE toxicity in neurodevelopment and brain activity may be mediated by mitochondrial dysfunction, we hypothesized that this dysfunction is driven by epigenetic changes caused

by exposure. In this study, we examined the effects of BDE-47 exposure during perinatal neuronal development on DNA methylation in the frontal lobes of rats. The frontal lobes of the cerebral cortex are involved in complex behavior, cognition, and language (Fuster 2002). Specifically, we measured the methylation of mitochondrial genes involved in respiration, i.e., cytochrome c oxidase I (*Mt-co1*), cytochrome c oxidase II (*Mt-co2*), and cytochrome c oxidase III (*Mt-co3*), in exposed and control rats. Altered cytochrome c oxidase activity has been implicated in cognitive function (Gu et al., 2014), neurodegenerative disease (Griguer et al., 2013) and brain damage (Novgorodov et al., 2014).

The stimuli received by the mitochondria can be transmitted to the nucleus to induce changes in the regulation of nuclear genes (Woodson and Chory 2008), including by epigenetic mechanisms (Smiraglia et al., 2008). Therefore, we also examined DNA methylation in the nuclear genome. We measured nuclear 5-hydroxymethylation (5hmC), an emerging alternative methylation marker, as well as 5mC methylation at the left end of rat L1Rn (long interspersed repeated), a marker of methylation in retrotransposons, the 'jumping' DNA sequences that have been shown to have key roles in neuronal plasticity (Jakovcevski and Akbarian 2012). To further understand neuronal epigenetic PBDE toxicity, we measured changes in the DNA methylation of nuclear candidate genes related to behavioral and brain functions. These included the brain-derived neurotrophic factor (*Bdnf*), corticotropin releasing hormone receptor 1 (*Crhr1*), melanocortin 2 receptor (*Mc2r*), nuclear receptor subfamily 3 group C, member 1 (*Nr3c1*), and alpha-synuclein (*Sncα*).

We observed that perinatal exposure to PBDE affects minor DNA methylation patterns in the brain in both the mitochondrial and nuclear genomes. However, the majority of DNA methylation patterns that we studied were not affected by PBDE exposure in the brain.

1.2 MATERIAL AND METHODS

1.2.1 Animals and Exposure

Seven-week-old Wistar female and male rats were received from Charles River Laboratories and then housed individually, except when breeding, in polypropylene cages (38×48×20) with sawdust bedding and under controlled temperature ($21 \pm 2^\circ\text{C}$), relative humidity ($50 \pm 10\%$), and 12-h light/dark cycle. Food (Rodent CHOW 5075, Charles River's Laboratories, Wilmington, MA) and tap water were provided *ad libitum*. All animals received care in compliance with the Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care, and all experimental protocols were approved by the institutional animal research ethics review board. Breeding pairs were established one week after acclimatization: each female rat was placed with a male overnight and copulation was confirmed in the morning by observation of vaginal plug. The day of detection was assigned as the first day of pregnancy [gestation day (GD) 0]. Dams were randomly assigned to three groups: 0.002 mg/kg body weight (4 dams) or 0.2 mg/kg body weight (5 dams) of neutral standard of 2,2', 4,4'-tetrabromodiphenyl ether (BDE-47, Chromatographic Specialties Inc., Brockville, Canada), or no exposure (control group, 4 dams) (Figure 1). The vehicle was administered to the control group according to the same protocol. We note that the control group also had background BDE-47 level (Suvorov and Takser 2011). A preparation of BDE-47 solution was used for all experiments to provide a consistent vehicle solution (990

μL peanut oil with 10 μL toluene) and administered at 500 μL/body weight each morning via oral gavage at GD 9 and 16 and at PND 1, 8, and 15 through stainless steel needles (Harvard Apparatus Inc., 16×4”). Dams were allowed to deliver and the litter size was not artificially altered. Dams and pups were kept together until weaning on PND 21. At PND 41, one male pup and one female pup per litter were selected randomly and killed by decapitation after stunning as described in the Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care (Canadian Council on Animal Care, 1993). Frontal lobe brain samples from control (4♀+4♂), 0.002 mg/kg (4♀+4♂), and 0.2 mg/kg (5♀+5♂) rats were isolated, snap-frozen in liquid nitrogen, and stored at -80°C until use (Figure 1).

1.2.2 DNA Extraction and Bisulfite Conversion

Genomic DNA was isolated using a Qiagen extraction kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions and kept at -80°C until use. Mitochondrial DNA was extracted from the frontal lobe of rat brains using the FOCUS™ Mitochondria prep kit (G-Biosciences, St. Louis, MO) according to the manufacturer's instructions. One microgram of genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. The final elution was performed with 30 μL of M-Elution Buffer. Bisulfite-treated DNA was aliquoted and stored at -80°C until use.

1.2.3 DNA Methylation Measurement

We developed bisulfite PCR pyrosequencing assays for rat mitochondrial genes (*Mt-co1*, *Mt-co2*, and *Mt-co3*), repetitive elements (L1Rn-ORF1 and L1Rn-5UTR), and nuclear genes (*Bdnf*, *Crhr1*, *Mc2r*, *Nr3c1*, and *Sncα*). Specific regions were chosen for being promoter regions or known regulators of gene expression. Primer sequences, PCR conditions, and genomic locations can be found in Supplemental Table S1. Between one and six CpGs were evaluated in each assay. PCR amplification was performed at standard conditions using the GoTaq® Hot Start Polymerase (Promega, Madison, WI). We used a PSQ Q96 MD Pyrosequencing System (QIAGEN, Valencia, CA), as previously described (Byun et al., 2013). Pyrogram peak patterns from every sample were visually inspected to confirm the quality of the reactions. The Pearson correlation coefficient between technical replication was between 0.44 (*Mt-co1*) and 0.99 (*Crhr1*). Pearson correlation coefficient for L1Rn-5UTR is $r=0.38$, $p=0.05$.

5hmC content was quantified using a MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek, NY, USA) according to the manufacturer's instructions. Briefly, after subtracting the negative control from the sample and the standard, the relative fluorescence units (RFU) value of 5hmC for each sample was calculated as a ratio of sample OD relative to the standard OD as follows: $5hmC\% = [(Sample\ OD - Negative\ control\ OD) / Slope \times Input\ DNA\ amount] \times 100$ (Li and Liu 2011). The Pearson Correlation coefficient between technical replication was 0.87 ($p<0.0001$).

1.2.4 Statistical Analysis

The 5mC data were generated through pyrosequencing, which may provide measures of multiple methylation CpG sites within each gene. We evaluated BDE-47's effect on

methylation at each CpG site as well as the average effect on all CpGs within each gene. To this end, we used mixed-effects regression models by adapting the methods described by Boeke et al. (Boeke et al., 2012). We used linear regression models to assess the effect of BDE-47 treatment (control, 0.002 mg /kg, and 0.2 mg/kg) on 5hmC methylation. We checked regression assumptions by performing diagnostic tests for each model, including the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality of residuals and predicted values graph for assessing heteroscedasticity and presence of outlier values.

All tests were two-sided and a p-value less than 0.05 was considered statistically significant. False discovery rate (FDR) was computed with the Benjamini-Hochberg's method. Average positions were not included in the FDR calculation. Results were considered significant at $FDR < 0.20$. All statistical analyses were performed in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA).

1.3 RESULTS

1.3.1 Effect of Perinatal BDE-47 Exposure on Mitochondrial DNA Methylation

Mitochondria were isolated from the frontal lobe of BDE-47 exposed rats and *Mt-co1* (1 CpG), *Mt-co2* (2 CpGs), and *Mt-co3* (3 CpGs) mitochondrial DNA methylation was measured. Mean methylation levels ranged from 0.74% (*Mt-co3* in position 1 of 0.002 mg/kg BDE-47 dose) to 4.28% (*Mt-co2* in position 1 of Control) (Table 1). One of the CpGs in the *Mt-co2* gene (position 1 in Table 1) showed a significant decrease in methylation at the 0.2 mg/kg BDE-47 dose compared to controls [difference vs. control in %5mC = -0.68, 95% confidence interval (CI) -1.17; -0.19, $p=0.01$, and $FDR=0.08$]. Other positions showed changes that were not statistically significant. The *Mt-co1* gene did not show any change in DNA methylation at the 0.002 mg/kg BDE-47 dose, nor at the 0.2 mg/kg BDE-47 dose (Table 1).

1.3.2 Effect of Perinatal BDE-47 Exposure on Global Nuclear 5hmC Methylation

The mean level of global nuclear 5hmC methylation was 0.48% (95% CI 0.33; 0.63) in controls, 0.38% (95% CI 0.24; 0.53) at the 0.002 mg/kg BDE-47 dose, and 0.69% (95% CI 0.56; 0.82) at the 0.2 mg/kg BDE-47 dose (Table 2). Global 5hmC levels in the 0.2 mg/kg group were non-significantly increased relative to the control group (difference in %5hmC = 0.21, 95% CI -0.02; 0.41, $p=0.08$). The 5hmC level following the 0.002 mg/kg BDE-47 dose was not different from controls (difference in %5hmC = -0.10; 95% CI -0.31; 0.11, $p=0.58$) (Table 2).

1.3.3 Effect of Perinatal BDE-47 Exposure on 5mC Methylation on Repetitive Elements L1Rn

We measured 5mC DNA methylation at two regions of repetitive elements L1Rn: the 5' untranslated region (5'UTR) and open reading frame (ORF) 1. Mean methylation in the L1Rn 5'UTR region was significantly decreased at the 0.002 mg/kg BDE-47 dose compared to controls (difference %5mC = -1.23; 95% CI -2.27; -0.18, $p=0.02$). The L1Rn UTR region did not show significant changes in mean methylation at the 0.2 mg/kg BDE-47 dose relative to controls (difference in %5mC = -0.40; 95% CI -1.40; 0.59, $p=0.41$). L1 ORF1

did not show significant changes in methylation at either dose (difference in %5mC= -0.22; 95% CI -1.03; 0.59, p=0.58 at 0.002 mg/kg, FDR=0.58; and difference in %5mC=0.41; 95% CI -0.36; 1.18, p=0.28, FDR=0.42 at 0.2 mg/kg) (Table 3).

1.3.4 Effect of Perinatal BDE-47 Exposure on Methylation of Neurodevelopment and Stress Nuclear Genes

We examined DNA methylation in genes associated with neuron development and neurotoxicity (three CpGs in *Bdnf* and two CpGs in *Snca*) and stress-related genes (five CpGs in *Crhr1*, two CpGs in *Mc2r*, and six CpGs in *Nr3c1*) in the frontal lobes of rats exposed to BDE-47. Of the three CpGs measured in *Bdnf*, position 2 showed significantly decreased methylation at both BDE-47 doses relative to controls (difference in %5mC= -1.34; 95% CI -2.61; -0.06, p=0.04, FDR=0.45 at 0.002 mg/kg and difference in %5mC= -2.13; 95% CI -3.34; -0.91, p=0.001, FDR=0.02 at 0.2 mg/kg) (Table 4). The average of the three *Bdnf* positions showed a marginal decrease in DNA methylation at the 0.2 mg/kg BDE-47 dose (difference in %5mC= -1.63; 95% CI -3.44; 0.17, p=0.07) (Table 4). Among all the CpG positions in *Nr3c1*, one CpG (position 4 in Table 4) showed a significant increase in DNA methylation at the 0.2 mg/kg BDE-47 dose relative to control (difference in %5mC= 0.41; 95% CI 0.13; 0.68, p=0.01, FDR=0.09); however, *Nr3c1* average methylation was not significantly changed at the same dose (difference in %5mC= 0.26; 95% CI -0.55; 1.07, p=0.50). None of the CpGs in *Nr3c1* showed significant changes in DNA methylation at the 0.002 mg/kg BDE-47 dose (difference in %5mC= 0.35; 95% CI -0.39; 1.08 p=0.32 for the average methylation of the six CpG sites). *Crhr1*, *Mc2r*, and *Snca* did not show any changes in DNA methylation in response to BDE-47 treatment (Table 4).

1.4 DISCUSSION

We investigated changes in DNA methylation in the frontal lobe of rats that were perinatally exposed to BDE-47. The prefrontal cortex of rats treated with BDE-47 showed decreased 5mC levels of the mitochondrially-encoded gene *Mt-co2*; decreased L1Rn 5mC levels, and alterations in DNA methylation in 5mC of nuclear encoded genes related to behavioral and brain functions (*Bdnf* and *Nr3c1*) at some of the CpGs. However, PBDE exposure did not affect the majority of DNA methylation patterns in the brain. These results indicate that perinatal exposure to BDE-47 at low doses comparable to those found in human populations (Suvorov and Takser 2011) does not have significant effects upon brain nuclear and mitochondrial DNA methylation patterns.

PBDE exposure has been shown to increase reactive oxygen species production (Blanco et al., 2014), to which mitochondria are particularly sensitive. Cytochrome c oxidase (COX) has three subunits encoded by mitochondrial DNA, and defects in COX structure can result in severe, and often fatal, metabolic disorders that predominantly affect tissues with high energy demands, such as the brain. We observed decreased methylation of mitochondrial COX-encoding genes with perinatal BDE-47 exposure. Although some regions showed significant and more robust decreases in methylation levels, the methylation changes were subtle.

DNA cytosine methylation is a key marker controlling gene transcription and has been increasingly studied in relation to environmental exposures such as air particles (Byun et al., 2013), metals (Doi et al., 2011), endocrine disruptors (Patel et al., 2013), and persistent organic pollutants (Rusiecki et al., 2009). DNA methylation, particularly 5mC, has been of primary interest for such studies. 5hmC has recently emerged as an alternative and functionally different form of methylation. Unlike 5mC, 5hmC has been associated with actively expressed genes (Chia et al., 2011). Relevant to this study, 5hmC is highly abundant in the brain, which may suggest a role in the epigenetic control of neuronal functions. In our study, perinatal exposures to low dose BDE-47 did not affect global 5hmC levels in the frontal lobe. However, further studies need to be done to understand the gene-specific changes in 5hmC levels.

Of the active repetitive elements in the brain, L1 elements are the most widely spread retrotransposons and constitute almost ~20% of the mammalian genome (Lander et al., 2001). L1 elements have retrotransposable ability in the central nervous system and are active during adult neurogenesis (Permiakov and Tskhovrebova 1988). A substantial number of newly inserted L1 elements in differentiated neurons are observed in the mouse brain *in vivo* (Muotri et al., 2009), and insertions can dramatically impact on neuronal differentiation affecting neural plasticity (Thomas and Muotri 2012). We observed that BDE-47 exposure did not decrease majority of L1 methylation in the frontal lobe of the brain. In a previous study, however, we showed decreased L1 RNA expression in the rat frontal lobe after BDE-47 exposure (Suvorov and Takser 2011). The source of this discrepancy should be further investigated in studies measuring both L1 methylation and expression in the same samples.

BDNF is a member of the nerve growth factor family. BDNF has important functions in the development of the nervous system and in brain plasticity-related processes such as memory, learning (Aid et al., 2007), and drug addiction, and it may play a role in the regulation of stress response (Roth et al., 2009) and in the biology of mood disorder (Aid et al., 2007). Altered brain *Bdnf* DNA methylation has been suggested as a cellular mechanism underlying the persistent cognitive deficits that are associated with the pathophysiology of post-traumatic stress disorder (Roth et al., 2011). Altered BDNF expression has been observed in perinatal hypothyroidism (Abedelhaffez and Hassan 2013). We observed decreased *Bdnf* promoter methylation at some CpGs following BDE-47 exposure, which may underlie the development of cognitive deficits and perinatal hypothyroidism. However, there was no effect of BDE-47 exposure on overall *Bdnf* promoter methylation.

Disruption of the *Nr3c1* gene expression is associated with neurodegeneration in rats (Murphy et al., 2002). A significant correlation between maternal prenatal stress, newborn birth weight, and newborn methylation in the promoter region of the glucocorticoid receptor *Nr3c1* has been observed in humans (Mulligan et al., 2012). Increased DNA methylation of the *Nr3c1* promoter region is also associated with increased stress in rat pups raised by dams exhibiting poor maternal care behaviors (McGowan et al., 2009). Thus, epigenetic alterations of *NR3C1* genes are associated with early neurobehavioral outcomes. In this study, perinatal exposure to BDE-47 was not linked to DNA methylation changes in *Nr3c1* in rat frontal lobe.

One of the limitations of our study, as in most bisulfite conversion-based DNA methylation studies, is that we were not able to discriminate 5hmC from 5mC levels. When we report 5mC levels, it includes a trace amount of 5hmC that technically cannot be distinguished by conventional bisulfite conversion. Another limitation is that we did not investigate gene expression or mitochondrial function with BDE-47 exposure in this study.

In summary, we demonstrated altered mitochondrial and nuclear DNA methylation in the frontal brain of rats treated with low doses of BDE-47. However, significant findings were observed and did not show consistent dose-response relationships. Further studies are needed to substantiate the role of DNA methylation—including both 5mC and 5hmC—as potential mechanism for PBDE toxicity on neuronal activity and development, and to link gene-specific changes in methylation levels to effects upon gene transcription, as well as to mitochondrial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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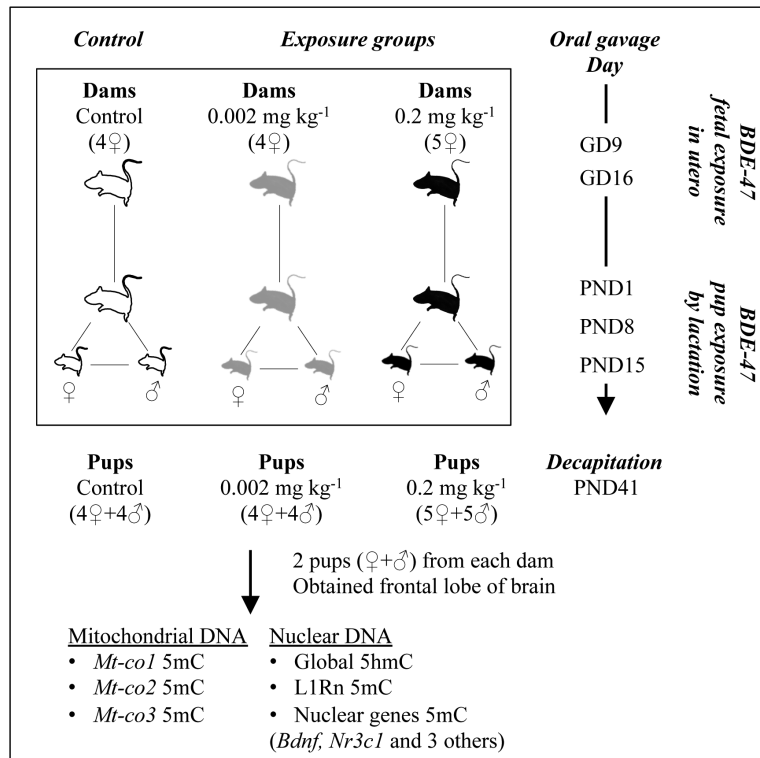


FIGURE 1. Experimental model of BDE-47 exposure in Wistar rats and measurements of DNA methylation

Oral gavage day: the day of BDE-47 exposure on dams by oral gavage, GD: gestational day, PND: postnatal day, ♀: female rat, and ♂: male rat.

TABLE 1

Effect of BDE-47 treatment on DNA methylation in the brain frontal lobe of mitochondrial Cox genes

Gene	CpG position	BDE-47 Dose						
		Control	0.002 mg kg ⁻¹		0.2 mg kg ⁻¹			
		Mean Estimate (95% CI)	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	p	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	p
<i>Mt-co1</i>	<i>Position 1</i>	1.78 (1.47; 2.09)	1.59 (1.28; 1.89)	-0.19 (-0.63; 0.24)	0.37	1.45 (1.17; 1.72)	-0.33 (-0.74; 0.08)	0.11
	<i>Position 1</i>	4.28 (3.91; 4.65)	3.96 (3.59; 4.33)	-0.32 (-0.84; 0.20)	0.21	3.60 (3.27; 3.93)	-0.68 (-1.17; -0.19)	0.01
<i>Mt-co2</i>	<i>Position 2</i>	1.11 (0.89; 1.32)	1.13 (0.92; 1.34)	0.03 (-0.27; 0.32)	0.86	1.20 (1.01; 1.39)	0.09 (-0.19; 0.38)	0.50
	<i>Average</i>	2.96 (2.63; 3.28)	2.83 (2.51; 3.15)	-0.13 (-0.58; 0.33)	0.57	2.61 (2.32; 2.89)	-0.35 (-0.78; 0.08)	0.11
<i>Mt-co3</i>	<i>Position 1</i>	0.85 (0.76; 0.93)	0.74 (0.65; 0.83)	-0.11 (-0.23; 0.02)	0.09	0.76 (0.68; 0.84)	-0.09 (-0.20; 0.03)	0.14
	<i>Position 2</i>	0.92 (0.80; 1.04)	0.76 (0.64; 0.88)	-0.16 (-0.33; 0.01)	0.07	0.84 (0.72; 0.95)	-0.08 (-0.25; 0.08)	0.32
	<i>Position 3</i>	3.70 (3.39; 4.00)	3.45 (3.15; 3.76)	-0.24 (-0.67; 0.19)	0.26	3.61 (3.34; 3.88)	-0.09 (-0.49; 0.32)	0.66
	<i>Average</i>	1.90 (1.74; 2.07)	1.70 (1.54; 1.87)	-0.20 (-0.43; 0.03)	0.09	1.80 (1.65; 1.95)	-0.10 (-0.32; 0.12)	0.35

TABLE 2

Effect of BDE-47 treatment on DNA methylation in the brain frontal lobe of global 5-hydroxymethylcytosine

	BDE-47 Dose					
	Control	0.002 mg kg ⁻¹		0.2 mg kg ⁻¹		p
	Mean Estimate (95% CI)	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	
Global 5hmc	0.48 (0.33; 0.63)	0.38 (0.24; 0.53)	-0.10 (-0.31; 0.11)	0.69 (0.56; 0.82)	0.21 (0.02; 0.41)	0.58

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TABLE 3

Effect of BDE-47 treatment on DNA methylation in the brain frontal lobe of repetitive elements L1Rn (long interspersed repeated)

Gene	CpG position	Control Mean Estimate (95% CI)	BDE-47 Dose					
			0.002 mg kg ⁻¹		0.2 mg kg ⁻¹		Relative to the Control group	
			Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	P	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	P
L1Rn (5'UTR)	<i>Position 1</i>	69.05 (68.43; 69.67)	68.37 (67.75; 68.99)	-0.68 (-1.55; 0.19)	0.12	68.54 (67.99; 69.09)	-0.51 (-1.33; 0.32)	0.22
	<i>Position 2</i>	40.51 (39.44; 41.58)	38.84 (37.77; 39.91)	-1.67 (-3.18; -0.16)	0.03	40.30 (39.34; 41.25)	-0.21 (-1.64; 1.22)	0.76
	<i>Average</i>	54.81 (54.07; 55.55)	53.59 (52.84; 54.33)	-1.23 (-2.27; -0.18)	0.02	54.41 (53.75; 55.07)	-0.40 (-1.40; 0.59)	0.41
L1Rn (ORF1)	<i>Position 1</i>	82.27 (81.69; 82.84)	82.05 (81.48; 82.62)	-0.22 (-1.03; 0.59)	0.58	82.68 (82.17; 83.20)	0.41 (-0.36; 1.18)	0.28

TABLE 4

Effect of BDE-47 treatment on DNA methylation in the brain frontal lobe of candidate nuclear genes

Gene	CpG position	BDE-47 Dose						
		Control	0.002 mg kg ⁻¹			0.2 mg kg ⁻¹		
		Mean Estimate (95% CI)	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	p	Mean Estimate (95% CI)	Relative to the Control group Difference (95% CI)	p
<i>Bdnf</i>	Position 1	2.91 (1.78; 4.04)	1.28 (0.15; 2.41)	-1.62 (-3.22; -0.03)	0.05	1.74 (0.72; 2.77)	-1.16 (-2.69; 0.36)	0.13
	Position 2	3.63 (2.73; 4.53)	2.29 (1.39; 3.19)	-1.34 (-2.61; -0.06)	0.04	1.50 (0.68; 2.31)	-2.13 (-3.34; -0.91)	0.001
	Position 3	10.28 (6.77; 13.80)	9.73 (6.21; 13.24)	-0.56 (-5.52; 4.41)	0.82	7.92 (4.8; 11.04)	-2.36 (-7.06; 2.34)	0.31
	Average	5.76 (4.42; 7.10)	4.49 (3.15; 5.83)	-1.27 (-3.17; 0.63)	0.18	4.13 (2.92; 5.33)	-1.63 (-3.44; 0.17)	0.07
<i>Crrh1</i>	Position 1	1.93 (79.62; 91.72)	3.59 (1.35; 5.82)	1.66 (-1.50; 4.82)	0.29	1.50 (-0.61; 3.60)	-0.43 (-3.50; 2.64)	0.78
	Position 2	1.91 (76.80; 95.52)	1.58 (-0.12; 3.27)	-0.33 (-2.73; 2.07)	0.78	0.31 (-1.29; 1.91)	-1.60 (-3.93; 0.73)	0.17
	Position 3	1.65 (93.05; 98.92)	1.11 (-1.12; 3.34)	-0.54 (-3.70; 2.61)	0.72	1.12 (-0.98; 3.23)	-0.53 (-3.59; 2.53)	0.72
	Position 4	2.68 (1.29; 3.13)	1.92 (0.43; 3.41)	-0.77 (-2.87; 1.34)	0.46	2.57 (1.17; 3.98)	-0.11 (-2.16; 1.94)	0.91
	Position 5	1.73 (0.49; 2.96)	1.09 (-0.14; 2.33)	-0.63 (-2.37; 1.11)	0.46	1.84 (0.68; 3.00)	0.11 (-1.58; 1.81)	0.89
Average	2.21 (1.20; 4.17)	1.62 (0.69; 2.54)	-0.59 (-1.89; 0.71)	0.36	1.59 (0.72; 2.46)	-0.62 (-1.88; 0.65)	0.33	
<i>Mc2r</i>	Position 1	95.99 (-0.58; 3.88)	95.82 (92.89; 98.75)	-0.17 (-4.31; 3.98)	0.93	94.53 (91.77; 97.3)	-1.45 (-5.48; 2.58)	0.46
	Position 2	86.16 (0.21; 3.61)	91.72 (82.36; 101.07)	5.56 (-7.67; 18.79)	0.39	89.50 (80.68; 98.32)	3.34 (-9.52; 16.20)	0.60
	Average	85.67 (-0.31; 4.16)	90.56 (84.50; 96.61)	4.88 (-3.67; 13.44)	0.25	89.74 (84.03; 95.44)	4.06 (-4.25; 12.38)	0.32
<i>Nr3c1</i>	Position 1	0.56 (0.35; 0.77)	0.53 (0.33; 0.72)	-0.03 (-0.32; 0.26)	0.82	0.71 (0.47; 0.95)	0.15 (-0.17; 0.47)	0.34
	Position 2	1.42 (1.19; 1.65)	1.46 (1.25; 1.67)	0.05 (-0.27; 0.36)	0.75	1.64 (1.39; 1.90)	0.23 (-0.12; 0.57)	0.18
	Position 3	1.51 (1.19; 1.83)	1.51 (1.22; 1.81)	0.00 (-0.43; 0.44)	0.99	1.54 (1.18; 1.91)	0.03 (-0.45; 0.52)	0.89
	Position 4	0.18 (0.00; 0.37)	0.17 (0.00; 0.34)	-0.01 (-0.26; 0.24)	0.93	0.59 (0.38; 0.80)	0.41 (0.13; 0.68)	0.01
	Position 5	1.90 (-1.64; 5.44)	3.84 (0.61; 7.08)	1.94 (-2.85; 6.74)	0.39	0.92 (-3.04; 4.88)	-0.98 (-6.29; 4.33)	0.69
	Position 6	1.22 (0.91; 1.53)	1.21 (0.94; 1.49)	0.00 (-0.42; 0.41)	0.98	1.12 (0.78; 1.46)	-0.10 (-0.55; 0.36)	0.66
	Average	0.94 (0.39; 1.48)	1.28 (0.79; 1.78)	0.35 (-0.39; 1.08)	0.32	1.20 (0.59; 1.80)	0.26 (-0.55; 1.07)	0.50
<i>Snca</i>	Position 1	3.73 (-0.19; 7.66)	4.73 (0.54; 8.93)	1.00 (-4.75; 6.74)	0.72	1.84 (-2.38; 6.06)	-1.89 (-7.66; 3.87)	0.50
	Position 2	6.69 (5.69; 7.69)	5.71 (4.64; 6.79)	-0.97 (-2.44; 0.50)	0.18	5.73 (4.72; 6.74)	-0.96 (-2.38; 0.47)	0.17

Gene	CpG position	BDE-47 Dose						
		Control		0.002 mg kg ⁻¹			0.2 mg kg ⁻¹	
		Mean Estimate (95% CI)	Mean Estimate (95% CI)	Relative to the Control group		Mean Estimate (95% CI)	Relative to the Control group	
		Difference (95% CI)	P		Difference (95% CI)	P		
<i>Average</i>		4.77 (2.95; 6.58)	5.48 (3.54; 7.42)	0.71 (-1.95; 3.37)	0.58	4.31 (2.36; 6.25)	-0.46 (-3.12; 2.20)	0.72

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