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Journal

Environmental Epigenetics, 2(1)

ISSN

2058-5888

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Publication Date

2016

DOI

10.1093/eep/dvv012

Peer reviewed

Published in final edited form as:

Environ Epigenet. 2016 March ; 2(1): . doi:10.1093/eep/dvv012.

DNA methylation: a mechanism linking environmental chemical exposures to risk of autism spectrum disorders?

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Abstract

There is now compelling evidence that gene by environment interactions are important in the etiology of autism spectrum disorders (ASDs). However, the mechanisms by which environmental factors interact with genetic susceptibilities to confer individual risk for ASD remain a significant knowledge gap in the field. The epigenome, and in particular DNA methylation, is a critical gene expression regulatory mechanism in normal and pathogenic brain development. DNA methylation can be influenced by environmental factors such as diet, hormones, stress, drugs, or exposure to environmental chemicals, suggesting that environmental factors may contribute to adverse neurodevelopmental outcomes of relevance to ASD via effects on DNA methylation in the developing brain. In this review, we describe epidemiological and experimental evidence implicating altered DNA methylation as a potential mechanism by which environmental chemicals confer risk for ASD, using polychlorinated biphenyls (PCBs), lead, and bisphenol A (BPA) as examples. Understanding how environmental chemical exposures influence DNA methylation and how these epigenetic changes modulate the risk and/or severity of ASD will not only provide mechanistic insight regarding gene-environment interactions of relevance to ASD but may also suggest potential intervention strategies for these and potentially other neurodevelopmental disorders.

Keywords

autism; bisphenol A; epigenetics; lead; neurodevelopment; PCBs

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by core deficits in social communication and interaction, restricted interests, and repetitive patterns of behavior. Symptoms typically present in the first 2 years of life, though there is

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Conflict of interest : None declared.

considerable clinical heterogeneity in severity, comorbidities, and response to treatment [1–3]. According to the autism and developmental disabilities monitoring network of the Centers for Disease Control (CDC), 1 in 68 eight-year-old children is diagnosed with ASD [4]. Although ASD affects both sexes, it is almost five times more common among boys (1 in 42) than girls (1 in 189) [4]. Alarmingly, the incidence of ASD continues to increase. Independent studies have reached the common conclusion that this trend cannot be explained in its entirety by increased awareness, broadening of diagnostic criteria, or improved detection of ASD [5–8]. In fact, these studies suggest that factors other than diagnostic drift likely account for more than one-half of new cases. Given that the economic cost of healthcare, schooling, and caregiver services for a child with ASD are estimated to start at $17, 000 more per year compared with a child without ASD [9], these sobering statistics underscore the need to identify factors that confer risk for ASD.

Evidence Suggesting Environmental Factors Influence ASD Risk

To date, much of the research on ASD etiology has focused on genetic factors [10, 11]. Although ASD is considered one of the most heritable neurodevelopmental disorders [12, 13], single genetic anomalies account for only a small proportion of affected cases [14, 15]. Furthermore, genes linked to ASD rarely segregate in a simple Mendelian manner [12]. These results have been interpreted as an indication that genetic mutations are not necessarily causal but rather act as modifying risk factors that singly or in combination contribute to ASD risk and/or severity. Numerous mechanisms have been proposed to explain how genetic mutations influence ASD, including inheritance of multiple gene variants with small to moderate effects on ASD, rare *de novo* single gene mutations, copy number variants, or alterations in the epigenome [16–21].

An alternative hypothesis that is gaining consensus in the field is that the genetic substrate confers increased susceptibility to environmental factors that interfere with normal neurodevelopment. It is the interaction between genes and the environment that determines individual ASD risk, clinical phenotype, and/or treatment outcome. Evidence supporting environmental contributions to ASD risk include observations of incomplete concordance for autism among monozygotic twins and incomplete penetrance within individuals expressing a given ASD-linked gene mutation, whereby a significant percentage of carriers do not express autistic phenotypes [14, 19, 22]. Two large, independent twin studies that examined the relative contributions of genetic heritability versus the shared environment similarly concluded that environmental factors were more predominant than genetic factors in determining autism risk [23, 24]. A significant role for environmental factors in determining ASD risk is consistent with the clinical heterogeneity that is a hallmark characteristic of these neurodevelopmental disorders and suggests a plausible explanation for the exponential rise in ASD cases over the past several decades.

Diverse environmental factors have been implicated as risk factors for ASD, including maternal stress and drug use, paternal age, nutritional status, hormones, and environmental chemicals [14, 25–29]. In this review, we focus on environmental chemicals. Environmental chemicals that have been implicated as risk factors for ASD include polychlorinated biphenyls (PCBs), lead, bisphenol A (BPA), mercury, and pesticides (Tables 1–2) [52–62].

However, mechanisms by which these environmental factors interact with genetic susceptibilities to confer individual risk for ASD remain largely speculative. Emerging evidence suggests that environmental chemicals can alter DNA methylation patterns in the developing brain, and these reports have led to a prevailing hypothesis in the field that environmental factors confer risk to genetically susceptible individuals via modulation of the developing brain methylome. Here, we review the evidence and the critical gaps in knowledge relevant to this hypothesis. In the following sections, we provide an overview of DNA methylation and its importance in neurodevelopment, then review experimental evidence demonstrating that environmental chemicals hypothesized to confer ASD risk alter the epigenome, specifically DNA methylation, using PCBs, lead, and BPA as examples (Table 1). We conclude with a discussion of the evidence linking effects of environmental chemicals on DNA methylation to increased risk of ASD.

An Overview of DNA Methylation and Its Importance in Neurodevelopment

Epigenetic modifications such as DNA methylation, histone protein modifications, and microRNAs function to regulate the transcriptional potential of a cell without altering its DNA sequence. The establishment, maintenance, and removal of epigenetic marks are critical during neurodevelopment and when disrupted can have significant impacts on neurodevelopment and cognitive function [63–66]. DNA methylation, the focus of this review is one of the most widely studied epigenetic modifications in development and disease, including ASD.

DNA methylation refers to the addition of a methyl group to the 5′ position of cytosine. This typically occurs at regions rich in CpG [67, 68]. DNA methylation is generally associated with transcriptional repression either through direct inhibition of transcription factor binding or the recruitment of methyl CpG binding domain (MBD) proteins, which interact with histone modifiers to confer a repressive chromatin state [69]. DNA methylation is catalysed by the DNA methyltransferase (DNMT) protein family. DNMT1 functions primarily in maintenance of DNA methylation whereas DNMT3A and DNMT3B are primarily involved in *de novo* DNA methylation [69]. Global deletion of mouse *Dnmt1, Dnmt3b,* or both *Dnmt3a* + *Dnmt3b* results in midgestation lethality [gestational day (GD) 9.5–11.5], while deletion of only *Dnmt3a* produces severe growth retardation and lethality by 4 weeks of age [70–72].

Pharmacological approaches and conditional deletion studies confirm roles for *Dnmts* in the developing central nervous system [73, 74]. Conditional deletion of *Dnmt1* in developing excitatory neurons and astroglia of the mouse cortex and hippocampus results in neuronal cell death between GD14.5 to 3 weeks postnatally and results in deficits in learning and memory in adulthood [63]. A fraction of hypomethylated neurons survive postnatally but exhibit increased dendritic branching and impaired excitability, likely through mechanisms related to neuronal layer specification, cell death, and ion channel function [63]. Mice lacking *Dnmt1* and *Dnmt3a* in postmitotic neurons show abnormal long-term plasticity in CA1 hippocampal neurons along with deficits in learning and memory [75]. Additionally, inhibiting DNMT activity increases miniature excitatory postsynaptic currents in cultured cortical neurons, suggesting that DNA methylation regulates glutamatergic synaptic strength

[76]. Together, these studies not only demonstrate the requirement for DNA methylation during neurodevelopment but also suggest that tight spatial and temporal regulation of *Dnmts* is important for activity-dependent synaptic plasticity. The relevance of these observations to ASD is indicated by recent advances in defining the molecular and cellular pathology of ASD that point to altered patterns of neuronal connectivity and synaptic plasticity in the developing brain as the neurobiological substrate underlying these disorders [11, 77, 78].

Epigenetic alterations can be stable and heritable and they can also be malleable and surprisingly dynamic in a spatially and temporally defined manner. The dynamic nature of DNA methylation is especially evident following fertilization in preimplantation embryos when a rapid wave of paternal genome demethylation occurs, followed by reestablishment of DNA methylation patterns to permit embryonic specification in the blastocyst [79, 80]. The ability to alter DNA methylation patterns in a cell- and stage-specific fashion is retained throughout life and is a key component of cell differentiation, specification, and maturation. Altered patterns of DNA methylation are often a hallmark of disease onset and progression [66, 69].

The fact that DNA methylation is malleable suggests that DNA methylation marks can also be removed. Identification of passive and active mechanisms by which DNA methylation marks can be lost has significantly impacted our understanding of transcriptional control of gene expression. Passive mechanisms of DNA demethylation include a reduction or loss of DNMT abundance or activity that reduce DNA methylation during subsequent rounds of DNA replication. In terms of active mechanisms of DNA demethylation, evidence points to MBD2, and even the DNMTs themselves, as having demethylase capability [81–83]; however, this is still controversial and does not preclude the participation of other factors leading to demethylation.

Significantly more is known about removal of DNA methylation through base modification of methylated cytosines followed by base excision and repair pathways [84, 85]. One example is the conversion of methylated cytosine to hydroxymethylcytosine. This is catalysed by members of the 10 eleven translocation (*Tet*) gene family and is the primary mechanism responsible for paternal erasure of DNA methylation during fertilization [84–88]. Hydroxymethylation is found at a relatively high level in neurons compared with other cell types and accumulates over time. Importantly, the *Tet* genes have been implicated in activity-dependent learning and memory [89–91]. DNA hydroxymethylation has also been shown to regulate gene expression in the cerebellum of patients with autism [92]. Our understanding of DNA hydroxymethylation is in its infancy and will no doubt evolve as previously unrecognized mechanisms are discovered, some of which may be important for understanding ASD etiology.

Although the dynamic nature of DNA methylation is necessary for normal development and differentiation, it also renders these events susceptible to modulation by environmental factors such as diet, hormones, stress, drugs, or exposure to environmental chemicals. In the following sections, we discuss: (i) the effects of environmental chemicals implicated as ASD risk factors on DNA methylation; (ii) evidence implicating DNA methylation as a critical

gene expression regulatory mechanism in ASD; and (iii) why environmentally induced changes in DNA methylation may underlie gene by environment interactions that determine individual risk of ASD (see also Tables 1–2).

Effect of Environmental Chemicals on DNA Methylation

Polychlorinated Biphenyls

PCBs are persistent organic pollutants that were initially synthesized in the 1930s for use in industrial mixtures as coolants and lubricants. Despite being banned from production in the 1970s, PCBs remain a current and significant public health risk due to the release of legacy PCBs from aging structures and landfills, and the inadvertent production of contemporary PCBs by industrial processes, primarily commercial paint pigments [93]. Recent studies have documented PCBs levels in excess of Environmental Protection Agency (EPA) standards in indoor air samples from elementary schools in USA [94], and the latest National Health and Nutrition Examination Survey (NHANES) data confirm widespread PCB exposures in women of childbearing age [58].

The weight of evidence from epidemiological studies supports a negative association between developmental exposure to PCBs and neuropsychological function in infancy and childhood [56, 95–99]. Identifying the mechanism(s) by which PCBs interfere with normal neurodevelopment has been confounded by the existence of 209 PCB congeners, which are grouped according to their molecular structure as dioxin-like (DL) and non-dioxin like (NDL). DL PCBs are so named because like dioxin, these congeners bind to and activate the aryl hydrocarbon receptor (AHR); in contrast, NDL PCBs have negligible AHR activity [100]. Although both DL and NDL PCBs are ubiquitous in the environment, recent evidence indicates that NDL PCBs predominate over DL PCBs in environmental samples and human tissues [101–103]. This is of significant concern because data from experimental models suggest that PCB developmental neurotoxicity is mediated predominantly by NDL PCBs [27, 104]. NDL PCBs are thought to disrupt normal neurodevelopment via modulation of signaling by biogenic amines, thyroid hormone, and intracellular calcium during critical windows of brain development [105]. Although PCBs have yet to be causally linked to ASD, several lines of evidence implicate PCBs as risk factors for ASD. First, studies in rodent models have shown that developmental PCB exposure causes deficits in social behavior [106]. Second, NDL PCB congeners modulate dendritic arborization and spine formation [107, 108], and similar changes in neuronal connectivity have been observed in the autistic brain [109, 110]. Third, NDL PCBs have been reported to activate calcium-dependent signaling pathways implicated in the pathogenesis of ASD [27].

PCBs and Altered DNA Methylation

Emerging evidence from *in vitro*, *in vivo,* and epidemiological studies suggest that PCB developmental neurotoxicity may be mediated in part by PCB effects on DNA methylation in the developing brain. Exposure to the NDL congener PCB 153 decreases DNMT activity in preimplantation mouse blastocytes [30], and decreases global DNA methylation levels in the N2A murine neuroblastoma cell line [31]. However, the latter finding may be unique to mouse cell lines since the DNA hypomethylating effects of PCB 153 were not observed in

the human SK-N-AS neuroblastoma cell line [31]. Animal studies also link PCBs to reduced *Dnmt* abundance. *In utero* and lactational exposure to a mixture of 14 NDL + DL PCBs at 1.1 mg/kg/day from GD1 to postnatal day (P) 21 reduced levels of the methyl donor S-adenosylmethionine as well as levels of *Dnmt1*, *3a,* and *3b* to 4, 54, and 17 of control values, respectively, in liver of prepubertal female Sprague-Dawley rats [32]. Similarly, postnatal exposure (P1, 5, 10, 15, and 20) to a 1000 × mixture of AHR agonists detected in human breast milk, including 3 DL PCBs (77, 126, and 169), reduced *Dnmt1* mRNA abundance levels to 32% of controls in the hypothalamus of P21 female Sprague-Dawley rats [33]. Although both of these studies demonstrate reduced *Dnmt* abundance following early life PCB exposure, it should be noted that the PCB mixtures used differed between the studies. The Desaulniers *et al.* [32] study employed a PCB mixture of NDL + DL PCBs comprised predominantly of NDL PCB congeners. In contrast, the Desaulniers *et al.* [33] study used a mixture containing three DL PCBs as well as non-PCB AhR agonists including polychlorinated dibenzodioxins and polychlorinated dibenzofurans. There was no overlap in the PCBs examined between the two studies. Whether the reduction in *Dnmt* mRNA abundance is a consequence of all or just a subset of PCB congeners remains to be determined. Since humans are exposed to complex PCB mixtures, this is an important consideration when analysing DNA methylation following PCB exposures.

PCB-associated changes in DNA methylation have been shown to influence sexual development and alter sex-specific patterns of gene expression in the brain [34]. This is important since many hormones are required for or have significant impacts on neurodevelopment. Indeed, endocrine disruption has been hypothesized to contribute to ASD, in part because ASD is more prevalent in males than females [111–113]. Exposure of Sprague-Dawley rats to Aroclor 1221, a technical mixture of PCBs, at 1 mg/kg on GD16 and GD18 alters gene expression in a sex-specific manner, perturbing reproductive function by delaying time to puberty in males and altering cyclicity of estrous in females [35]. This study also examined impacts of PCB exposure on DNA methylation in the anteroventral periventricular (AVPV) nucleus and arcuate nucleus, which are regions of the brain known to regulate reproductive function. In female rats, PCB exposure increases gene expression profiles from P15–90 such that they are more similar to vehicle-treated male rats [35]. This masculinization pattern is seen in *Dnmt1* expression as well, with PCB treatment increasing expression of *Dnmt1* from P15–90 in the female rat AVPV to levels that are more typical of male expression [35]. The functional consequence of increased AVPV *Dnmt1* transcript abundance in this study is unclear. Although PCB-induced changes in promoter DNA methylation were not detected in two genes upregulated by PCBs, including the androgen receptor (*Ar*), DNA methylation at 4 CpG sites of *Ar* was positively correlated with *Ar* mRNA expression uniquely in the AVPV of females exposed to PCBs versus control females [35]. These results are confounded by the fact that DNA methylation levels for genes expressed in the AVPV at P15 are already low, which could limit levels of detection [35]. Nonetheless, these results indicate that PCB-dependent changes in DNA methylation may impact endocrine function with consequences on gene expression in the brain.

The growing body of evidence from *in vitro* and experimental animal models indicating that PCBs alter the methylome extends to humans. In a cohort of 399 healthy Japanese women, serum levels of NDL PCBs 17, 52, 69, 74, 183, and DL PCB 114 were inversely associated

with global DNA methylation levels in leukocytes [36]. A similar trend was observed in a second population of healthy Koreans for NDL PCB 153, 183, and 187 [37] and in a population of Greenlandic Inuits [38]. Conversely, in a separate study of 524 elderly men and women (70 years of age) living in Uppsala, Sweden, high levels of DL PCBs were associated with global DNA hypermethylation [39]. While there were significant differences in age, geographical location, and lifetime exposure levels to different PCBs between these study populations, these studies raise the possibility that the composition or congener profile of the PCB exposure is an important determinant of the outcome on the methylome [114]. There is evidence to suggest that the DL and NDL PCBs have opposing actions on DNA methylation, with DL PCBs shifting the balance toward DNA hypermethylation, as was observed in the Sweden study [39], and NDL PCBs favoring DNA hypomethylation as observed in the Korean and Inuit studies [36, 38]. This possibility is further supported by reports that 2,3,7,8-tetrachlorodibenzo-p-dioxin induces DNA hypermethylation [30, 115]. Although it is known these NDL and DL PCB congeners act through different signaling pathways, the question of whether they differentially alter DNA methylation remains to be carefully investigated.

Lifetime exposure levels are also likely confounding variables in epidemiological studies. For example, in healthy Koreans, exposure to PCBs exhibits an inverted U-shape dose–response relationship with DNA methylation of the promoter region of the DNA repair gene, O6-methylguanin-DNA methyltransferase [116]. Interestingly, a nonmonotonic dose–response relationship has also been reported for NDL PCB effects on dendritic arborization of cultured rat hippocampal neurons [107] and learning and memory deficits in rats exposed throughout gestation and lactation to NDL PCBs in the maternal diet [117]. This raises the interesting question of whether there may be a link between PCB effects on DNA methylation and PCB effects on neurodevelopmental outcomes of relevance to autism.

Lead

Common sources of lead exposure include paint, household items, air, and water. Children are often exposed to higher levels than adults, with an estimated 535 000 US children aged 1–5 years of age having blood lead levels higher than the reference level set by the CDC [118]. Studies of lead exposure in children provide evidence of impaired executive function and attention [119]. Animal models of developmental lead exposure also indicate changes in behavioral and neurochemical endpoints similar to those seen in children with ASD [119, 120].

Lead and Altered DNA Methylation

There is evidence to implicate DNA methylation as a potential mechanism by which developmental lead exposure alters neurodevelopment and function throughout life [121, 122]. In cultured human embryonic stem cells, physiologically relevant concentrations of lead (0.4–1.9 μM) cause dose-dependent changes in DNA methylation of 1275 genes during neural progenitor differentiation, with the majority displaying DNA hypomethylation [41]. The top hypomethylated genes are involved in neurological system processes, calcium ion import, and actin cytoskeleton arrangement while the top hypermethylated genes belong to

families responsible for calcium ion import and development of neuronal projections [41]. These are pathways that are also dysregulated in ASD [27, 105, 123].

Lead-induced changes in DNA methylation are stage specific, with the greatest number of changes observed in differentiating human embryonic stem cells relative to undifferentiated human embryonic stem cells or neural progenitor cells [41]. Consistent with this evidence of stage-specific changes in DNA methylation, lead exposure also produces differential effects on neurite outgrowth dependent upon the developmental stage at the time of exposure. Lead exposure during neural rosette formation produces shorter neurites and reduces branching compared with controls, whereas lead exposure during later developmental stages increases the number and length of neurites [41]. These findings are important for two reasons: (i) they reveal a sensitive developmental window during which lead exposure produces a greater number of changes in DNA methylation and (ii) they link altered DNA methylation to changes in neuronal morphology. Whether lead-induced changes in DNA methylation are causally linked to effects on neurite morphology has yet to be determined. Additional questions that remain include the functional consequences of lead-induced changes in DNA methylation and neurite morphology in terms of synaptic connectivity or higher orders of function, such as learning and memory or social interactions. Despite these challenges, the observation that lead changes DNA methylation in a human embryonic stem cell line supports the hypothesis that epigenetic mechanisms underlie lead developmental neurotoxicity in humans.

The findings of Senut *et al.* [41] in terms of developmental windows of lead sensitivity are corroborated by experimental animal studies. Analyses of *Dnmt1* expression in rats exposed to lead throughout gestation and lactation versus only during lactation reveal that dose, developmental age during which exposure occurs, and sex influence the lasting impacts of lead on DNMT expression. Exposure to lead (150, 375, 750 ppm) *in utero* and throughout lactation significantly reduced DNMT1 protein abundance by ~25% uniquely in the female P55 Long Evans rat hippocampus [42]. In contrast, lactational exposure only (P1–21) had no effect on DNMT1 abundance in the female P55 hippocampus. However, in the male P55 hippocampus, lactational exposure to lead diminished DNMT1 expression by 18–23% at the lower doses of 150, 375 ppm and enhanced DNMT1 expression by 20% at the highest dose of 750 ppm [42]. Sex- and stage-specific changes are also observed for DNMT3A and methyl CpG binding Protein 2 (MECP2) [42]. A serious caveat with these studies is that the levels of lead used are not relevant to most human lead exposures. However, consistent with these findings, developmental exposure to more physiologically relevant levels of lead (3, 30 ppm) has been reported to cause differential DNA methylation in male versus female cortex and hippocampus of young adult mice [43]. In this study, developmental lead exposure (3, 30 ppm) resulted in over 1000 differentially methylated CpG sites, predominantly DNA hypermethylation, in regions corresponding to 117 unique genes in the adult female mouse hippocampus whereas no changes were observed in male mice [43]. Importantly, differential DNA methylation is retained when blood levels of lead from developmentally exposed animals have returned to levels of unexposed control animals [43]. Thus, developmental exposure to lead is sufficient to induce persistent changes in DNA methylation in a sex- and brain region-specific fashion, with female mice showing greater changes than male mice, and hippocampus showing greater changes than cortex. The functional consequence of lead-

induced DNA hypermethylation in this context has yet to be defined but may account for changes in gene expression important for synapse and memory formation [43].

Epidemiological studies also suggest that lead can induce changes in DNA methylation [44, 46, 124]. In men, patellar lead levels are inversely associated with global *LINE-1* DNA methylation levels in blood [44]. Similarly, maternal lead levels are inversely correlated with genomic DNA methylation of the *LINE-1* element in umbilical cord blood [45]. Gene-specific alterations in DNA methylation are also linked to lead exposure. In a study of adult men exposed to lead, those with the highest blood lead levels had complete DNA methylation of the *p16* gene, a tumor suppressor gene involved in neurodegeneration [125]. In contrast, men with lower lead levels had partial to no DNA methylation of the *p16* gene [46]. Thus, *p16* DNA methylation may serve as a biomarker of lead exposure. These data also raise the intriguing hypothesis that changes in *p16* expression in the brain may contribute to neurodevelopmental and/or neurodegenerative effects of lead. Together, these observations support the possibility that lead-induced changes in DNA methylation may play a role in developmental neurodevelopmental disorders, possibly by altering genes important for calcium ion import, neuron projection development, actin cytoskeletal arrangement, and neurodegeneration.

BPA

BPA is found in household plastics and other products, including food and beverage cans [126]. According to 2003–04 NHANES data, detectable levels of urinary BPA were found in over 92% of people 6 years of age or older sampled in USA [127]. Alarmingly, levels were highest among children [127]. BPA is thought to be an endocrine disruptor that acts as an estrogen mimetic [128–131], thus research has focused largely on its effects in reproductive tissues. However, BPA exposure has also been linked to effects in the developing brain, including altered synapse formation and abnormalities in neurite and dendrite morphology [132], and it is associated with cognitive and social impairments in rodents [48, 133–135]. In a cohort of 198 children ages 3–5, high levels of maternal BPA were associated with altered emotional reactivity including increased aggressive behavior in boys [136]. Furthermore, in a recent report of 46 children with ASD and 52 age-matched neurotypical control children, total urine BPA concentrations were higher in children with ASD compared with controls [137].

BPA and Altered DNA Methylation

Epigenetic alterations have been implicated in BPA-associated changes in pathology and function in several hormone-responsive tissues including the brain [49, 130, 138–141]. In embryonic hypothalamic mouse cell lines, BPA (200 μM) decreases *Dnmt1* and *3a* expression by ~30% but increases *Dnmt3b* abundance nearly 2-fold relative to control levels [47]. This is an interesting observation considering micromolar concentrations of BPA are also capable of decreasing synaptic density in cultured rat hypothalamic neurons [142]. Whether these two observations are causally linked is unknown but raises the intriguing hypothesis that DNA methylation mediates the effects of BPA on neuronal connectivity.

In mouse models, gestational BPA exposure (1.25 mg/kg in the maternal diet; resulting in 5 micrograms of BPA ingested daily) selectively decreased *Dnmt1* abundance in the GD18 female mouse brain while male levels were unaltered [48]. Importantly, this exposure produced blood BPA levels within the range detected in humans. Changes in *Dnmt1* abundance do not necessarily lead to changes in global or gene-specific DNA methylation but this question was not examined in this study. However, under this experimental paradigm, BPA exposure increased expression of the glutamate transporter *Scl1a1* in female but not male brain at GD18 [48]. Gestational exposure to BPA also increased sex-dependent changes in social interaction, uniquely increasing social interaction among juvenile female mice [48]. Whether changes in *Slc1a1* DNA methylation drive changes in protein expression and/or behavior can not be concluded from these studies but raises the possibility. An interesting observation in this study is that BPA uniquely impacted *Dnmt3a* expression in female but not male mouse brain, but unlike its effects on *Dnmt1*, BPA prevented female-specific reduction in *Dnmt3a* expression [48]. Thus, in the female GD18 brain, BPA exposure resulted in *Dnmt3a* expression typical of male mice. This observation is consistent with BPA acting as an endocrine disruptor and is also reminiscent of masculinization phenotypes observed with PCB exposure as mentioned earlier [35]. The consequences of increased *Dnmt1* but decreased *Dnmt3a* expression are not known but are likely gene, tissue, and stage specific.

Although not conclusive, these are among the first studies to provide evidence that there may be sex-dependent differences in sensitivity to BPA during brain development that translate to altered *Dnmt* gene expression and behavior in juvenile animals. Subsequent studies support this possibility. *In utero* exposure to BPA was shown to cause sex-, dose-, and brain region-specific changes in *Dnmt* expression [49]. *In utero* BPA exposure (2, 20, 200 μg/kg/day) significantly decreased *Dnmt1* expression in the prefrontal cortex of both male and female mice. This same exposure paradigm produced a nonmonotonic dose response for *Dnmt1* expression in the hypothalamus and *Dnmt3a* expression in the prefrontal cortex. Interestingly, a U-shaped dose–response relationship was observed in female mice, whereas an inverted U-shaped dose–response relationship was observed in male mice [49].

BPA exposure also alters social exploratory and anxiety-like behaviors in young adult mice (P30–70) by disrupting sexually dimorphic behaviors. Exposure to BPA reduced chasing behavior in males to levels similar to that of females and reversed the sex-dependent differences in open field behavior in distance traveled and inner area time such that each parameter was reduced in females and increased in male mice [49]. High doses of BPA are also associated with increasing aggressive behavior [49]. These sexually dimorphic changes were linked to BPA-induced alterations in DNA methylation and expression of estrogen Receptor 1 in the brain [49]. Why this study observed unique changes in male and female mice while the earlier study [48] only observed alterations in female mice is likely due to differences in BPA administration (dietary versus oral dosing), strain of mice used (C57Bl/6 versus BALB/c), the developmental ages examined, and the endpoints measured. However, both studies are consistent in demonstrating that *in utero* exposure to BPA has epigenetic effects on the brain that are associated with permanent sex-dependent differences in *Dnmt* expression and behavior in mice. Considering ASD is more prevalent in boys than girls, examining mechanisms underlying sex-dependent differences in *Dnmts* and DNA

methylation are warranted. These studies further confirm a nonmonotonic dose–response relationship in regard to changes in *Dnmt* expression and mouse social behavior, important points to consider when conducting and analysing these types of studies.

Epigenetic "Memory" of Past Environmental Exposures

Environmental exposures have been linked to epigenetic mechanisms of transgenerational changes in gene expression and behavior. Transgenerational inheritance is considered a permanent alteration in the epigenome of the germ line that results in heritable transmission [143]. Evidence of transgenerational effects of environmental chemical exposures that are relevant to neurocognitive function come from studies using BPA and vinclozolin, an endocrine disruptor with antiandrogenic effects. One study examined mate preference in rats, a task that relies on multiple brain regions including amygdala, hippocampus, olfactory bulb, cingulated cortex, entorhinal cortex, and preoptic area-anterior hypothalamus [144, 145]. Third generation female (F3) descendents of rats exposed to vehicle or vinclozolin (100 mg/kg) from GD8–14, preferred F3 vehicle lineage male rats versus F3 vinclozolin lineage male rats, suggesting differential mate preference [144]. F3 vinclozolin lineage male and female rats exhibited sexually dimorphic disruption of transcription in the hippocampus and amygdala, including changes in pathways involved in axon guidance and long-term potentiation [146]. Since these brain regions are associated with learning, memory, and anxiety, it is not surprising that vinclozolin transgenerational exposure is also linked to behavior. F3 vinclozolin male rats displayed a decrease in anxiety-like behaviors while F3 vinclozolin female rats exhibited an increase in anxiety-like behaviors [146]. Thus, epigenetic reprogramming of the germline by environmental exposures can alter the brain transcriptome and influence behavior.

In utero BPA exposure has also been implicated in transgenerational effects on rodent brain development and behavior. In one study, compared with controls, F3 juvenile mice from the BPA exposed line (5 mg/kg diet) showed increased locomoter activity in the open field test and increased investigation of a stimulus mouse upon subsequent trials [147]. Despite intact olfactory senses, F3 mice from the BPA lineage did not become habituated to a familiar stimulus mouse and did not switch their interaction preference after the introduction of a novel mouse [147]. Reduced expression of estrogen receptor, oxytocin, and vasopressin in the brain were observed and postulated to underlie the deficits in behavior of mice in the BPA lineages [148]. Together, these results suggest that BPA exposure has transgenerational effects on brain transcript abundance and social recognition tasks in mice.

Neuroanatomic consequences have also been linked to transgenerational epigenetic reprogramming and altered learning and memory. Female F2 descendents of mice exposed to BPA (1, 10 mg/kg) on GD6–17 displayed a decreased number of newly generated hippocampal cells compared with vehicle lines [149]. This change was associated with deficits in learning and memory. Although Morris water maze testing did not reveal significant differences between treatment groups, F2 mice of the BPA lineage did exhibit reduced cross over latency in passive avoidance testing, suggesting impaired ability to remember past foot shock [149]. These mice also displayed deficits in brain-derived neurotrophic factor (BDNF), phosphorylated cAMP response element binding protein (p-

CREB) and phosphorylated extracellular signal-regulated kinase, which were accompanied by changes in DNA methylation of the CREB regulated transcription factor coactivator 1 gene [149]. These data are important because they establish the link between environmental exposures and transgenerational impacts on the brain transcriptome coincident with altered behavior.

The observation that effects of environmental exposures can be transgenerationally inherited via the germline epigenome further strengthens the hypothesis that the epigenome mediates the effects of gene by environment interactions on adverse neurodevelopmental outcomes of relevance to ASD. Further, it suggests the possibility that autism risk can change over generations. Understanding the complex epigenetic changes occurring in animal models will undoubtedly shed light on the etiology of brain development and ASD.

DNA Methylation Changes Observed in ASD

Several genetic disorders with high penetrance of ASD, including Rett, Fragile-X, Prader-Willi, and Angelman syndromes, result from alterations in genes involved in epigenetic modifications. For example, Rett syndrome is associated with mutations in the *MECP2* [150, 151]. Independent of specific genetic mutations, changes in global DNA methylation and *DNMT* expression have also been observed in patients with ASD. In the cerebellum of autistic patients, *DNMT1, 3A,* and *3B* expression are elevated compared with neurotypical controls [92, 152], which aligns with findings of increased global DNA methylation and hydroxymethylation in these patients [92, 152]. Additionally, there are numerous reports linking changes in DNA methylation to altered gene expression in patients with ASD versus neurotypical controls. Some examples are highlighted later and readers are referred to recent reviews on the topic [65, 151, 153–155].

Altered DNA methylation has been linked to reduced expression of genes in the GABAergic inhibitory system, a neurotransmitter system implicated in the pathophysiology of ASD [156]. Two examples include glutamate decarboxylase 67 (*GAD1*), which decarboxylates glutamate to form gamma-aminobutyric acid (GABA), and reelin, a gene expressed in GABAergic neurons that functions in neural migration and cortical lamination during development [157]. Both genes are reduced in patients with ASD relative to neurotypical controls and are associated with changes in DNA methylation and hydroxymethylation marks within the promoter region leading to MECP2-dependent repression [158].

Imbalances in synaptic connectivity have also been posited as a mechanism underlying ASD pathogenesis [77, 159] and may provide a biological substrate for enhanced susceptibility to environmental factors [27, 77, 159]. The synaptic protein SH3 and multiple repeat domains 3, SHANK3, is a postsynaptic scaffolding protein of excitatory glutamatergic synapses. Translocation and breakpoint mutations in SHANK3 have been consistently implicated in developmental delays and ASD [160]. *SHANK3* expression in brain and other tissues is regulated by DNA methylation [161, 162], and increased levels of *SHANK3* DNA methylation, corresponding to decreased isoform-specific expression of SHANK3, have been observed in postmortem brain tissues of ASD patients compared with neurotypical control tissues [162].

Neonatal levels of *BDNF*, a critically important gene in neural development, neuronal connectivity, and activity-dependent synaptic plasticity [50, 163], are reduced in children later diagnosed with ASD compared with age-matched controls [164]. However, when examined in older children (4 and 11 years of age), serum levels of BDNF are elevated in children with ASD versus neurotypical controls [165, 166]. The reason for the discrepancy in these two findings is unknown but may be specific to the developmental stage examined. *BDNF* transcription is regulated by DNA methylation [167, 168] and altered patterns of *BDNF* DNA methylation has been found in patients with cognitive impairments [167, 169, 170]. Whether changes in *BDNF* DNA methylation contribute to altered *BDNF* expression observed in ASD patients has yet to be determined.

DNA methylation is complex and not always directly associated with decreased gene expression. For example, overexpression of engrailed 2 (*EN2*), another gene implicated in autism, is associated with DNA hypermethylation in the cerebellum of ASD patients [171]. Although seemingly counterintuitive, follow-up studies to distinguish DNA methylation from hydroxymethylation, confirmed elevated *EN2* DNA hydroxymethylation in ASD cerebellum relative to controls [92]. The authors further showed that repressive MECP2 binding was reduced in areas of DNA hydroxymethylation, likely due to MECP2's lower affinity for DNA hydroxymethylation versus DNA methylation [172]. This observation provides a plausible mechanism for the elevated *EN2* expression and increased DNA methyl marks in ASD cerebellum. These results are important because they highlight the complex interaction between DNA methyl marks and gene expression and serve as a reminder that elevated DNA methylation is not necessarily inconsistent with elevated gene expression.

Studies of monozygotic twins provide additional evidence that epigenetic mechanisms play a role in ASD etiology [21, 173]. Among 50 pairs of disease discordant monozygotic twins, several genes were found to be differentially methylated between the twin diagnosed with ASD and the nonsymptomatic twin, including genes previously implicated in ASD pathology such as *GABRB3*, *AFF2*, *NLGN2*, *JMJD1C*, *SNRPN*, *SNURF*, *UBE3A,* and *KCNJ10* [21]. Further, there were significant DNA methylation differences between autistic twin pairs discordant for autistic traits (social, restrictive repetitive behaviors and interests, and communication) [21]. The changes in DNA methylation at differentially methylated CpG sites also correlated with total childhood autism symptoms test scores [21]. Together, these studies support a role for epigenetic mechanisms, and in particular, DNA methylation, in determining ASD susceptibility and raise new questions as to how environmentally mediated changes in the epigenome contribute to autism etiology.

DNA Methylation: Bridging the Gap between Environmental Exposure and ASD Susceptibility

In the sections earlier, we highlighted evidence demonstrating that: (i) environmental factors contribute to determining individual ASD risk and/or severity; (ii) developmental exposures to environmental chemicals can alter DNA methylation in multiple tissues, including the brain; and (iii) changes in DNA methylation have been documented in autistic individuals and implicated in ASD pathogenesis. The question remaining is whether these events are

causally linked. Currently, evidence pointing to changes in DNA methylation as a mechanism by which environmental chemicals contribute to ASD risk is limited (Table 2) but the few studies that have addressed this question have potentially significant implications regarding the importance of environmental epigenetics in the etiology of ASD. Perhaps most intriguing are recent data suggesting a link between PCB exposure, DNA methylation, and autism risk. The goal of this study [40] was to quantify levels of specific PCB and polybrominated diphenyl ether (PBDE) congeners in postmortem brain tissues from neurotypic controls versus patients with autism of unknown etiology and autistic patients comorbid for other neurodevelopmental disorders with a known genetic cause such as maternal Chromosome 15 q11–q13 duplication (15q duplication). Of the eight PCB congeners examined, the only environmental chemical that varied significantly between groups was the NDL congener PCB 95. 15q duplication was the strongest predictor of PCB 95 exposure and these individuals also exhibited DNA hypomethylation of the *LINE-1* element [40]. Although it has yet to be determined whether there is a causal relationship between PCB 95 exposure and 15q duplication, and if so the nature of the relationship (e.g. did the PCB 95 exposure increase the risk of 15q duplication or did the genetic anomaly contribute to increased accumulation of PCB 95 in the brain), these findings are consistent with the hypothesis that complex genetic, epigenetic, and environmental factors interact to determine risk for autism. They further support the possibility that the epigenome may be a convergence point for effects of environmental neurotoxicants like PCBs on genes that confer susceptibility for ASD or other neurodevelopmental disorders.

In animal models, *in utero* exposure to BPA (200 μg/kg/day) produces sex-dependent alterations in DNA methylation and expression of mouse hippocampal genes [50]. Exposure to BPA increased hippocampal expression of *Bdnf* in female P28 mice but decreased it in male mice, and these effects persisted to at least P60 [50]. Concurrently, changes in *Bdnf* expression were associated with sex-specific changes in DNA methylation driven by male-induced hypermethylation of a CpG site within the *Bdnf* promoter [50]. BPA-induced changes in hippocampal gene expression and DNA methylation were accompanied by decreased exploration of a novel object [50], an endpoint used to indicate deficits in learning and memory. BPA-induced changes in *BDNF* DNA methylation are also observed in humans. *BDNF* DNA methylation is higher in cord blood from boys whose mother had higher levels of BPA during pregnancy [50]. Intriguingly, these boys at 3–5 years of age displayed increased aggressive behavior and their emotionally reactive symptom scores were 1.62 times higher compared with boys with low prenatal BPA concentrations [136]. Thus, *BDNF* DNA methylation may serve as a biomarker for BPA exposure, and potentially as an indicator of behavioral deficits in children [50]. These results corroborate findings that total BPA concentrations are positively associated with *LINE-1* global DNA methylation in human placenta [51].

Together, these results link exposure to the environmental chemicals PCBs and BPA to changes in DNA methylation, gene expression, and behavior. Whether these events are causally linked is unknown but future studies aimed at addressing this important question are warranted.

Challenges for the Future

The studies highlighted in this review identify a common theme: developmental exposures to environmental chemicals decrease *Dnmt* expression or decrease global DNA methylation levels (Table 1). This suggests two possible mechanisms by which environmental chemicals change DNA methylation: (i) altering *Dnmt* expression or activity or (ii) altering DNA base modifications and repair mechanisms known to participate in reducing DNA methylation. Since these processes themselves are not completely understood, how environmental chemicals produce these changes remains a significant knowledge gap in the field. These changes likely occur in a sex-, stage,- and gene-specific fashion providing a further challenge to understanding the functional consequences of the full battery of epigenetic changes elicited by environmental exposures during neurodevelopment.

The importance of addressing the impact of environmentally induced changes in the methylome on neurodevelopmental outcomes is heightened by the observation that the directional change in DNMT expression/DNA methylation upon exposure to environmental chemicals is not always consistent with that observed in ASD patients. These discrepancies highlight the necessity for moving away from assessment of global methylation toward assessment of gene-specific changes. Addressing these questions will be challenging, in part because of limitations in the tools currently available to address these questions. Pharmacological inhibitors of DNA methylation are available but lack gene or cell type specificity and can have off-target effects. Genetically modified animals that enable conditional deletion of *Dnmts* are available and have proven invaluable for understanding the role of *Dnmts* in a cell type and developmental stage-specific fashion; however, they do not provide the ability to alter DNA methylation in a gene-specific fashion. These limitations notwithstanding, studies examining the effects of environmental chemical exposures in these genetically modified animals would likely provide useful insights. Additionally, extending environmental epigenetic studies focused on neurodevelopmental outcomes to animal models such as guinea pig and nonhuman primates with primarily postnatal brain development will be important for addressing issues related to species differences in prenatal versus postnatal brain development [174, 175]. Finally, future epidemiological studies focused on environmental exposures, global DNA methylation, gene-specific DNA methylation in the brain, and ASD severity in cohorts of ASD patients versus neurotypic controls are needed. As the field of epigenetics continues to grow, integration of new techniques with proven approaches will no doubt enhance our understanding of epigenetic mechanisms underlying gene by environment interactions in ASD.

As indicated earlier, a critical knowledge gap is the paucity of evidence indicating whether environmental chemical effects on DNA methylation target genes specifically implicated in ASD. In other words, are DNA methylation changes induced by developmental exposures to environmental chemicals causally linked to adverse neurodevelopmental outcomes via altered expression of ASD susceptibility genes? Additionally, with a heterogeneous disease like ASD, how is the degree of impairment determined? This is an important area of future study with clinical significance. Finally, it is important to remember that differential DNA methylation is only one of a number of epigenetic mechanisms that may play a role in determining ASD risk.

Conclusion

The epigenome may mediate effects of environmental risk factors on the developing brain, especially during developmental stages when epigenetic patterns are being established. These early life perturbations can have lasting impacts on gene expression and behavior and, thus, provide a plausible mechanism by which environmental factors converge on existing genetic mutations to determine the risk and severity of ASD.

The malleability of the epigenome is both negative, in that it increases susceptibility to the neurotoxic effects of environmental chemicals, and positive, in that the very fact that it can be modulated raises opportunities for therapeutic interventions. On the other hand, the dynamic nature of the epigenome suggests that each individual likely has a unique combination of epigenetic marks based on timing of exposures, frequency and dose of exposure, and the combination of environmental exposures, which in turn interacts with the individual's unique genetic substrate. This makes an approach to reverse abnormal epigenetic marks very difficult and would likely manifest in a heterogeneous population response to any given therapeutic strategy. Nonetheless, one such approach has been to intervene with DNA methylation through modifying the availability of methyl donors in the diet. Folic acid along with methionine, choline, and others are essential methyl donors in the reaction catalysed by *Dnmts* to add methyl groups to DNA. Therefore, by altering levels of available methyl donors, changes in DNA methylation can be studied along with their downstream consequences. The use of diet in modulating ASD pathogenesis is an active area of research and readers are referred to reviews on the topic [153, 176]. The fact that chemical exposures are more readily controlled than genetic factors to prevent or mitigate deleterious traits related to neurodevelopmental disease, coupled with the fact that the epigenome is malleable, underscore the relevance, and potentially significant impact of investigating epigenetic mechanisms of environmentally induced adverse neurodevelopmental outcomes in ASD.

Acknowledgments

The authors thank Dr Chad Vezina (University of Wisconsin-Madison) for comments on earlier versions of the article. This work was supported by the National Institutes of Health (grants ES014901, ES011269 to P.J.L.) and the US Environmental Protection Agency (grant R833292). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the United States Environmental Protection Agency.

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Table 1

summary of major studies included in this review implicating DNA methylation as a target of environmental chemicals

Publication	Environmental chemical	Exposure period	Effect on DNA methylation
Wu *et al.* [30]	PCB 153	Preimplantation mouse blastocytes	Decreases DNMT activity.
Bastos Sales *et al.* [31]	PCB 153	N2A mouse and human SK-N-AS neuroblastoma cell line	Decreases global DNA methylation in N2A cell line.
Desaulniers *et al.* [32]	PCBs 52, 99, 101, 128, 138, 153, 170, 180, 183, 187, 28, 105, 118, 156	Rats; *in utero* and lactational	Reduces *Dnmt1*, *Dnmt3a,* and *Dnmt3b* expression in liver of female offspring.
Desaulniers *et al.* [33]	PCBs 77, 126, 169, and a mixture of PCDD and PCDF	Rats; P1, 5, 10, 15, 20	Reduces *Dnmt1* abundance in hypothalamus of female rats.
Matsumoto *et al.* [34]	Hydroxy metabolites of PCB 30 and 61	Red-eared slider turtle; developing embryos	Prevents female gonad loss of aromatase promoter DNA methylation under female producing incubation temperature.
Walker *et al.* [35]	Arochlor 1221	Rats; GD16 and 18	Increases *Dnmt1* expression in female rat AVPV nucleus similar to levels observed in vehicle-treated male rats.
Itoh *et al.* [36]	PCBs 17, 52, 69, 74, 183, 114	399 Japanese women	Serum levels of PCBs are inversely associated with global DNA methylation levels.
Kim *et al.* [37]	PCBs 153, 183, 187	86 Koreans	Serum levels of PCBs are inversely associated with global DNA methylation levels.
Rusiecki *et al.* [38]	PCBs 28, 52, 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187	70 Greenlandic Inuit	Serum levels of PCBs are inversely associated with global DNA methylation levels.
Lind *et al.* [39]	PCB 126	524 elderly Swedish	Serum levels associated with global DNA hypermethylation.
Mitchell *et al.* [40]	PCB 95	107 postmortem human brain tissues	15q duplication was the strongest predictor of PCB 95 exposure and these individuals also exhibited DNA hypomethylation of the LINE-1 element.
Senut *et al.* [41]	Lead	Human embryonic stem cells	Stage and dose-dependent changes in DNA methylation of genes during neural progenitor differentiation, with the majority displaying DNA hypomethylation.
Schneider *et al.* [42]	Lead	Rats; *in utero* and lactational	Reduces *Dnmt1* abundance in adult female rat hippocampus.
Schneider *et al.* [42]	Lead	Rats; lactational exposure	No change in *Dnmt1* abundance in adult female rat hippocampus but *Dnmt1* is diminished in male rat hippocampus at low lead doses (150, 375 ppm) and increased at high doses (750 ppm).
Sanchez-Martin *et al.* [43]	Lead	Mice; *in utero* and lactational	Produces 1000 differentially methylated CpG sites—predominantly DNA hypermethylation—in regions corresponding to 117 unique genes in the female mouse hippocampus.
Wright *et al.* [44]	Lead	517 men in normative aging study	Patellar lead levels are inversely associated with global *LINE-1* DNA methylation levels in blood.
Pilsner *et al.* [45]	Lead	103 Mexican women	Maternal lead levels are inversely correlated with genomic DNA methylation of the *LINE-1* element in umbilical cord blood.
Kovatsi *et al.* [46]	Lead	19 individuals	Highest blood lead levels had complete DNA methylation of the *p16* gene, a tumor suppressor gene also involved in neurodegeneration while those with lower lead levels had partial to no DNA methylation of the *p16* gene.

Publication	Environmental chemical	Exposure period	Effect on DNA methylation
Warita *et al.* [47]	BPA	Embryonic hypothalamic mouse cell lines	Decreases *Dnmt1* and *3a* expression but increases *Dnmt3b* abundance relative to control levels.
Wolstenholme *et al.* [48]	BPA	Mice; gestational	Selectively decreases *Dnmt1* abundance in the GD18 female mouse brain while male levels are unaltered.
Kundakovic *et al.* [49]	BPA	Mice; *in utero*	Decreases *Dnmt1* expression in prefrontal cortex in both male and female mice. Produces a nonmonotonic dose response for *Dnmt1* expression in the hypothalamus and *Dnmt3a* expression in the prefrontal cortex with a U-shaped dose–response relationship observed with females whereas an inverted U-shaped dose–response relationship is observed with male mice. Also alters DNA methylation of estrogen Receptor 1 in the brain.
Kundakovic *et al.* [50]	BPA	Mice; *in utero* Human; cord blood	Induces BDNF hypermethylation in blood and brain of male mice. BDNF DNA methylation is higher in cord blood from boys whose mother had higher levels of BPA during pregnancy, suggesting BDNF DNA methylation may act as a biomarker for BPA exposure and potentially as an indicator of altered neurodevelopment.
Nahar *et al.* [51]	BPA	36 human fetuses	BPA concentrations are positively associated with *LINE-1* global DNA methylation in human placenta.

Abbreviations: BPA, Bisphenol A; BDNF, Brain-derived neurotrophic factor; DNMT, DNA methyltransferase; PCBs, Polychlorinated biphenyls; PCDD, Polychlorinated dibenzodioxins; PCDF, Polychlorinated dibenzofurans.

Table 2

summary of major studies included in this review linking altered DNA methylation as a result of environmental chemicals to ASD relevant endpoints

Publication	Environmental chemical	Exposure period	Environment + DNA methylation + ASD link
Mitchell *et al.* [40]	PCB 95	107 postmortem human brain tissues	Patients with 15q duplication, a genetic determinant of autism, predicts PCB 95 exposure and these patients exhibit DNA hypomethylation of the *LINE-1* element.
Senut *et al.* [41]	Lead	Human embryonic stem cells	Lead-induced changes in DNA methylation occur in genes involved in calcium ion import and actin cytoskeleton arrangement-pathways altered in ASD. Also link changes in DNA methylation to altered neuronal morphology.
Sanchez-Martin *et al.* [43]	Lead	Mice; *in utero* and lactational	Lead induced differential CpG methylation sites—predominantly DNA hypermethylation—in regions corresponding to genes implicated in ASD which function in neurogenesis, memory formation, neurite outgrowth, and axon formation.
Wolstenholme *et al.* [48]	BPA	Mice; gestational	BPA-induced changes in DNA methylation and gene expression are linked to altered social behavior—an endpoint with face validity to ASD.
Kundakovic *et al.* [49]	BPA	Mice; *in utero*	BPA-induced changes in *Dnmt* expression and DNA methylation are linked to altered social exploratory and anxiety-like behaviors in young adult mice by disrupting sexually dimorphic behaviors.
Kundakovic *et al.* [50]	BPA	Mice; *in utero* Human; cord blood	BPA-induced changes in BDNF DNA methylation and expression are linked to decreased novel object recognition in mice, indicating deficits in memory formation.

Abbreviations: BPA, Bisphenol A; BDNF, Brain-derived neurotrophic factor; DNMT, DNA methyltransferase; PCBs, Polychlorinated biphenyls.