CHAPTER 21

NEURODEVELOPMENTAL TOXICOLOGY AND AUTISM SPECTRUM DISORDERS

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21.1 INTRODUCTION

This chapter discusses the evidence that neonatal exposure to environmental chemicals of concern to human health including metals (e.g., mercury, lead, arsenic, and cadmium), and organohalogens (e.g., polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), may play a role in the etiology of neurodevelopmental disorders such as autism and may contribute to the largely unexplained rise in the number of children diagnosed with autism worldwide [1–3]. A description of the major features of autism is followed by a discussion of research on the effects of neonatal exposure to common persistent toxicants on neurodevelopment and the possible contribution of such exposure to the etiology of autism. Major animal models used to model specific aspects of autism are also discussed.

21.2 AUTISM

Autism spectrum disorders (ASD) have been characterized as a spectrum of disorders in brain development that manifest in altered development of social interactions,

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disruptions to communication, and restricted and repetitive behavior. Included in this spectrum are childhood or infantile autism, Asperger syndrome, and pervasive developmental disorder—not otherwise specified (PDD–NOS), and Rett syndrome (RTT) [4]. Fragile X syndrome (FXS) is included in this chapter as well, because it has a very similar neurocognitive profile to ASD, and often results in ASD. These disorders within ASD share a core set of behavioral and cognitive deficits that are used as diagnostic criteria for ASD. Of the core deficits in ASD, social deficits are the primary distinguishing factor that separates ASD from other neurodevelopmental disorders, but the stereotyped behavioral patterns and deficient communication are also part of the behavioral spectrum. Mental retardation (full-scale IQ < 70) is present in some but not all cases of ASD [5, 6].

In brief, the social deficits present in ASD include a lack of interest in other children, failure to attend to social stimuli or properly identify social situations, less eye contact than typically developing toddlers, and less response to their own name. The pattern of social deficits in ASD, however, is not constant between individuals; some may exhibit hypersocial behavior in some arenas and hyposocial behavior in others, but an alteration to normal social interaction remains in one form or another [6–8]. The communication deficits often seen in autism include echolalia (immediate repetition of sounds or words), delayed onset of babbling, diminished responsiveness, odd gestures, and vocal patterns. At 3–5 years of age, autistic toddlers often show inappropriate word combinations, less frequent usage of language, and tend to reverse pronouns (e.g., refer to themselves as "you" and to another person as "I" or "me"). Again, there is a large amount of heterogeneity in communication deficits: not all autistic individuals are nonverbal, but all individuals with ASD show some form of abnormal communication [5, 9]. Stereotyped or repetitive behaviors include the following: inappropriate toy usage (e.g., playing with a toy car by turning it upside down and feeling the underside), fascination with twirling or spinning objects, obsession with ritual and schedule in daily tasks, restricted behavior, or excessive focus on a single or small subset of items in the world around them, and, at times, self-injury [5–7, 10–12].

The diagnosis of autism is primarily behavioral, relying on diagnostic tests such as the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R). For individuals to be diagnosed with ASD, they have to exhibit six major symptoms, including at least two qualitative impairments in social interaction, one qualitative impairment in communication, and one symptom of repetitive/stereotyped behavior. Symptoms include lack of social or emotional reciprocity, stereotyped and repetitive use of language or idiosyncratic language, and persistent preoccupation with parts of objects. Onset must be prior to 3 years of age, with delays or abnormal functioning in either social interaction, language as used in social communication, or symbolic or imaginative play. Due to the behavioral as opposed to physical or neuroanatomical manifestation of ASD, diagnosis is difficult and often diagnosis does not occur until 5–8 years of age, even though diagnosis is possible as early as 14 months in select cases [5–11, 13].

High heritability of ASD is indicated by the 60%–90% concordance rate between monozygotic twins and the prevalence of ASD among siblings with autistic disorder ranges from 2%–6% to as high as 14% [14–16]. Of additional interest is the

consistent finding that there is a very unequal male:female ratio in ASD prevalence, with recent reports calculating a 4.3:1 male:female ratio that is dependent upon cognitive impairment, roughly 2:1 when mental retardation is present, and nearly 5.5:1 when mental retardation is absent [17]. Despite the high heritability, autism has a heterogeneous genetic basis, with multiple genes and chromosomes suspected to be involved. The number of genetic loci implicated in autism continues to increase each year, suggesting that mutation of a single locus is unlikely explain the genetics underlying the majority of ASD cases [18–23]. The investigation of autism genetics, like that of other neuropsychiatric disorders, has moved away from a prediction of "common disease, common variant" to a "common disease, rare variant" prediction that suggests that many alternative rare variants may disrupt overlapping pathways of pathogenesis [24].

There are cases where an increased prevalence of ASD within a family is associated with a single gene mutation or chromosomal rearrangement. Examples of this include mutations in neuroligin genes [25, 26], Rett syndrome with mutated *MECP2* [27], 15q chromosomal duplication leading to alterations in GABAergic neurotransmission [28], and 22q11 deletions [29] among others.

Estimates of the prevalence of autism have risen from 7.1:10,000 in the 1980's to 20.6:10,000 individuals in 2007, with the prevalence of the more inclusive ASD estimated to be 1 in 110 individuals [1, 2, 16]. Potential reasons for this increased incidence include the possibility that more children may indeed have ASD, raising the specter of an as-yet unidentified etiological agent in operation. However, additional reasons for the increase include greater recognition and more efficient diagnosis in recent years leading to higher incidence rates, diagnosis at earlier ages contributing to short-term inflations in the rate of incidence, or an alteration to diagnostic criterion between the DSM-III and DSM-IV that may have broadened the criterion and thus included a greater number of individuals that would have been excluded previously [1, 2, 16, 17].

Although the latter possibilities undoubtedly contribute to the rise in the prevalence and incidence of ASD, it has been demonstrated that the rise in incidence is only partially explained by these known factors and that the other unknown factors contribute significantly [1, 2]. As an explanation for the increase not explained by the above criterion, numerous groups have suggested that a range of environmental factors may serve as causative agents for autism: ranging from foods and food allergies, pesticides, thalidomide, measles–mumps–rubella (MMR) vaccinations, heavy metals such as methyl mercury or, more recently, ethyl mercury (as thimerosal present in childhood vaccines), or lead poisoning, PCB and PBDE derivatives, and other environmental toxicants [30–35]. Several studies have now failed to find links between the MMR vaccine and thimerosal in vaccines and autism [35–38]. However, there are presently insufficient data to evaluate the importance of the other potential environmental influences in autism, and this should be a focus of future studies.

To better characterize and determine the validity of these hypotheses, rodent models have been developed, including neuroligin-3 knockout mice as a model of autism, *Mecp2* mutant mice modeling Rett syndrome, and *Fmr1* mutant mice to model fragile X syndrome (FXS). These models provide opportunities to test hypotheses directly that involve genetic–environmental interactions and how they

may or may not contribute to ASD. A remaining difficulty is the lack of a truly adequate rodent model of ASD, an "autistic" mouse or rat has yet to be characterized [39]. Behavioral testing procedures have been developed to assess social interactions, social communication, perseverative, and stereotypic behaviors in rodents and these are now being applied to research on environmental factors and gene–environment interactions in abnormal development of behaviors relevant to autism [39–42]. An alternate outcome measure is to correlate neuroanatomical and/or neurochemical alterations in mice along with behavioral abnormalities with the findings from human ASD cases [43].

21.3 NEUROANATOMICAL AND NEUROCHEMICAL ALTERATIONS IN AUTISM

In line with the heterogeneity of behavioral deficits underlying a diagnosis of ASD, findings concerning the neuroanatomical and neurochemical correlates of ASD are also highly variable and difficult to understand. Despite the heterogeneity in neural correlates of ASD, *in vivo* magnetic resonance imaging (MRI) and postmortem histological studies have provided a wealth of knowledge concerning the pathology of ASD. Based on these findings, neuroanatomical criterion for ASD subtypes have been proposed [44, 45].

21.3.1 Brain Volume

Several studies have reported increased brain volume in children with ASD compared to age and IQ-matched conspecifics [46]. However, other studies have failed to reach the same conclusion [47], and it has been suggested that the precise age at which each scan is taken is extremely important, and differences in scanning age may account for these disparate findings. There is now a consensus that brain overgrowth in early life occurs in autism, but this ends prematurely, resulting in normal to slightly smaller brain volume at older ages. Few studies have followed individuals longitudinally, which has made validation of this hypothesis difficult [48–50]

21.3.2 Cortex

Another consistent finding in autism is alterations of gray and white matter in the cortex. Several studies have reported thickened white matter and slightly thinned gray matter in a number of cases [47], although in other cases this finding is reversed. In some studies, the density of the white matter appears compromised, having a lower density but a larger volume, perhaps contributing to the macrocephalic phenotype [51–53].A more recent finding that has now been confirmed in several studies is abnormal folding patterns of the gyri on the surface of the neocortex [54, 55]. There are also reports of altered patterns of connectivity in ASD brains relative to typically developing individuals. Specifically, there appears to be a decrease in the density

of long-distance connectivity (i.e., temporal–frontal connections) but increased local connectivity (i.e., frontal–frontal connections) throughout the brain [56–58].

21.3.3 Corpus Callosum

In autism, there are numerous morphological disturbances to the corpus callosum, with reports of near complete dysgenesis in some cases [49, 59, 60]. In the most rigorous study of corpus callosum morphology, ASD associated with macrocephaly had a larger total corpus callosum size, whereas those with normal brain size had smaller corpus callosum volume, especially the midbody and genu, suggesting some heterogeneity in the phenotype [49, 59–61].

21.3.4 Thalamus

It has been reported that the thalamus of autistic individuals is smaller in comparison to the total brain volume than expected. This may be due to either a smaller thalamus or a larger total brain volume driven by increased cortical volume in these studies and those discussed above. Of particular interest is the finding that in autism there appears to be an increase in thalamocortical connectivity, which is remarkable considering the thalamus is potentially undersized in these subjects [62–65].

21.3.5 Cerebellum

The cerebellum of ASD patients has long been of interest to researchers. It has been shown that the cerebellar cortex in ASD is undersized, perhaps due to a poorly formed vermis (lobules VI–VII are reduced in size), but the cerebellar hemispheres are often normal or, counterintuitively, they may be enlarged [66, 67]. There have also been reports of a reduction in Purkinje cell number in roughly half the cases studied in one lab in two separate stereological evaluations [68, 69] as well as white matter abnormalities throughout the deep cerebellar nuclei, and, in rare cases, in the cerebellar peduncles themselves [66, 67, 69–73].

21.3.6 Hippocampus

It has been shown that the hippocampus is affected in ASD in some, but not all, studies. This discrepancy may be due to the age of the ASD patients studied and to methodological differences. Based on longitudinal studies by laboratories reporting abnormalities it has been suggested that the dentate gyrus and hilus in the hippocampus are undersized in youth but of relatively normal volume in adults with ASD [74, 75]. No differences in pyramidal cell layers were observed, but since the time of this study, advances have been made in the resolution of MRI scanning techniques that may be able to identify such abnormalities more readily. Additionally, the areas surrounding the subiculum in ASD have been shown to show an inward deflection (reflective of reduced cell packing or localized decrease in cell number into which the subiculum deflects), and the severity of this abnormality had a relationship with the

severity of the ASD symptomology. Other studies have shown consistent enlargement of the hippocampus in ASD with no influence of age [74–77].

21.3.7 Amygdala

Reports concerning anatomical abnormalities in the amygdala in ASD have been varied, in some cases even between studies carried out by the same laboratory. In imaging studies, the amygdala has been reported to be identical to that of control cases, larger, or smaller—all depending upon the age and severity of the cases of ASD studied by the individual laboratories [76, 78]. In one case, the amygdala was smaller early in life but had normal volume later in life [77]. It has been reported, however, in postmortem stereological studies that the amygdala was of normal size but with a significant reduction in the number of cells present [76–79].

21.3.8 Caudate

It has been reported that the caudate in children with ASD is enlarged and this enlargement has been correlated to difficulties in motor learning and the tendency toward repetitive behaviors in children with ASD. In fact, this enlargement is unique to the caudate as the same studies evaluated the globus pallidus and putamen but failed to find increases in size for these regions [80]. In addition, the caudate appears to have a more diffuse connectivity with the cortex in ASD than in the general population [80–82].

21.4 NEUROCHEMICAL ABNORMALITIES

Early findings in studies of the biology underlying ASD found that there were increased levels of serotonin and serotonin metabolites in the serum of ASD patients. These findings led researchers to investigate the possible role of serotonin synthesis and transport machinery in the pathogenesis of autism. For a time, it appeared that ASD might be explained by a serotonergic hypothesis, at least until other neurotransmitter systems were evaluated [83–87]. Further analysis of other neurotransmitter systems revealed a potential role for the cholinergic system, glutamatergic system, dopaminergic system, noradrenergic system, and GABAergic system [88–90].

The most consistent findings to date appear in the GABAergic system as many of the studies have involved immunohistochemistry on post mortem tissue. Postmortem studies of ASD have shown decreases in the expression of two isoforms of glutamate decarboxylase (*GAD*). Lower levels of *GAD65* mRNA in the dentate nucleus of the cerebellum and *GAD67* mRNA in the Purkinje cell layer compared to unaffected individuals. In contrast, an excess of *GAD67* mRNA expression was reported in cerebellar interneurons [91–93]. There also have been studies suggesting the $GABA_A$ receptor $\beta 3$ subunit has an abnormal distribution in ASD. One study has found a decrease in $GABA_A$ receptor $\beta 1$ and $\beta 2$ subunits in the cerebellum. A similar study found $GABA_A$ receptors were similarly decreased, with this study finding an increase

in $GABA_A$ receptor $\alpha 1$ being altered in the frontal cortex. GABA binding in the hippocampus was also reduced, whereas glutamatergic binding appeared undisturbed [88, 91–96].

21.5 ENVIRONMENTAL AGENTS AND AUTISM

The possibility that environmental factors could be playing a role in the etiology of neurodevelopmental disorders such as autism is of considerable concern [3]. The developing nervous system is particularly vulnerable to exposure from chemicals in the environment [97–103]. This vulnerability is due to several factors including differences in drug metabolism, drug elimination rates, binding affinity of proteins, and a lack of a mature blood–brain barrier [100]. Because children are often exposed to toxic chemicals beginning early in development [104, 105] they are at increased risk for damage to their developing nervous system, which may result in impairments that may not become evident until later in life.

During early development, the nervous system is exposed to many chemicals raising the possibility that neonatal exposure to some may be negatively impacting brain development and may contribute to the etiology of neurodevelopmental disorders. Several of these chemicals are able to enter the body and cross the placental barrier to reach the developing fetus [98, 106–108]. Furthermore, many compounds, including PCBs and PBDEs, can be transferred postnatally via lactational exposure [109, 110]. Due to these routes of exposure and the high bioavailability of these compounds, the developing nervous system is under constant assault and the consequences of these exposures during child development needs to be better understood and characterized.

A brief overview is presented of two important classes of environmental pollutants: heavy metals and persistent organic pollutants. The effects of these compounds on the developing nervous system as well as their possible involvement in the etiology of neurodevelopmental disorders such as autism are discussed. Two of the most studied and best-characterized neurotoxic compounds for each class are described within each section. The role these compounds have in the increased prevalence of neurodevelopmental orders, including autism, is still unknown but the likelihood that they are contributing factors cannot be ruled out.

21.5.1 Heavy Metals

21.5.1.1 Lead The developmental neurotoxicity of lead is well established [111]. Lead (Pb) is a widely distributed heavy metal not usually found naturally in the environment but one that is extracted from ores used in a variety of consumer goods. Lead has been used in such products as paint, gasoline, solders, and water systems, but its use has steadily declined over the past 30 years due to recognition of its toxicity in humans, and in children in particular [111–113]. Lead is still found in harmful quantities in batteries, paints, metal products, and ceramic glazes [114] and is still detected in the environment in air, water, and soil.

Lead exposure can produce a variety of adverse effects such as encephalopathy, peripheral neuropathies, anemia, and renal failure [115–117]. However, the developmental neurotoxicity associated with lead exposure has been of greatest concern. Children are particularly sensitive to lead exposure due to the fact that they absorb 30%–75% of ingested lead compared to 11% in adults [118], mainly because the blood–brain barrier is not completely functional during early development. These factors allow metals such as lead to accumulate in the brain. Lead is capable of causing toxicity at relatively low levels, with significant toxicity produced at very low levels in developing children [111,113,119]. Currently the Center for Disease Control has set the lowest adverse level of lead exposure for children at 10 μg/dL. However, studies have shown that even lower levels of lead can damage the developing nervous system [119–121].

A large body of research has documented the adverse neurodevelopmental effects of exposure to lead. Children exposed to lead show significant reductions in IQ, behavioral disturbances, and altered endocrine function [113, 114, 119, 122]. These studies also show that lead-induced brain damage preferentially occurs in the prefrontal cortex, the cerebellum, and the hippocampus—brain regions important for cognitive function, motor skill, and memory. Experiments using nonhuman primate and rodent models have also consistently shown neurodevelopmental deficits due to lead exposure. Impairments in higher-order learning have been shown in rhesus monkeys exposed both pre- and postnatally to lead [123, 124]. In rodents, lead exposure can also result in deficits in complex behaviors including learning and memory and mechanisms of attention [125, 126].

The mechanism of lead toxicity is not fully understood. The most commonly suggested mechanism involves interference with in calcium homeostasis and calcium-regulated pathways [127–129]. The chemical properties of lead are similar to those of calcium. Therefore lead can compete with calcium for common binding sites and can be incorporated into calcium transport systems in the nervous system that are required for neurotransmitter release and regulation. Lead is also capable of activating Ca^{2+}-regulated molecules and thus can have drastic effects on cell signaling pathways. For example lead can activate protein kinase C (PKC), an enzyme involved in many cell signaling pathways, by increasing intracellular calcium levels or by mimicking the action of Ca^{2+} itself [130]. Increased intracellular calcium levels due to lead exposure can also result in excessive calcium influx into mitochondria, leading to the production of toxic free radicals and reactive oxygen species (ROS) [131]. Furthermore, environmentally relevant levels of lead exposure during neurodevelopment decreases the expression of the NR1 subunit of the *N*-methyl-D-aspartic acid (NMDA) receptor in rats, providing a molecular mechanism by which lead can alter synaptic plasticity and impair cognitive function [127,132]. Similarly, perinatal lead exposure decreases nNOS activity in rats that regulates long-term potentiation and other physiological processes, providing another mechanism for altered neuroplasticity [133].

There is, at present, no direct evidence that developmental lead exposure contributes to the etiology of autism or ASD. However, there have been reports of increased levels of lead in the blood of autistic children as well as the suggestion that lead exposure may have contributed to the onset or accelerated the development of

symptomatology in autism [134]. Evidence has already been presented that children with autism may have trouble excreting sulfhydryl-reactive metals, including lead, arsenic, and cadmium, resulting in higher body burdens of these metals and possibly contributing to the symptoms of autism [135]. An association between the risk of developing ASD and developmental exposure to a variety of metals, including mercury, cadmium, and nickel has also been reported. Data showing that ASD and TD children display different associations between mRNA transcript levels and low levels of blood lead were presented recently, and it was suggested that this might relate to the underlying genetic differences between the two groups [136]. Therefore, continued research on the role of lead exposure for neurodevelopmental disorders, including autism, is warranted.

21.5.1.2 Mercury Inorganic mercury is present naturally within the earth's crust and is widespread in the environment due to several factors described by the World Health Organization: volcanic emissions, burning of waste and fossil fuels, use in electrical, medical, and laboratory instrumentation and the extraction of gold. Mercury can be found in three basic forms in the environment: elemental, inorganic, and organic. Studies of the neurotoxicity of mercury have focused on organic forms of mercury, which are the most damaging, particularly to the developing nervous system [137]. The best characterized form of organic mercury is methylmercury (MeHg), which has been studied in depth due to its neurotoxic potential [99]. Methyl mercury is typically formed because of bacterial transformation of inorganic mercury that has leached into aquatic environments because of human activities. Once methylated, mercury can enter into living organisms and travel up the food chain accumulating at higher and higher concentrations (e.g., bioaccumulation). For this reason, most human exposures to mercury are the result of dietary consumption [138]. Episodes of human mercury poisoning have been reported in Japan during the 1950s and 1960s and in Iraq in the early 1970s. These poisonings were due to consumption of contaminated food, and research on those exposed to mercury during these episodes have provided crucial information about the dangers of mercury exposure at all ages of development [139, 140]

When MeHg is consumed, it is almost completely absorbed through the gastrointestinal tract and rapidly enters the bloodstream where about 95% is taken up by red blood cells and then distributed through the whole body (Fig. 21.1) [137]. In pregnant women, MeHg readily crosses the placenta and. because it has high affinity for fetal hemoglobin, fetal blood levels are about 25% higher then that of the mother [141]. Once MeHg enters the body, its main target for toxicity is the brain. In adults, early neurotoxicity is seen in the cerebral cortex and cerebellum where there is neuronal destruction [142]. In the fetus exposure to mercury leads to disruption of normal development, including alterations in neurobehavioral parameters as well as impaired cognitive function [99, 107, 143–145].

The mechanisms by which mercury exposure results in neurodevelopmental toxicity are still not fully understood, but research suggests that damage may occur at several physiological levels. MeHg has a high affinity for sulfhydryl (SH) residues in cysteine containing molecules, a common feature of many molecules, and most

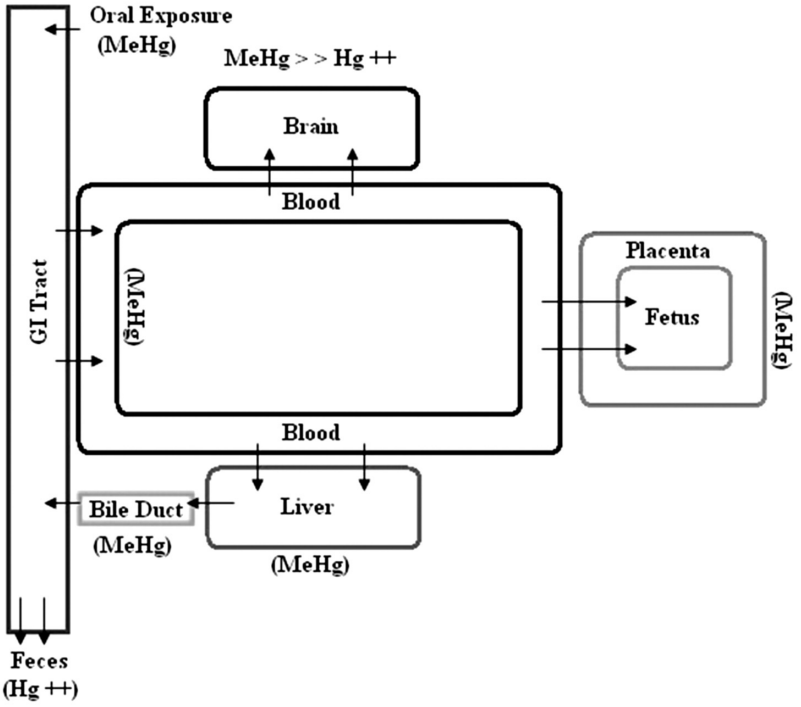

FIGURE 21.1 Distribution of MeHg within the body. Orally ingested methyl mercury (MeHg) enters the gastrointestinal (GI) tract and is either metabolized into elemental mercury (Hg^{++}) and excreted or is transferred into the blood. Once in blood, MeHg can distribute throughout the body, including the brain as either MeHg or H^{++}, or to the liver where it then enters the GI tract through the bile duct. In pregnant women, MeHg can also cross the placental barrier as well as pass through the umbilical cord, exposing the fetus to MeHg where it can damage development during gestation. Diagram adapted from Clarkson et al. [137].

proteins. This has led to the hypothesis that MeHg exerts it toxic activity by suppression of cell growth through nonspecific inhibition of these molecules [146]. Other research has proposed that MeHg produces neurotoxicity through effects on synaptic transmission by interference with Ca^{2+} signaling pathways. The role of MeHg in alterations of synaptic transmission is of particular interest due to the deficits in motor function seen in episodic mercury poisonings. Studies into this phenomenon indicate that MeHg is capable of altering synaptic release of acetylcholine (ACh) at both the neuromuscular junction as well as within cholinergic pathways in the brain [147–149]. In addition it is believed that MeHg is also capable of blocking excitatory postsynaptic potentials through suppression of calcium entry into nerve terminals [150–152]. Other possible mechanisms by which MeHg may cause toxicity include disruption of mitochondrial function through inhibition of respiration and ultrastructural changes [153–159] and production of ROS through membrane lipoperoxidation

[153, 158–162], and interference with normal cell cycle activity [163]. This wide array of neurotoxic effects caused by MeHg shows that this heavy metal could be a significant contributor to the rise in neurodevelopmental disorders seen within the general population and thus must be included as a possible environmental factor in their etiology. In addition, there is also a large body of research that was carried out on the ethylmercury-containing preservative thimerosal. Thimerosal has been used as a preservative in several childhood vaccines. It has been hypothesized that ethylmercury toxicity may play a causal role in some cases of autism. Both ethylmercury and thimerosal themselves have been shown to modulate Ca^{2+} signaling events when tested in a variety of *in vitro* preparations [164]. Arguably, the most sensitive targets of thimerosal are the microsomal Ca^{2+}-release channel family comprised of ryanodine receptors (RyRs) and inositol 1,4,5-trisphophate receptor (IP_3R) families [165–167]. For example, studies of dendritic cells isolated from mouse bone marrow indicate that nanomolar concentrations of thimerosal uncouple ATP-mediated Ca^{2+} signaling and dysregulate IL-6 secretion [168]. Thimerosal neurotoxicity is highly associated with deletion of cellular glutathione [169, 170] and the latter is known to tightly regulate the function of RyRs and IP3R [167]. Despite the evidence that thimerosal is a potent toxicant in cellular studies, epidemiological and *in vivo* laboratory research have not supported a causality between thimerosal exposures and autism [35–38]. In a recent report, 2- to 5-year-old children with and without ASD participating in the case control epidemiological study known as Childhood Autism Risks from Genetics and the Environment (CHARGE) [171] were not found to differ significantly in their mean total mercury levels in blood [172]. Nevertheless, autistic children from the same study differed from those with typical development in how their blood mercury levels were correlated with differential expression of genes regulating specific pathways, including genes involved in TGFβ, α-adrenergic, and Map38 signaling cascades.

21.5.2 Persistent Organic Pollutants

21.5.2.1 Polychlorinated Biphenyls Polychlorinated biphenyls (PCBs) are a widely distributed class of environmental pollutants that have been detected in virtually all environmental samples, and in human and wildlife tissue samples [110]. Almost every person in most populations examined has detectable levels of PCBs in their blood [173]. Because of their chemical properties, these compounds were used in industrial products such as capacitor and transformer oils, hydraulic fluids, plasticizers, and lubricating oils. The production of PCBs, which began late in the nineteenth century, reached its peak in the 1970s when the adverse health effects of these compounds were recognized and greater regulation of their manufacture and use was instituted. Since then, there as been a steady decline in the presence of these compounds within the environment, but their continued bioaccumulation and known toxicity continues to focus toxicological research on their role in the etiology of neurodevelopmental disorders.

The neurotoxicological properties of PCBs became evident after several well-documented accidental exposures that resulted in severe human health problems including numbness and weakness of extremities and decreased peripheral nerve

conduction velocities in exposed adults [108]. The developmental neurotoxicity of PCBs, however, was best characterized after the large-scale consumption of PCB-contaminated rice oil that occurred in 1968 in Japan and in 1979 in Taiwan [174,175]. From these exposures, it was clear that PCBs produced adverse effects on the developing brain with exposed offspring displaying behavioral abnormalities as well as significantly lower verbal and full-scale IQs [97]. This correlation between PCB exposure and abnormal neurodevelopment led to the targeting of these compounds as possible environmental contributors to the increased occurrence of neurodevelopmental disorders seen within the general population [106, 173].

The PCB family contains 209 congeners that differ by the number and placement of chlorine atoms around the biphenyl ring structure (Fig. 21.2). The placement and geometry of the chlorine atoms is closely related to the toxicological properties of the specific congener. PCBs can be further subdivided into two groups: coplanar (dioxinlike) and noncopolanar (nondioxinlike). Coplanar congeners exhibit potent overt toxicities with both acute and chronic exposures, and these are primarily mediated through activation of the Aryl hydrocarbon receptor (AhR). Activation of AhR signaling enhances transcription of dioxin-responsive genes, particularly CYP 450 enzymes that catalyze oxidation of xenobiotic molecules [102]. The noncoplanar congeners exhibit less overt acute toxicity but have the ability to bioaccumulate readily and have been shown to disrupt cell signaling pathways and hormone homeostasis [176–178].

Research into this class of environmental pollutants has produced many significant findings implicating these compounds as important developmental toxicants. Most consistently, PCBs have been shown to produce developmental neurotoxicity by altering executive function activity [179]. This process, which regulates cognitive abilities necessary for goal-directed behavior including abstract thought processing and planning, is also commonly altered in children who suffer from autism [180]. The level of toxicity that results from exposure to PCBs is also closely linked to the time of exposure with prenatal exposures resulting in significant neurodevelopmental toxicity due to the vulnerability of the developing brain during this period [97,179,181]. Along with human epidemiological studies, animal studies have also reported damaging affects of exposure to PCBs on learning and memory, motor function, and the appearance of hyperactivity, especially for exposures occurring during early developmental periods [178, 182–184]. From this body of research, three main mechanisms by which PCBs are believed to cause neurodevelopmental effects have been proposed: alterations in neurotransmitter level, alterations in intracellular signaling pathways, and alterations in hormone balance.

Studies examining at the effects of PCBs on neurotransmitter levels *in vivo* have shown that dopamine (DA) levels can be significantly reduced by exposure to PCBs and these reductions are associated with neurobehavioral changes [185–188]. Serotonergic, cholinergic, and noradrenergic systems were also shown altered by exposure to PCBs [189–191] but to a lesser degree than the dopaminergic system. *In vitro* studies looking at the possible direct mechanism of PCBs on neurotransmitter levels have shown that PCBs have the ability to inhibit tyrosine hydroxylase [187], an important enzyme in the production of DA, and to inhibit vesicular monoamine transporters

thus reducing synaptosomal dopamine content [192, 193]. Specific *in vitro* effects of PCBs on other neurotransmitter systems have not yet been examined systematically.

Implications for alterations in intracellular signaling pathways are due to deficits in learning and memory documented in exposed humans [106]. Learning and memory is associated with a process of synaptic facilitation called long-term potentiation (LTP) which is modulated through second messenger systems including calcium, inositol phosphates, protein kinase C (PKC), arachidonic acid (AA), and nitric oxide synthase (NOS) [183, 194, 195], Several studies have been conducted to determine the effects of PCBs on these systems associated with synaptic plasticity. The results of both *in vivo* and *in vitro* studies show that PCBs uniformly alter calcium homeostasis within neurons [177, 178, 196, 197], and that these effects can lead to further downstream actions producing abnormal neuronal activity and abnormal neurodevelopment [177, 198–200]. The pivotal roles of Ca^{2+} signals in regulating movement, metabolism, growth, proliferation, gene transcription, and protein translation in virtually all cell types is well established. A selective receptor-targeted mechanism was proposed based on the stringent structure-activity relationship of PCBs for enhancing the activity of RyRs [201–204]. Exposure to PCBs during development therefore can have drastic effects on the maturing nervous system and its ability to carry out experience-dependent plasticity (i.e., LTP) required for memory storage and learning.

Finally, PCBs are also known to affect regulation of thyroid hormones (TH). Thyroid hormones contribute to the regulation of important neurodevelopmental processes, including neuronal proliferation and differentiation [205, 206], as well as overall brain organization [207–210]. PCB exposure can decrease circulating TH levels during development [211, 212] by at least three possible mechanisms, including direct interaction of PCBs with the thyroid gland decreasing TH synthesis [213], increased biliary excretion of THs through the induction of phase two metabolic pathways [213–215], and displacement of THs at the level of receptor binding [211, 216]. Toxicity, however, has been more commonly attributed to displacement of thyroid homone from its carrier proteins and receptors by PCBs. This binding increases retention of PCBs in the body, the likelihood of placental transport to the fetus, and the risk for neurodevelopmental disorders.

21.5.2.2 Polybrominated Diphenyl Ethers PBDEs have recently become an important class of environmental pollutants due to their structural similarities to PCBs (Fig. 21.2) [182]. The widespread presence of PBDEs in the environment is the result of their extensive use as flame retardants in a wide variety of commercial goods such as electronics, furniture, textiles, carpets, and clothing. PBDEs are not stably bound to the polymers in which they are added [217,218] and easily leach into the environment. As a result, they are present in alarmingly high concentrations in both the environment and in human tissue samples all over the world [98, 219–221]. Levels of PBDEs in the environment, unlike many of the PCBs, have continued to increase over the past 30 years [222, 223]. This, in conjunction with recent reports of neurodevelopmental toxicity of several PBDEs, raises concern about these environmental contaminants [224–226].

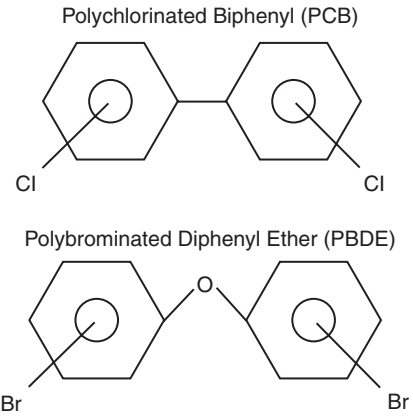

FIGURE 21.2 Structural differences between PCBs and PBDEs. Both chemicals are made up of two phenyl rings; however, they differ in their bonding between the two phenyl rings and their halogenated constituents.

The PBDE family, like PCBs, is made up of 209 congeners. The specific toxicological activity of each congener depends on the placement, geometry, and number of bromine atoms around the diphenyl ether structure [227]. Because PBDEs are similar in both structure and chemical nature to PCBs, a large body of research exists examining the adverse health effects of these compounds as they relate to developmental neurotoxicity (Fig. 21.2). These studies, most of which were carried out in animal models, show that PBDEs are capable of causing long-lasting behavioral alterations in both motor activity and cognitive behavior within animal models [224–226] as well as altering thyroid hormone homeostasis and function [228, 229].

Behavioral studies in rodents looking at the effects of PBDEs administered postnatally or perinatally show that several PBDE congeners are capable of producing long-lasting changes in spontaneous locomotor behavior with a lack of habituation to novel environments [225, 230–234]. The lack of habituation could be due to increased anxiety in exposed animals as a result of PBDE exposure leading to less exploration early in the trial period compared to later, or it may be the result of hyperactivity following exposure that interferes with habituation [98]. In addition to alterations in locomotor activity, exposure to some PBDE congeners results in impairments in cognitive function in tasks designed to assess learning and memory [230–232, 235]. The impairments in these learning and memory tasks are also indicative of possible alterations in second messenger signaling pathways necessary for synaptic plasticity, such as those altered by PCB exposure, and studies on PBDEs and brain function could provide important mechanistic insights into the mechanisms by which PBDEs produce neurodevelopmental disorders. For example, research conducted in this area has shown that LTP and postsynaptic protein levels are altered as a result of exposure to PBDEs [224]. Other studies have shown that PBDEs can elicit a number of effects on cell signaling and cell function, including reduced neurotransmitter

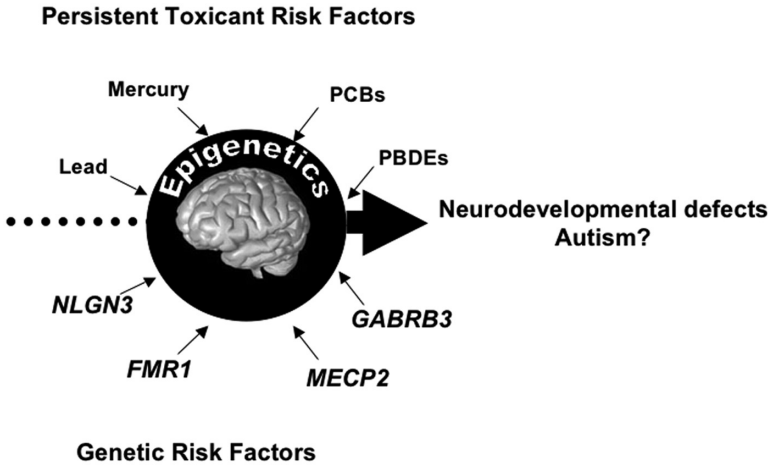

FIGURE 21.3 Interaction of genetic risk factors and environmental toxicant risk factors for epigenetic effects on neurodevelopment.

uptake and altered signaling via PKC and glutamate–nitric oxide–cGMP pathways [200, 227, 236, 237]. More research is needed to provide more conclusive information as to the precise mechanisms by which early exposure to PBDEs can result in neurodevelopmental toxicity. With regard to autism, it has been reported that the immune/cytokine response of peripheral blood monocytes to exposure to PBDE47 is altered in children with ASD compared to age-matched typically developing controls, supporting the possibility for a role of environmental toxicants in immune system abnormalities reported in autism [238].

Alterations in thyroid hormone levels also occur with exposure to PBDEs [226]. Several studies have found that PBDEs can disrupt the thyroid system in both adults as well as developing offspring [228, 229, 239–242]. Because thyroid hormones are important in brain development [243, 244] and hypothyroidism has been associated with a large number of neuroanatomical and behavioral effects [245], the action of PBDEs on this system is of particular interest. Such studies have consistently shown that exposure to PBDEs can cause decreased levels of circulating T4 following exposure. However, the actual mechanism by which this occurs has yet to be determined. Some hypothesized mechanisms include increased metabolism and excretion of T4 because of PBDE exposure or a direct interaction of PBDEs with thyroid hormone transporters. Again, further research is needed to understand the mechanisms by which PBDEs disrupt thyroid hormone function.

What remains unclear is whether these neurotoxicants contribute directly or indirectly to incidences of autism or autismlike behavioral per se. A great deal is now understood about autism, and much is known about how various environmental toxicants affect neurodevelopment. Unfortunately, scientists and clinicians to date have not been able to identify clearly populations with known genetic susceptibility to autism or populations of individuals with increased susceptibility to environmental

toxicants. This contributes greatly to the difficulty in identifying environmental agents that may be particularly toxic to individuals predisposed genetically for autism.

To better study the genetics of autism, mouse models have been developed which mimic the genetic anomalies identified in a few isolated human populations with autism. This facilitates research on the role genetic susceptibility plays in the pathogenesis of autism in the absence of environmental factors. Inbred mouse strains have been similarly studied to assess the role of environmental agents in neurodevelopment without the confound contributed by genetic heterogeneity in the human population. What has not been as actively pursued, however, is the methodical study of the interaction between these factors (e.g., gene and environmental interactions in neurodevelopment). For example, roughly 30% of individuals with fragile X fall within the autism spectrum. What is it about those 30% that predispose them to be autistic and the other 70% not? One possible explanation is that gene–environmental interactions are important determinants of the effects of perinatal exposure to an environmental contaminant on nervous system development. These issues can begin to be evaluated in the lab using mouse models.

21.6 ANIMAL MODELS OF AUTISM

Some of the most promising research in the field of neurodevelopmental disorders, including autism, have come from the establishment of animal models of human neurodevelopmental disorders such as Rett syndrome and fragile X, as well as animal models to investigate gene mutations associated with increased risk of autism including neuroligin and $GABA_A$ receptor mutations. These animal models offer the possibility of understanding the molecular mechanisms that may underlie each of these disorders, as well as providing model systems for studying their natural history and developing rational treatments for the disorders. Furthermore, these mouse models are unique in that they are models of single gene loci mutations that have been identified in humans, and thus offer a potential to study gene-environment interactions in the laboratory. The development of behavioral test batteries for mice that assess complex social behaviors, including social recognition and dyadic social interaction, as well as anxiety, sensorimotor gating, perseverative behaviors, and stereotypes has also greatly facilitated studies of neurodevelopmental disorders in these animal models [39, 40, 246, 247].

21.6.1 Neuroligin Mutations

Mutations in genes encoding neuroligins (*NLGN3/NLGN4*) were found in two pairs of Swedish brothers with ASD [248]. Neuroligins are cell adhesion proteins expressed on the surface of postsynaptic neurons that bind to presynaptic proteins called neurexins, providing a physical connection between the pre- and postsynaptic membranes. In addition, neuroligins promote recruitment of neurotransmitter receptors to the synapse, and may therefore regulate the balance of excitatory and inhibitory activity in the brain, which has been hypothesized to be abnormal in autism [249]. Humans

express five neuroligins, *NLGN1–NLGN5* [250]. The mutations found in individuals with ASD include missense and nonsense mutations in *NLGN3* and *NLGN4* [248]. One of these mutations, a substitution of Arg^{451} to $cysteine^{451}$, has been created in mice by gene targeting [251] to study the role of neuroligin mutations in brain function and autism. These mice show a ∼90% loss of neuroligin-3 protein in the forebrain, evidence for increased levels of markers for inhibitory synaptic transmission (e.g., numbers of vesicular GABA transporters), increased inhibitory synaptic strength, and impaired social interactions [251]. The increase in inhibitory activity was unexpected as autism has typically been thought to be associated with a decrease in inhibitory synaptic drive [176, 252]. This mouse model has provided important insights into the molecular mechanisms of ASD, at least for familial autism in those rare families with neuroligin mutations. While promising as a model of autism, a recent study did not find evidence for deficits in social interactions in *Nlgn3* mutant mice, although minor developmental differences were found in somatic growth, motor activity and pup ultrasonic vocalizations [253]. Evidence for a possible role for neuroligin-2 in neurodevelopmental disorders has also been recently described [254]. Neuroligin-2 is enriched at the inhibitory synapses and appears to play an important role in regulating the excitatory/inhibitory balance of activity in the brain. Mice overexpressing *Ngnl2* in several brain areas, including the cortex, amygdala, and hippocampus, show a variety of abnormalities [250]. These include reduced life span, lower body weight, abnormal synaptic morphology, enhanced acoustic startle, stereotypic jumping behaviors, spike–wave discharges at 7 Hz in frontal-parietal cortex, increased anxiety in the open field, and decreased social interaction. Interestingly these mice also showed limb clasping, a behavior associated with the Rett syndrome mouse model.These findings again point to an important role for neuroligins in normal development as well as their possible relevance for understanding their function in neurodevelopmental disorders such as autism.

21.6.2 Fragile X Syndrome

The most common form of inherited mental retardation is FXS [255] with a prevalence of approximately 1:4,000 males and 1:6000 females. FXS is due to an expanded (i.e., >200 repeats) CGG trinucleotide repeat in the 5′-untranslated regions of the *FMR1* gene, resulting in hypermethylation of the gene, silencing of the gene, and a resulting absence of fragile X mental retardation protein (FMRP). In addition to mental retardation, between 15%–33% of individuals within the FXS population meet the criteria for autism [256], and between 60%–90% of individuals with FXS show some of the behaviors associated with autism, including avoidance eye gaze, repetitive behaviors, speech perseveration, avoidant eye gaze, and impairments in complex social interactions [256, 257]. In order to study FXS and the role of FMRP in brain development an *Fmr1* knockout mouse was created that lacks Fmrp, which is required for normal brain development [258]. This mouse displays poor motor performance, hyperactivity, impaired learning and memory in some tasks, and altered social interactions [259, 260] . The mice also show immature appearing dendritic spines in the neocortex similar to that reported in postmortem tissue from the neocortex of humans

with FXS [261].These knockout (KO) mice also show enhanced long-term synaptic depression (LTD) in the hippocampus, a mechanism important for synaptic pruning during development and experience-dependent learning and memory [262, 263]. Enhanced LTD in *Fmr1* KO mice was hypothesized to result from increased AMPA receptor internalization triggered by mGluR5 stimulation due to an absence of Fmrp, and recent evidence now supports this hypothesis [264]. The *Fmr1* KO mouse has been used to explore therapeutic strategies that could be used to reverse or reduce the cognitive deficits associated with FXS. Aberrant social behaviors and impaired sensorimotor gating abnormalities have been shown to be rescued in *Fmr1* KO mice carrying a human *FMR1* transgene [259, 260], demonstrating the potential reversibility of FXS. More recently, it was reported that two mGluR5 antagonists, MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochloride) and Fenobam, could rescue the behavioral phenotype and abnormal neuronal morphology in *Fmr1* KO mice [265]. Continued research on *Fmr1* KO mice should result in new insight into the molecular and behavioral mechanisms of FXS, as well as provide an animal model that could be used to examine the contribution of neonatal exposure to environmental toxicants on brain development regulated through *FMR1*-related mechanisms.

21.6.3 Rett Syndrome

RTT is a neurodevelopmental disorder that results from mutations on the *MECP2* gene that codes for methyl-CpG–binding protein 2 (MECP2), a transcriptional modulator (reviewed in [**?**]). The disorder primarily affects girls, with a prevalence rate of 1 in 10,000. Inheritance of RTT is X-linked dominant, which accounts for the skewed sex distribution. Although prenatal and perinatal development is apparently normal until about 6–8 months of age, a late infancy regression in acquired developmental skills is observed. RTT results in severe cognitive and physiological impairments, including loss of skills in communication and purposeful hand use, stereotyped hand movement, breathing difficulties, EEG abnormalities, feeding problems, and autistic-like behaviors. Although mutations in *MECP2* are rare in autistic individuals, recent evidence indicates that single nucleotide polymorphisms (SNP) around *MECP2* may confer increased risk for autism and ASD [267]. In addition, reduced MeCP2 expression is observed in 79% of autism cortex samples and correlated with increased *MECP2* promoter methylation in autistic males [268, 269]. No cure exists for RTT syndrome, but experimental genetic and pharmacological therapies have been proposed to bypass or reverse the effects of *Mecp2* mutations. As reviewed in Ricceri et al [270] these strategies have been tested in one of several mouse models of RTT created by either deleting exon 3 and 4 from the *Mecp2* gene, resulting in a null mutant referred to as the *Mecp2*$^{\text{Bird}}$ mouse [271], by deleting exon 3 conditionally in the brain [272] or by truncating *Mecp2* (*Mecp2*$^{308/y}$) [273] similar to mutations seen in RTT. *Mecp2*$^{\text{Bird}}$ mice, both males and females, appear normal until approximately the third postnatal week after which time males show uncoordinated gait, reduced movements, irregular breathing, and hindlimb clasping. Male *Mecp2*$^{\text{Bird}}$ mice lose weight and die around the twelfth postnatal week, whereas females typically develop less severe symptoms beginning after 12 weeks of age. Targeted deletion of exon 3 results in abnormal gait in male mice as early as 4 weeks of age, with loss

of body weight and tremors by 5 weeks of age. Heterozygous mutant females appear normal at 4 months of age and develop ataxia and reduced activity later. Mice with the truncated *Mecp2*$^{308/y}$ mutation show milder effects of gene mutation with no abnormalities evident until approximately 6 weeks of age; visible tremors are present at 4 months. Most *Mecp2*$^{308/y}$ mice survive to at least 1 year of age, with heterozygous females showing milder and more variable symptoms starting at approximately 1 year of age. Little information is available concerning social behaviors in RTT model mice, except for *Mecp2*$^{308/y}$, which has been reported to show low social motivation, reduced social interaction [274], and deficits in spatial memory [275]. A variety of pharmacological and dietary treatments have been tested in RTT mice, including tricyclic antidepressants, ampakines, and dietary supplements with choline, folate, and betaine (see review by [270]). Recent studies have also reported that tamoxifen-induced reactivation of *Mecp2* expression in transgenic mice, when expression was originally silenced by insertion of a tamoxifen dependent "stop-lox" construct, could rescue the normal phenotype. This provides evidence that some of the deleterious effects of RTT might be at last partially reversible with the appropriate therapeutic strategy [276].

21.6.4 $GABA_A$ Receptor Mutant Mice

Chromosome 15q11-13 duplications are observed in approximately 1%–3% of autism cases, usually observable as a supernumerary chromosome (idic15) [277]. In addition to parentally imprinted genes responsible for the 15q11-13 deletion syndromes, Angelman and Prader-Willi syndromes, a cluster of three $GABA_A$ receptor subunit genes are located in 15q11-13 and implicated in social behavioral defects and seizures in Angelman syndrome [278, 279]. The $\beta 3$ $GABA_A$ receptor subunit (*GABRB3*) is reduced in 56% of autism cortex samples [280] and has been genetically linked to inheritance of autism in several studies [281–284]. *Gabrb3* KO mice are considered a potential model of autism (reviewed in [278]). *Gabrb3*-deficient mice exhibit impaired social and exploratory behaviors, defects in nonselective attention, increased risk assessment, hypotonia, and hyperactivity [285, 286]. Although 15q11-13 duplication has been predicted to increase *GABRB3* expression, expression of the three 15q *GABAR* genes were reduced in cortex of an adult with idic15 and autism, supporting an epigenetic dysregulation and a general down-regulation of GABRB3 in autism [287].

21.7 EPIGENETICS OF AUTISM: THE INTERFACE BETWEEN GENETIC AND ENVIRONMENTAL RISK FACTORS

Many of the genes implicated in autism and reviewed in the previous section are known to be regulated by epigenetic mechanisms (reviewed in [288]). Epigenetics can be defined as inheritable and reversible modifications to DNA or chromosomes that do not alter the sequence but can alter expression of genes. Two well-characterized examples of epigenetic mechanisms are parental imprinting, in which maternal versus paternal inheritance alters gene expression (as in 15q11-13), and X chromosome

inactivation, in which females inactivate one copy of most X-linked genes for dosage compensation. *NGNL3*, *NGNL4*, *FMR1*, and *MECP2* are all X-linked genes subject to X chromosome inactivation in both human and mouse models. Although *GABRB3* is located within the imprinted locus of 15q11-13, it is biallelically expressed in controls but expressed monoallelically in a subset of Rett and autism brain samples showing epigenetic dysregulation [289].

Epigenetic modifications can come from environmental toxicants (Fig. 21.3), such as endocrine-disrupting pesticides [290] and bisphenol A [291]. Although epigenetic effects of environmental toxins are expected of being genomewide, a hypothesis is emerging that some genetic loci may be particularly sensitive to epigenetic alterations because of their location to the X chromosome or parentally imprinted chromosomal loci. Using available mouse models for genetic factors implicated in autism, together with experimental exposures to one or more of the suspected toxicants, future experiments can be designed to test the epigenetic and behavioral outcomes of combined genetic and environmental toxicant risk factors in the development of social behavior and cognition in mouse models.

21.8 SUMMARY

Autism spectrum disorders (ASD) are devastating disorders that appear to be increasing at great cost to the affected individual, the family, and society. As a result, autism has become a major focus of biomedical research in the neurosciences. A great deal of progress has been made in the classification and early diagnosis of ASD, but the lack of specific biomarkers of autism with clear and diagnostic physiological indices of ASD is hampering development of research in the field. Continued research to delineate and better define brain anomalies in autism should be a high priority. Research on the role of perinatal exposure to environmental contaminants, including metals such as mercury, lead, and arsenic, as well as organic pollutants such as PCBs and PBDEs in autism, is timely and important. Establishment of a role for such agents in autism could result in important regulation of their manufacture, use, and environmental contamination that could reduce exposure in the future. The development of highly relevant animal models of genetic risk factors in ASD to study epigenetics, pathophysiology, and behavioral underpinning of ASD is moving forward at an accelerating rate, promising to provide necessary tools to understand the causes and to develop effective treatments for ASD and ASD-related neurodevelopmental disorders.

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