9 Environmental Pressures on Transgenerational Epigenetic Inheritance

An Evolutionary Development Mechanism Influencing Atypical Neurodevelopment in Autism?

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9.1 INTRODUCTION

Research in developmental neuropsychiatric conditions has revealed morphological and functional divergences in the brain. In some cases, the divergences occur due to one or two highly penetrant genomic mutations. In case such as autism, mutations in varied sets of genes may produce a convergent autism behavioral phenotype. It is thus likely that there may be other forms of non-genomic regulation of gene expression during development affecting behavioral outcome. Epigenetic gene regulation is one such mechanism that can permanently switch on or switch off gene expression, and these epigenetic changes can be inherited from one cell stage to another during differentiation, mimicking the effects of genomic mutations. Epigenetic gene regulation occurring during early developmental stages of cellular differentiation, which are highly sensitive to environmental cues, is the primary mechanism responsible for the phenomenon known as *evolutionary development* or "evo-devo." This chapter discusses these mechanisms in the context of autism and the environmental factors that influence it. We also discuss the latest technologies that are used to study these mechanisms and the impacts they might have on our current understanding of neuropsychiatric conditions and their association with evolutionary development.

Autism spectrum disorders (ASDs), as defined by the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; APA, 2013), are neurodevelopmental conditions diagnosed on the basis of a dyad of behavioral impairments: impaired social communication, alongside unusually narrow and repetitive interests and activities. Atypical language development, which was a third behavioral impairment defined in DSM-IV, was removed in DSM-5, and is now classified as a co-occurring condition. The DSM-5 criteria also emphasize the dimensional nature of autism. Although the term ASD is frequently used, the term autism spectrum condition is sometimes preferred, as it also indicates a biomedical diagnosis requiring individual support and recognizes affected individuals as different, but without the negative connotations of the term "disorder" (Lai et al., 2014).

Many genes have been identified as having an association with an autism diagnosis (Bourgeron, 2015; O'Roak et al., 2011, 2012). The Simons Foundation Autism Research Initiative (SFARI) database catalogues the most relevant of these genes according to the type of genetic variations from whole-genome sequencing studies, rare genetic mutations, and mutations causing syndromic forms of autism. It was first published in 2009 (Basu et al., 2009), and an up-to-date reference for all known genes can be found at https://gene.sfari.org/autdb/HG_Home.do. This catalogue currently contains >800 genes with a significant association with autism, 99 of which are classified as "high-confidence" genes (being independently replicated, and most of which are rare genetic variants). Although common genetic variants are thought to contribute around 50 percent of the variance (Bourgeron, 2015), many common genetic variants have not been successfully replicated. Indeed, genome-wide association studies of autism have only replicated five common genetic variants linked to autism, even though the actual number is likely to be hundreds (Bourgeron, 2015). The large number of associated genes has made it a complicated task to identify pathways and mechanisms. One subset of autistic individuals[1] may carry a single mutation in a highly penetrant gene, while another subset may carry many common variants, each of small effect, while in a subset of typical individuals a deleterious mutation in the same gene(s) might not be enough to produce autism at all (Bourgeron, 2015). This extreme genetic heterogeneity has resulted in research into the environmental and hormonal associations of autism in an effort to understand if these environmental or epigenetic factors can help explain such diverse genetic mechanisms and if these implicate a common final pathway. Preliminary

[1] We use "identity-first" language (autistic individuals) rather than person-first language (individuals with autism) to reflect the preference of the autism community (http://autisticadvocacy.org/about-asan/identity-first-language).

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estimates suggest environmental factors to account for up to 38.1 percent of all cases (Sassone-Corsi & Christen, 2015).

Although autism environmental research is still in its infancy, there have been many epidemiological studies demonstrating significant associations of various environmental agents with autism, and the timing of exposure (Figure 9.1) seems to be of importance to an autism outcome (Modabbernia et al., 2017; Rossignol et al., 2014). Preconception factors include advanced parental age (Wu et al., 2017) and exposure to toxic substances in the period prior to conception (McCanlies et al., 2012). Gestational factors include exposure to insecticides and pesticides (Rauh et al., 2006; Roberts & English, 2013), air pollution (Volk et al., 2011), drugs (Gentile, 2014; Kobayashi et al., 2016), high levels of fetal and maternal steroids (Baron-Cohen et al., 2015; Kosidou et al., 2016), and maternal stress and immune activity (Ronald et al., 2010; Masi et al., 2015). Events occurring at birth such as birth injury, trauma, and hypoxia are also strongly associated (Gardener et al., 2009, 2011; Modabbernia et al., 2016), while perinatal and childhood factors include exposure to inorganic mercury (Yoshimasu et al., 2014), heavy metals (Price et al., 2010), air pollution, and particulate matter (PM) (Windham et al., 2006). Many of the environmental and hormonal factors associated with autism have the ability to cause sweeping gene regulatory effects through transcriptional and epigenetic mechanisms (Abel & Zukin, 2008; Bao & Swaab, 2011; McCarthy & Nugent, 2013; Tsai et al., 2009). We should note that until these environmental factors are well replicated and can be established as being of "high confidence," we should keep an open mind that some of these may be spurious associations (http://tylervigen.com/spurious-correlations).

Human behavioral divergences associated with autism may involve developmental mechanisms causing permanent transgenerational inheritance during cellular differentiation in a phenomenon referred to as "evolutionary development" or evolution of development (or "evo-devo") (Carroll, 2008). This phenomenon is not well understood, as it involves several molecular mechanisms that produce wide-ranging phenotypes originating from a single genomic template (or genotype). It is a form of evolution that does not arise out of natural selection of genomic mutations, but by the selection of phenotypic features that mimic the effects of genomic mutations. This occurs as organisms undergo morphogenesis by differentiation of cells during development. Non-genomic factors such as regulatory proteins and RNA periodically switch on and switch off gene expression to produce heritable gene expression states analogous to the effects of genomic mutations. This selection of phenotypic features during development through the action of such non-genomic factors can also be triggered by atypical environmental stimuli. This brings about cellular divergences during development that are responsible for permanent phenotypic alterations during adulthood. The ability of the environment to alter and select cellular developmental mechanisms that result in permanent phenotypic divergences during adulthood is an example of how "evo-devo" mechanisms of transgenerational inheritance work in multicellular organisms during development.

One of the mechanisms of "evo-devo" involves epigenetic regulation that is later consolidated as heritable changes (Gerhart & Kirschner, 1997). This chapter reviews studies that have shown how environmental influences during development cause behavioral divergences with respect to autism and associated neurodevelopmental conditions, as well as providing a description of cutting-edge technologies that have started to investigate this heritable gene regulatory mechanism. This chapter also discusses these mechanisms in the context of autism and how the environment applies selection pressure to produce phenotypes that are missing heritability factors. As these processes are observable within a short time frame, they can be easily studied in humans using a laboratory model such as the induced pluripotent stem cell (iPSC) model, combining this with cutting-edge sequencing-based methods to study epigenetic regulation. The study of the role of the environment in bringing about

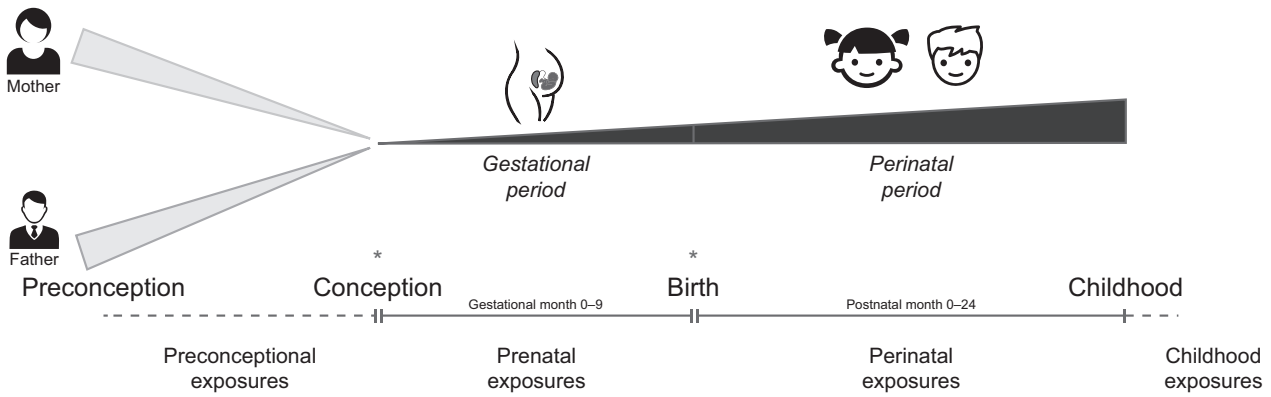

Figure 9.1 Timing of environmental exposures associated with autism. *Exposure to toxic substances or traumatic events around conception and birth are strongly associated with an autism outcome.

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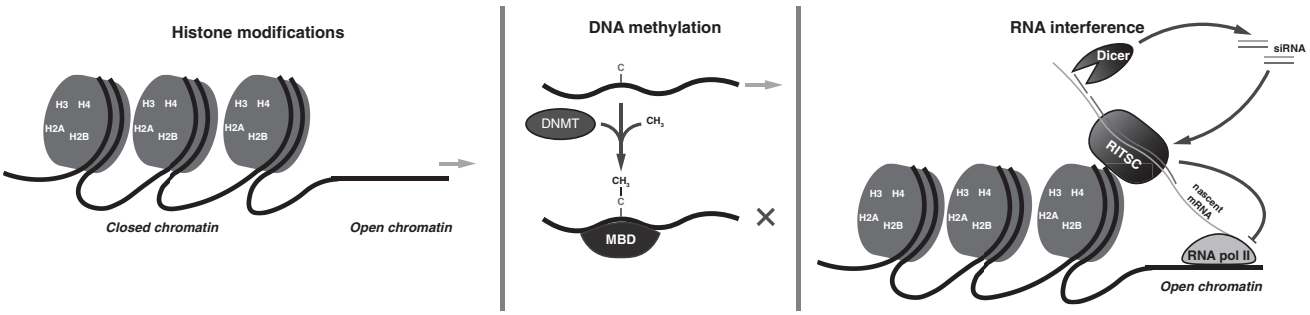

Figure 9.2 Major cellular epigenetic mechanisms responsible for transgenerational inheritance. siRNA: small interfering RNA.

permanent changes in brain development, especially with respect to autism, may provide novel insights into our understanding of the development of the human brain, a research theme that will likely predominate over the next decade. When studies using the above methods are performed in other animals, it will also give us a better understanding of the "evolution of function" (Carroll, 2008) of epigenetic proteins and the origin of epigenetic regulation as an agent of "evo-devo."

9.2 TRANSCRIPTIONAL AND EPIGENETIC REGULATORY MECHANISMS

Transcription factors are proteins that form complexes or may act alone to bind DNA and control the rate of gene expression (Latchman, 1997). They are also involved in the process of transcribing DNA into RNA, the first step in the central dogma of molecular biology (Crick, 1970). This transfer of sequential information from DNA to RNA then to protein is crucial to transforming genomic information into visible phenotypes. As a consequence, manipulation or disruption of this process can alter this sequential transfer of information, affecting the phenotype and often manifesting in a "disease" state. Most transcription factors act in a transient fashion, and removal of stimuli generally dismantles the transcription factor complex. However, certain classes of transcription factors, known as epigenetic factors, can produce a more long-term effect on gene regulation by a mechanism known as imprinting (Berger et al., 2009). Epigenetic factors can activate or repress genes permanently in a configuration that is often inherited into the next generation.

The first transcription factors discovered were polymerases between 1955 and 1961 (Hurwitz, 2005). Polymerases are the most fundamental component of any transcription factor complex, such as the DNA-dependent RNA polymerase, which transcribes DNA into RNA. Different transcription factors have dedicated functions in different tissues of the body. For example, MEF2, Pax6, CREB, and NF-κB are known to drive gene expression in the developing brain (Mao et al., 1999; O'Neill & Kaltschmidt, 1997; Rammes et al., 2000; Warren et al., 1999). NF-κB is one of the best-characterized transcription factors, which responds to changing levels of neurotransmitters as well as modulating the immune response against infection and injury in the brain (O'Neill & Kaltschmidt, 1997). Responses to different kinds of stimuli, including various environmental ones, are convergent functions of transcription factors. Epigenetic factors, which alter DNA conformation, act like regulating mechanisms for transcription factor activities.

This is an important step in determining cell fate and lineage, as it is epigenetic factors that determine which chromosomal regions are expressed based on stimuli received from the surrounding environment. This results in activation of a subset of genes, namely those that are required for determining a particular cell lineage. The combined activities of epigenetic factors and transcription factors are responsible for giving rise to the thousands of different cell types in the human body from a single set of genetic information. The difference between epigenetic and transcription factors is that the effects of the former are inherited in each sister cell after mitosis, while the latter act more on the instructions set by epigenetic factors depending on the lineage of the cells. It is believed that cancer cells show atypical epigenetic signatures, often de-repressing lineage-nonspecific genes, causing genomic instability and cancer progression (Kanwal et al., 2015).

There are three types of epigenetic modifications: (1) DNA methylation; (2) histone regulation; and (3) gene silencing by noncoding RNA (Figure 9.2) (Handy et al., 2011). How some of these mechanisms are dysregulated to produce atypical phenotypes in autism is described in Section 9.3.

9.3 TIMING OF ENVIRONMENTAL EXPOSURE

Every cell in the body has a unique epigenetic signature. The epigenetic signatures of some cells may be more closely related than others depending on the tissues they form and the functions they carry out. Stem cells have an open epigenetic state, as opposed to differentiated cells (e.g., neurons, muscle, blood, and liver), which have an ON-memory epigenetic state that maintains the stability of the mechanisms necessary for cellular identity (Hormanseder et al., 2017). The temporal differences in transcriptional regulation mean that the stage of cellular

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development at which certain environmental exposures occurs is crucial in determining the phenotypic fate. This section discusses the timeline of exposures known to be associated with a diagnosis of autism. Possible cellular/molecular mechanisms resulting from these exposures are discussed in Section 9.4.

9.3.1 Preconception Exposure

Exposure of parents to toxic chemicals prior to conception is significantly associated with autism in children. A 1976 study found 26 percent of families with an autistic child (n = 78) had parents who were exposed to toxic chemicals at work, compared to only one percent of parents with typically developing (TD) children (Coleman, 1976). Parents of children with autism (n = 93) were more likely to have been exposed to lacquer, varnish, solvents, and asphalt from their workplace during the three months prior to conception, compared with parents of TD children (n = 81) (McCanlies et al., 2012). However, the overall significance of these studies is limited by their small sample sizes, and further investigation is required to these establish association in a wider population in case these are spurious correlations.

9.3.2 Prenatal Exposure

The fetus is in the most vulnerable stage of human development, and any chemical imbalances in the mother or atypical exposure to toxins can affect fetal development. Some of the most toxic substances for the fetus are pesticides, and prenatal exposure to organochlorine pesticides is associated with heightened likelihood of autism, with the highest likelihood in mothers who lived within 500 m of the source (Roberts et al., 2007).

Children with higher exposure to organophosphate insecticides are also significantly more likely to develop symptoms of pervasive developmental disorders (Rauh et al., 2006). Maternal exposure to air pollution during pregnancy is also highly associated with autism, and one study showed pregnant mothers living near freeways were more likely to have children with autism (Volk et al., 2011). Air pollutants associated with higher risk of autism include quinoline, styrene, ozone, nitrogen dioxide, and PM including mercury, lead, nickel, manganese, diesel particulates, and methylene chloride (Becerra et al., 2013; Kalkbrenner et al., 2010; Roberts et al., 2013; Volk et al., 2013). Again, which of these findings are true or spurious correlations needs to be investigated.

Steroids are important factors in brain development, and higher levels of circulating sex steroids in the male fetus are positively associated with autism (Baron-Cohen et al., 2011, 2015). Mothers with polycystic ovary syndrome, characterized by hyperandrogenism and elevated levels of androgens during pregnancy, are also at greater risk of having children with autism (Kosidou et al., 2016; Pohl et al., 2014). Prenatal stress is another trigger for a range of postnatal outcomes associated with autism, including core symptoms such as hypersensitivity and repetitive behavior, cognitive deficits, and abnormalities in immune function (Kinney et al., 2008). The molecular mechanisms responsible for increased susceptibility to prenatal factors is not well understood.

9.3.3 Perinatal and Childhood Exposure

The period around birth and the first 24 months of a child's life are also susceptible periods for toxic environmental exposures, although to a lesser degree than the fetal stage. Exposures to pesticides such as phosphine (a fungicide) and dichlorodiphenyltrichloroethane (DDT) during childhood have been linked with increased prevalence of autism (Audouze & Grandjean, 2011; Garry et al., 2002). Childhood exposure to air pollution appears to be an especially important factor associated with autistic symptoms. A survey of children at ~24 months (2 years of age) identified cadmium, nickel, trichloroethylene, vinyl chloride, and diesel PM to be highly correlated with an autism diagnosis (Windham et al., 2006). Heavy metal exposure during childhood is also associated with autism prevalence. The role of mercury exposure in autism is not clear (Palmer et al., 2006), although some studies have found a strong positive association with autism (Blanchard et al., 2011; Palmer et al., 2009). However, childhood exposure to lead has been reported to have a high positive association with autism (Price et al., 2010). The perinatal period is also associated with the establishment and stabilization of healthy gut microbiota, and the disruption of this process either through birth by cesarean section, preterm birth, or the use of antibiotics can lead to atypical microbiota colonization, affecting brain development and behavior (Clarke et al., 2014; Diaz Heijtz et al., 2011). Epidemiological studies have revealed an association between autism and atypical perinatal microbiota (Finegold et al., 2002; Mittal et al., 2008), while altered microbiota colonization patterns have also been associated with studies in animal models of autism (de Theije et al., 2014b). Which of these putative environmental factors will emerge as "high confidence" (i.e., well replicated) remains to be seen.

The association of childhood toxic exposure with autism diagnosis demonstrates that the developing brain during the first two years of life is almost as susceptible to environmental insults as the fetal brain. The large body of epidemiological studies discussed in this section have identified an exposure window extending from preconception until two years after birth, demonstrating that the mechanisms responsible for the appearance of autism phenotypes can be triggered through exposures from parents, transmitted through the placenta, or transmitted through direct exposures after birth. At the prenatal, neonatal, and postnatal stages, the problem for scientists is to disentangle possible causal agents from mere correlational factors, since the epidemiological design that has

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revealed the majority of these agents and factors does not allow one to achieve such a separation. The strength of the iPSC model system is that causality can be directly tested by experimental manipulation.

9.4 MOLECULAR MECHANISMS TRIGGERED BY ENVIRONMENTAL AGENTS

The effect of environmental risk factors on mental health was not recognized until recent epidemiological studies demonstrated associations with neurodevelopmental conditions such as autism. The influence that these environmental agents have on the molecular machineries of the developing brain is still not fully understood. Some molecular mechanisms have been identified through pharmacological studies using animal models (Table 9.1), and they have revealed two major effects of environmental agents on animal cell biology, as discussed in this section: (1) activation of oxidative stress, the immune system, and inflammation; and (2) modulation of epigenetic gene regulation. It is interesting to note that the genes involved in both biological mechanisms are strongly associated with autism (Bourgeron, 2015); however, currently no evidence exists to suggest that environmental agents act via these mechanisms to trigger the condition. This section discusses the prominent molecular mechanisms activated by environmental risk factors, aiding understanding of their effects and their potential involvement in the triggering of neurological symptoms.

9.4.1 Pesticides

Pesticides are chemical compounds used to control, repel, or kill plants or animals that are considered to be "pests" (Corsini et al., 2013). They include herbicides, fungicides,

Table 9.1 Major environmental agents and mechanisms affected.

Environmental factor categories	Physiological system affected	Environmental agent	Mechanisms of action	References
Pesticides	Immune system	Organophosphates	Reduces T-cell proliferation, inhibits NK cells, LAK cells, and CTLs Dysregulates cell death	Galloway & Handy, 2003; Li, 2007
		Organochlorides	Immunosuppression Disrupts ability to inactivate or eliminate pathogens Aberrant T- and B-cell regulation Cytokine secretion	Cooper et al., 2004; Hermanowicz & Kossman, 1984; McConnachie & Zahalsky, 1992; Reed et al., 2004; Schaalan et al., 2012; Vine et al., 2000
	Cellular epigenetics	Organophosphates	Induces alkaline damage and DNA damage	Ray & Richards, 2001
		Organochlorines	Alters DNA methylation patterns Dysregulates histone acetylation Affects male and female reproductive systems Mimics epigenetic regulation by endogenous hormone Disrupts hypothalamic gene expression	Desaulniers et al., 2009; McLachlan et al., 2006; Shutoh et al., 2009; Song et al., 2010, 2011; Stouder & Paoloni-Giacobino, 2011; Zama & Uzumcu, 2009
Metals	Cellular oxidative stress	Cadmium	Replaces iron and copper in proteins Causes DNA damage in the CNS	Price & Joshi, 1983; Waalkes, 2000; Watjen & Beyersmann, 2004
		Lead	Inhibits trace element absorption Elevates oxidative stress	Gurer & Ercal, 2000; Hoffman et al., 2000;

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Table 9.1 Cont.

Environmental factor categories	Physiological system affected	Environmental agent	Mechanisms of action	References
			Directly increases ROS Depletes antioxidant pools	Hunaiti & Soud, 2000; Patrick, 2006
		Nickel	Hydroxyl radical formation Oxidative DNA damage Elevate sister-chromatid exchanges during cell division Disrupts histone–DNA binding	Bal et al., 2000; Dally & Hartwig, 1997; M'Bemba-Meka et al., 2007
		Mercury	Accumulates as organomercuric compounds Crosses placental barrier and blood–brain barrier Hypoxia, oxidative stress, disruption of brain development	Guzzi & La Porta, 2008
	Cellular epigenetics	Cadmium	Increases DNMT activity Dysregulates DNA methylation Induces genomic imprinting	Doi et al., 2011; Somji et al., 2011; Takiguchi et al., 2003
		Nickel	Inhibits DNA repair Histone deacetylation Histone ubiquitination Disrupts histone–DNA binding Influences binding of MeCP2 to DNA	Borochov et al., 1984; Chen et al., 2006; Ji et al., 2008; Karaczyn et al., 2006, Ke et al., 2008; Martinez-Zamudio & Ha, 2011; Yan et al., 2003
		Mercury	Alters histone methylation and acetylation Reduces BDNF expression	Onishchenko et al., 2008
		Lead	Affects DNA methylation	Bollati & Baccarelli, 2010; Wright et al., 2010
Air pollution	Cellular oxidative stress and innate immunity system	UFPM	Neuro-inflammation Delivers toxic substances into the CNS Stimulates pro-inflammatory cytokines Induces microglial activation	Block & Calderon-Garciduenas, 2009; Long et al., 2007; Nemmar & Inuwa, 2008; Oberdorster et al., 2004; Tin Tin Win et al., 2008; Valavanidis et al., 2008; Wang et al., 2007, 2008
		Ground-level ozone	Oxidative stress on proteins and lipids Brain lipid peroxidation Dopaminergic neuron death Morphological damage in neurons Stimulates pro-inflammatory cytokines	Angoa-Perez et al., 2006; Araneda et al., 2008; Guevara-Guzman et al., 2009; Pereyra-Munoz et al., 2006
	Cellular epigenetics	PAH	Dysregulated methylation of genes associated with neural impairments	Bellavia et al., 2013; Breton et al., 2012; Liang et al., 2012;

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Table 9.1 Cont.

Environmental factor categories	Physiological system affected	Environmental agent	Mechanisms of action	References
			Dysregulated cell death and survival mechanisms	Madrigano et al., 2012; Pavanello et al., 2010; Rossnerova et al., 2013; Tang et al., 2010
		PM	Decreased methylation of iNOS gene	Breton et al., 2012; Sofer et al., 2013
GI exposures	Immune system	Atypical bacterial gut flora	Maternal immune activation Affects neurogenesis Dysregulation of secondary messengers	Choi et al., 2016; Emanuele et al., 2010
		Microbe-released metabolites	Activates immune responses	Rieder et al., 2017
		Prolonged antibiotic treatment	Reduces monocyte numbers	Mohle et al., 2016
	Gut–brain axis	Prolonged antibiotic treatment	Atypical GABA receptor expression in PFC neurons Decreased neurogenesis	Mohle et al., 2016; Sharon et al., 2016
		Microbe-released metabolites	Autism symptoms Anxiety-like behaviors Bacteria-derived glutamate directly affect neurotransmission Atypical GABA signaling Dysregulation of synaptic transmission genes and neurophysiological mechanisms	Ding et al., 2017; Favre et al., 2013; Foley et al., 2014; Kuo & Liu, 2017; Mohle et al., 2016; Persico & Napolioni, 2013; Rieder et al., 2017; Sharon et al., 2016; Sheldon & Robinson, 2007; Wang et al., 2012

NK: natural killer; LAK: lymphokine-activated killer; CTL: cytotoxic T lymphocyte; CNS: central nervous system; ROS: reactive oxygen species; DNMT: DNA methyltransferase; MeCP2: methyl CpG binding protein 2; BDNF: brain-derived neurotrophic factor; UFPM: ultrafine particulate matter; PAH: polycyclic aromatic hydrocarbon; PM: particulate matter; iNOS: inducible nitric oxide synthase; GI: gastrointestinal; GABA: γ-aminobutyric acid; PFC: prefrontal cortex.

and insecticides. Pesticides have widespread uses, including heavy use in agriculture worldwide, and their pervasive presence in living and working environments is a major public health concern. Because of their widespread use, there are always low levels of pesticides prevalent in the circulation.

9.4.1.1 Immune System Modulation

Pesticide exposure has been associated with autism. Several studies have identified the immune system to be very susceptible to pesticide exposure, with children especially vulnerable because of their immature immune system (Corsini et al., 2008; Repetto & Baliga, 1997; Wigle et al., 2008). Pesticides can cause acute alterations in immune responses in children, making them susceptible to infection and immune disorders (Phillips, 2000). Organophosphates affect the immune system by reducing T-cell proliferation and inhibiting natural killer (NK) cells, lymphokine-activated killer cells, and cytotoxic T lymphocytes (Galloway & Handy, 2003; Li, 2007). Several immune activation pathways are affected, and proteases responsible for inducing programmed cell death in target cells are suppressed (Galloway & Handy, 2003). Organochlorine pesticides have immunosuppressive effects. DDT can alter immunoglobulin levels (Cooper et al., 2004; Vine et al., 2000), disrupting its ability to inactivate or eliminate pathogens and thus altering resistance to infections (Hermanowicz & Kossman, 1984). Organochlorines are also responsible for aberrant peripheral T- and B-cell regulation (McConnachie & Zahalsky, 1992), decreased NK cell activation (Reed et al., 2004), cytokine secretion (Schaalan et al., 2012), and autoimmune activation (McConnachie & Zahalsky, 1992).

9.4.1.2 Epigenetic Modulation

In addition to immune dysregulation, pesticides are also able to modify epigenetic gene regulatory functions. DDT and other organochlorine pesticides are known to alter

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DNA methylation patterns in the hippocampus in animal studies, and CpG islands have been found to be hypomethylated compared to typical controls (Desaulniers et al., 2009; Shutoh et al., 2009). One organochlorine pesticide, methoxychlor (MXC), is part of the environmental endocrine disruptor family, which mimic endogenous hormones and cause epigenetic dysregulation (McLachlan et al., 2006). MXC affects both the male and female reproductive systems (Stouder & Paoloni-Giacobino, 2011), disrupts expression of the hypothalamic genes responsible for normal reproductive function (Stouder & Paoloni-Giacobino, 2011), and downregulates estrogen receptor β expression in ovaries (Zama & Uzumcu, 2009). MXC also alters DNA methylation patterns of paternally as well as maternally imprinted genes (Stouder & Paoloni-Giacobino, 2011). Organochlorines Paraquat and Dieldrin have been associated with exacerbating Parkinson's disease symptoms. Exposure to Paraquat induces histone H3 acetylation and reduces histone deacetylase (HDAC) activity in N27 dopaminergic cells (Song et al., 2010, 2011). Dieldrin has also been associated with a time-dependent increase in histone H3 and H4 acetylation and upregulation of histone acetyltransferases, and prolonged Dieldrin exposure in rats has been shown to induce histone "hyperacetylation" in the striatum and substantia nigra (Song et al., 2010). Organophosphates are also able to cause alkaline damage and induce DNA methylation (Ray & Richards, 2001).

9.4.2 Metals

Metal ions are important elements that carry out a wide range of biological functions in the body. A delicate metal-ion homeostasis is maintained in cells with the help of protein transporters. However, as metals form strong ionic bonds, a breakdown of metal-ion homeostasis by the introduction of metals foreign to the body may result in strong binding of these metals to ligands designed for a different metal, and this may result in oxidative stress and deterioration of protein function, leading to cellular death and disease in the organism (Jomova & Valko, 2011).

9.4.2.1 Oxidative Stress

As discussed in Section 9.3.3, exposure to metals such as mercury, lead, nickel, manganese, and cadmium are associated with autism. Toxic exposure to cadmium can replace iron and copper in various proteins, thereby increasing free iron and copper ions, which can cause oxidative stress (Price & Joshi, 1983). Cadmium-induced free copper ions break down hydrogen peroxide into free radicals that are able to cause DNA damage in the central nervous system (Waalkes, 2000; Watjen & Beyersmann, 2004). Lead, a heavy metal, can damage cellular components by elevating oxidative stress, and it can competitively bind to protein enzymes, inhibiting trace element absorption (Patrick, 2006). Lead also deactivates certain classes of antioxidants (Patrick, 2006). Lead has a dual mechanism of action to elevate oxidative stress: it directly increases reactive oxygen species (Gurer & Ercal, 2000; Hunaiti & Soud, 2000), while it also depletes antioxidant pools through inhibition of enzymes such as glutathione reductase and δ-aminolevulinic acid dehydrogenase (Hoffman et al., 2000).

Nickel is another metal that causes oxidative stress. Under different physiological conditions and ion concentrations, it can cause hydroxyl radical formation and oxidative DNA damage or induce elevated sister-chromatid exchanges during cell division (Dally & Hartwig, 1997; M'Bemba-Meka et al., 2007). Oxidation–reduction (redox) activity of nickel can disrupt histone–DNA binding (Bal et al., 2000).

Mercury accumulates inside the body in the form of organomercury compounds (Guzzi & La Porta, 2008). Ethylmercury and methylmercury can also cross the placental barrier and blood–brain barrier (BBB), but are not excreted through urine. The brain is the major organ affected by mercury compounds, where its accumulation causes hypoxic conditions, oxidative stress, cell death, and disruption of fetal brain development during gestation. Elemental mercury also accumulates in the brain.

9.4.2.2 Epigenetic Modulation

Metals ions can modulate transcriptional regulation through epigenetic modifications. Cadmium increases DNA methyltransferase (DNMT) activity and DNA hypermethylation-induced gene silencing in rats (Takiguchi et al., 2003). In the chicken embryo, short exposures of cadmium can have a hypomethylating effect (Doi et al., 2011). Cadmium can induce genomic imprinting, leading to heritable changes in chromatin structure (Somji et al., 2011). Nickel ions can modulate the DNA methylation status of cells. After exposure to nickel, cells that are transformed show silencing of DNA-repairing gene O6-methylguanine DNA methyltransferase (MGMT) expression by DNA hypermethylation of its promoter, in addition to histone H3 deacetylation and H3K9me2 enrichment (Ji et al., 2008). DNA–histone interactions can also be disrupted, leading to abnormal condensation of higher-order chromatin structures (Borochov et al., 1984) and global deregulation of gene expression (Martinez-Zamudio & Ha, 2011). Some studies have observed histone H2A/H2B ubiquitination after nickel exposure (Karaczyn et al., 2006; Ke et al., 2008). Nickel also influences the binding characteristics of MeCP2, an autism risk gene (Chen et al., 2006; Yan et al., 2003).

Mercury is the third metal showing an association with autism prevalence, and in its methylmercury form interacts with histone-modifying factors to increase levels of H3K27me3 and decrease levels of H3Ac in promoter regions of neurodevelopmental genes such as brain-derived neurotrophic factor (BDNF). BDNF, a gene associated with autism, is one of the promoters most affected, resulting in reduced expression in the dentate gyrus, with appearance of depression-like symptoms in mice (Onishchenko et al.,

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2008). Lead has unknown effects on DNA methylation and epigenetic regulation; however, one study has shown lead to be associated with reduced DNA methylation levels (Wright et al., 2010). In vitro studies have also shown lead to affect DNA methylation (Bollati and Baccarelli, 2010).

9.4.3 Air Pollution

Air pollution is a heterogeneous mixture of suspended particles, gases, and fumes that have adverse effects on the human body. It is composed of PM, gases (e.g., carbon monoxide, sulfur oxides, nitrogen oxides, ground-level ozone), suspended organic compounds (e.g., aromatic hydrocarbons, endotoxins), and metals (e.g., mercury, lead, nickel, manganese, vanadium) (Block & Calderon-Garciduenas, 2009). Components of air pollution are strongly linked with autism occurrence (Becerra et al., 2013; Kalkbrenner et al., 2010; Roberts et al., 2013; Volk et al., 2013). Unsurprisingly, dominant molecular mechanisms triggered by air pollution include increased oxidative stress, altered innate immunity, and chronic neuro-inflammation (Block & Calderon-Garciduenas, 2009), and, in some cases, epigenetic modulation of gene expression (Ji et al., 2016; Lin et al., 2016, Silveyra & Floros, 2012).

9.4.3.1 Oxidative Stress and Innate Immune System Modulation

Unlike pesticides and metals, the effect of air pollution on the human brain has been the focus of many studies after it was found that air pollution could cause ischemic stroke (Thomson et al., 2007) and neurodegeneration (Calderon-Garciduenas et al., 2008a, 2008b). Air pollution can trigger the release of cytokines, which are major neuro-inflammatory agents and act as a communication links between the brain and peripheral immune system. Triggering of pro-inflammatory signals in peripheral organs such as the lungs, liver, and the cardiovascular system results in a systemic innate immune response. Blood cytokine concentrations of IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor are elevated, while also elevating immune cell populations of marrow-derived neutrophils and monocytes in both human and animal models (Mills et al., 2009).

Cytokines are able to cross the BBB to produce neuro-inflammation, neurotoxicity, and vascular damage (Ling et al., 2006; Manousakis et al., 2009; Perry et al., 2007; Qin et al., 2007; Rivest et al., 2000). Ultrafine PM (UFPM) can penetrate into neurons and the brain through several routes, producing direct air pollution-induced neuro-inflammation by activation of the brain's immune system (Block & Calderon-Garciduenas, 2009; Valavanidis et al., 2008). Experimental inhalation of UFPM in rodents has revealed efficient translocation of these nano-sized particles in the systemic circulation and the brain (Nemmar & Inuwa, 2008; Oberdorster et al., 2004). UFPM is able to cross traditional barriers in the lungs, be carried by erythrocytes, cross the BBB, and finally end up in neurons (Block & Calderon-Garciduenas, 2009).

The nasal epithelium is another portal of entry for UFPM, and inhaled particles can be traced through the nasal olfactory pathway, trigeminal nerves, brainstem, and hippocampus (Wang et al., 2007, 2008). Once in the brain, UFPM may trigger an immune response in two ways: (1) being made up of nanocarbon-based particles, UFPM adsorbs toxic organic compounds that are easily delivered into the central nervous system, in the same way as designer nanoparticles deliver custom drugs. Metals, hydrocarbons, and lipopolysaccharides have been associated with UFPM-adsorbed chemical-induced neuro-inflammation; and (2) UFPM themselves may stimulate innate immunity in the brain, and rodent studies have shown that carbon black particles (model of UFPM without adsorbed compounds) elevate pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ) in the olfactory bulb (Tin Tin Win et al., 2008) and induce activation of microglial cells (Long et al., 2007).

Sometimes, emissions generated from industry and vehicles are able to react with each other to form ground-level ozone, which is a strong oxidizing agent when inhaled. Ozone reacts with proteins and lipids, inducing oxidative stress. It is unlikely that ozone physically reaches the brain, as it is a reactive molecule with a short half-life, but breakdown products can reach the brain, and studies in rats have shown that chronic exposure to ozone leads to brain lipid peroxidation, dopaminergic neuron death, and neuron morphological damage (Angoa-Perez et al., 2006; Guevara-Guzman et al., 2009; Pereyra-Munoz et al., 2006). Astrocytes located near brain capillaries in rats have also been shown to have enhanced ozone-induced expression of pro-inflammatory factors (Araneda et al., 2008).

9.4.3.2 Epigenetic Modulation

Some cellular/molecular changes brought about by air pollution are mediated through epigenetic regulation of transcription. Air pollutants such as polycyclic aromatic hydrocarbons (PAHs) including benzo[*a*]pyrene (B[a]P), by-products of incomplete fossil fuel combustion, and PM can induce hypomethylation and hypermethylation of specific genes associated with increased risk of neural impairments (Madrigano et al., 2012), cell death and survival mechanisms (Bellavia et al., 2013; Pavanello et al., 2010), and the immune system and inflammation (Breton et al., 2012). One study found air pollution induced hypomethylation in 58 CpG loci in different genes with a greater than 10 percent difference in children in a highly polluted area than in a non-polluted area (Rossnerova et al., 2013). PAH exposure was associated with hypomethylation in the promoters of MGMT, p53, and IL-6, genes involved in the cell death and survival mechanisms, and the immune system (Bellavia et al., 2013; Pavanello et al., 2010).

Another important gene that is able to detoxify PAHs, glutathione S-transferase Mu2 (GSTM2), is hypermethylated at its promoter region by PAHs, thus inhibiting its

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transcription, leading to suppressed detoxification processes (Tang et al., 2010). B[a]P induced promoter methylation of the retinoic acid receptor β2 (RAR-β2) gene (Ye & Xu, 2010), and also decreased acetylation of histone H3K14 associated with the steroid acute regulatory (StAR) protein (Liang et al., 2012). Increased levels of PM are also associated with decreased methylation within the CpG island of the inducible nitric oxide synthase (iNOS) gene, while increased methylation in endothelial nitric oxide synthase (eNOS) disrupted nitric oxide (a key biological messenger) production in the body (Breton et al., 2012). One study also found carbon black to be significantly associated with aberrant methylation patterns in 31 genes (Sofer et al., 2013).

9.4.4 Gastrointestinal Exposure

The gut microbiota play a primary role in gastrointestinal (GI) physiology and is able to modulate microbiota–gut–brain axis signaling (Vuong & Hsiao, 2017). Animal studies have demonstrated the importance of the establishment of symbiotic and commensal bacterial colonies in the gut, and germ-free rodents have been shown to exhibit altered behavior, most often exhibiting reduced social exploration (Arentsen et al., 2015; Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2014) and abnormal transcriptomic profiles across the frontal cortex, striatum, amygdala, and hippocampus (Diaz Heijtz et al., 2011). Altered behavior can be restored by the manipulation of the microbiome by probiotic treatment (Coiro et al., 2015; Hsiao et al., 2013; Shi et al., 2003). In humans, the gut microbiota establish themselves within the first two years after birth (Diaz Heijtz et al., 2011), and disruption in this process can lead to atypical interaction of microbiota with gut-associated immune cells and the enteric nervous system (Quigley, 2016). Atypical gut microbiota may trigger an immune reaction, stimulating the body's immune cells to release inflammatory cytokines, or in certain cases, compounds released by microbiota may enter the bloodstream, and some of these compounds, such as γ-aminobutyric acid (GABA) and 5-hydroxytryptamine (5-HT), are neurotransmitters that can directly affect the central nervous system and alter behavior (Li et al., 2017; Quigley, 2016).

Large numbers of GI problems and microbiota anomalies have been reported in individuals with autism. The most consistent GI observations have been diarrhea, constipation, and abdominal pain, as well as the occurrence of "leaky gut." The Childhood Autism Risks from Genetics and Environment (CHARGE) study showed greater association of GI symptoms with social withdrawal, stereotypy, irritability, and hyperactivity (Chaidez et al., 2014). Fecal bacterial profiling in individuals with autism has revealed a greater abundance of certain bacterial species such as *Clostridium* (Finegold et al., 2002; Parracho et al., 2005; Song et al., 2004), *Sutterella* (Wang et al., 2013), *Lactobacillus*, and *Desulfovibrio* (Tomova et al., 2015), with reduced *Bacteroidetes/Firmicutes* ratio (Tomova et al., 2015), *Prevotella, Coprococcus*, and *Veilonellaceae* (Kang et al., 2013; Williams et al., 2011). This section discusses the mechanisms and effects of gut-mediated immune system modulation and microbiota-released metabolites, as well as their relationship with neuropsychiatric conditions such as autism.

9.4.4.1 Immune System Modulation

The gut microbiota are able to interact with the immune system, altering circulating levels of brain inflammatory factors associated with autism. Elevated IFN-γ, IL-β, IL-6, IL-12p40, TNF-α, monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-β (TGF-β), and chemokine (C–C motif) ligand 2 (CCL-2) have been shown to be associated with autism (El-Ansary & Al-Ayadhi, 2014; Jyonouchi et al., 2001; Suzuki et al., 2011; Xu et al., 2015). Elevated bacterial endotoxin levels have also been found in individuals with autism (Emanuele et al., 2010). Cellular immune response has also been shown to be elevated in autism. For example, an animal model study has shown that maternal immune activation, which is known to be associated with the development of autism, results in the production of Th17 immune cells and secretion of IL-17α, but treatment with the commensal bacteria species *Bacteroides fragilis* is able to correct many of its adverse effects (Choi et al., 2016).

Experiments in animals have also shown that gut microbiota can affect neurogenesis and essential processes inside neurons, including transcriptional regulation of secondary messengers, synaptic transmission genes including postsynaptic density 95 and synaptophysin, and neurophysiological mechanisms (Diaz Heijtz et al., 2011). Long-term antibiotic treatment of adult mice can induce decreased neurogenesis, which can be rescued by probiotic treatment and voluntary exercise (Mohle et al., 2016; Sharon et al., 2016). This occurs as a result of a reduced number of monocytes associated with prolonged antibiotic treatment (Mohle et al., 2016). Gut microbiota have also been associated with atypical GABA receptor expression in prefrontal cortex neurons (Bravo et al., 2011). Several of these mechanisms that are highly correlated with autism are yet to be studied in humans.

9.4.4.2 Gut Microbiota-Released Metabolites Activity

Gut microbiota are known to release metabolites that can directly affect brain function. These include short-chain fatty acids (SCFAs), phenol compounds, and free amino acids (FAAs), peptidoglycan, and lipopolysaccharide (Li et al., 2017; Sharon et al., 2016). For example, elevated SCFAs have been found in the fecal matter of children with autism (Wang et al., 2012). In mice, subcutaneous administration of a SCFA – propionic acid – was shown to result in anxiety-like behavior (Foley et al., 2014). Another SCFA – butyrate – is an inhibitor of HDACs (De Vadder et al., 2014), similar to valproic acid, which is a major autism risk factor (Favre et al., 2013; Kuo & Liu, 2017). Studies using a valproate animal model for autism have

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demonstrated dysregulation of gut microbiota (e.g., an increase in *Firmicutes/Bacteroidetes* species) and also elevation in butyrate levels (de Theije et al., 2014a, 2014b).

However, butyrate is also a ligand for certain G-protein-coupled receptors and is involved in ATP production. It may have a neuroprotective effect (Bourassa et al., 2016), and in one autism animal model study using BTBR mice, butyrate has been shown to improve autism-associated behavioral deficits (Kratsman et al., 2016). Gut bacteria also synthesize phenol compounds such as *p*-cresol from tyrosine, which is easily absorbed through the GI tract and has been postulated to worsen the symptoms of autism (Persico & Napolioni, 2013). High levels of FAAs are observed in fecal samples of children with autism, particularly glutamate, which is easily absorbed and acts as a neurotransmitter. An excess of glutamate may impact behavior and lead to neuronal death (Ding et al., 2017; Sheldon & Robinson, 2007). Peptidoglycans from bacterial cell walls and lipopolysaccharides, which are bacterial endotoxins, are also able to enter through the gut and activate immune responses and atypical neural pathways (Rieder et al., 2017).

Studies so far have identified numerous ways by which environmental pressures increase autism susceptibility and generally increase the propensity for mental health issues. The environmental agents act via multiple transcriptional and epigenetic mechanisms either through direct interaction with molecular machinery or through the activation of immune-responsive cellular pathways and oxidative stress. Most findings have unfortunately been made in animal models that do not properly recapitulate human neurodevelopment or neuropsychiatric conditions. To find out how transcriptional and epigenetic mechanisms imprint gene expression signatures on human cell lineages, research will need to be done on a comparable human model system. Section 9.5 discusses the state of current research that includes animal-based model systems and the future of neuropsychiatric research using iPSC technology.

9.5 METHODS FOR STUDYING TRANSCRIPTIONAL AND EPIGENETIC REGULATION

Studying the interaction between protein and DNA has unique challenges. This is why we know very little about how proteins control gene expression regulation, which in some cases are also inherited from one cell generation to the next. Recent findings are, however, pointing toward a greater role of these gene-regulatory proteins, known as transcription and epigenetic factors. For example, there is greater acknowledgment of the fact that epigenetic gene regulation during development gives rise to the plethora of different cell types of the body from just one starting embryonic stem cell containing only one set of genomic information. The concerted regularity in which genes are "switched on" and "switched off" in different cell types during development, or the aberrant switching on/off of nonspecific genes in cancer cells, has also given rise to the question of how much of an impact the environment can play on this cellular machinery. This section deals with how genes are regulated inside cells and the methods that can capture this unique phenomenon (Table 9.2) to enable the study of typical and disease-state epigenetic gene expression regulation.

9.6 PROTEINS AND RNA INVOLVED IN EPIGENETIC REGULATION

The primary epigenetic proteins in the eukaryote cells are histones. There are four classes of histones: H1, H2 (H2A, H2B), H3, and H4. Histones H2A, H2B, H3, and H4 form

Table 9.2 Methods for studying epigenetic regulation.

Method	Epigenetic mechanism studied	References
Chromatin immunoprecipitation sequencing (ChIP-seq)	Protein–DNA interaction, especially histone–DNA	Hayashi, 1975; Johnson et al., 2007; Kuo & Allis, 1999; Madisen et al., 1998; Sen et al., 1976,
Chromosomal conformation capture (3 C)	Structural properties and spatial conformation of chromosomes	Dekker et al., 2002
Hi-C	Modification of 3 C method using deep sequencing to identify protein-linked DNA sequences	Belton et al., 2012; Hsieh et al., 2016; Lieberman-Aiden et al., 2009; Nagano et al., 2013; Ramani et al., 2017,
Chromatin interaction analysis by paired-end tag (ChIA-PET)	Combines ChIP and 3 C to enable detection of short- and long-range chromatin interactions by protein of interest	Davies et al., 2017; Fullwood & Ruan, 2009; Fullwood et al., 2009,

Continued

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Table 9.2 Cont.

Method	Epigenetic mechanism studied	References
Chip-based methylation arrays	Targeted approach to studying methylated cytosines in the genome	Infinium BeadChip arrays from Illumina
Whole-genome bisulfite sequencing (WGBS)	Detects methylated CpG islands in the whole genome	Gu et al., 2011
Reduced representation bisulfite sequencing (RRBS)	Targeted detection of methylated CpG islands by restriction digestion using MspI	Gu et al., 2011; Guo et al., 2015; Nwankwo & Wilson, 1988; Xu et al., 2005,
MethylC-seq	All methylated cytosines, including non-CpG island methylated cytosines	Urich et al., 2015
Chromatin affinity purification with mass spectrometry (ChAP-MS)	Collective assembly of gene expression regulators at DNA regulatory regions	Byrum et al., 2012
Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)	Open chromatin regions that can be accessed by the hyperactive Tn5 transposase	Buenrostro et al., 2013

an octamer around which DNA is packaged into structural units called nucleosomes (Vardabasso et al., 2014). DNA packaged in this way is inactive. However, histones are able to undergo covalent modifications to unpack the DNA in a precise, coordinated manner to bring about long-term changes in gene expression. Such changes are often inherited from one cell generation to the next (Feil & Fraga, 2012; Henikoff & Shilatifard, 2011; Law & Jacobsen, 2010). Another class of epigenetic proteins make direct modifications on the DNA to influence gene expression regulation. This class of proteins, called DNMTs, is known to methylate cytosine found as part of CpG dinucleotides (Law & Jacobsen, 2010). Methylated CpG islands (mCG) can then bind with methyl-CpG-binding domain proteins MBD1, MBD2, MBD4, and MeCP2, which mediate the repression of gene expression (Stroud et al., 2017). More recent studies have revealed a non-CG methylation site, a CA sequence that can also be methylated at the cytosine (mCA). CA and CG methylation by methyltransferases seems to occur at a high rate and at similar levels in adult neurons, and DNMT3A seems to be primarily responsible (He & Ecker, 2015; Lister et al., 2013; Stroud et al., 2017).

Other than epigenetic proteins, there are also RNA molecules that are able to regulate gene expression by inhibiting translation or degrading cytoplasmic mRNA (Castel & Martienssen, 2013). This form of gene regulation is known as RNA interference (RNAi) and involves microRNAs, small interfering RNAs, and PIWI protein-interacting RNAs. These regulatory RNA molecules are processed by enzymes such as Dicer and Drosha. These enzymes are known to interact with the environment and epigenetic factors to process this form of gene regulation through RNAi, which can be inherited from one cell generation to the next.

In mammals, many of these epigenetic factors have been found to be present at high levels during early embryonic stages of development, which is a possible explanation for cells at various stages of pluripotency being especially susceptible to environmental triggers. The stem cell differentiation process also involves systematic transitions from one epigenetic state to the next, and this most likely makes it vulnerable to epigenetic modifications from external cues (Feil & Fraga, 2012). The endocrine system and its disruptors are also able to influence germ line epigenetic modifications that can be perpetuated through transgenerational inheritance.

9.6.1 Molecular Methods for Epigenomic Studies

Studying epigenetic regulation of gene expression involves studying protein–DNA interactions, or more precisely, studying the interaction of epigenetic factors with the DNA, then identifying the binding DNA sequence to locate the gene that the epigenetic factor was regulating. But proteins such as histones have an extremely dynamic relationship with the DNA, and only transiently interact with the genome through weak chemical bonds. To be able to extract these proteins, they need to be cross-linked with the DNA, then identified using an antibody tag specific to the protein of interest. Cross-linking agents such as formaldehyde have been successfully used for this purpose, and this method of cross-linking protein–DNA to study epigenetic interactions is known as chromatin immunoprecipitation (ChIP) (Kuo & Allis, 1999). These types of ChIP cross-linking studies have been performed since the 1970s (Hayashi, 1975; Sen et al., 1976). However, the interest in ChIP waned in the 1980s due to the lack of quality antibodies. More selective antibodies were subsequently developed for looking at various configurations of

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histones (Kuo & Allis, 1999; Madisen et al., 1998). With the emergence of high-throughput sequencing, the sequence of the binding DNA fragment could be easily identified, and soon ChIP-seq became one of the most widely used methods for studying epigenetics (Johnson et al., 2007).

Using the same protein–protein and protein–DNA cross-linking abilities of formaldehyde, methods such as chromosomal conformation capture (3 C) have been devised to study the structural properties and spatial conformation of chromosomes (Dekker et al., 2002). While local chromosomal conformations (using ChIP) have been established as playing a big role in gene expression regulation, global structural features play lesser-known but equally important roles in gene expression and DNA recombination (Dekker et al., 2002). In this method, DNA fragments covalently linked together with proteins are purified and their sequences investigated using polymerase chain reaction (PCR), or more recently using deep sequencing in a high-throughput method known as Hi-C (Belton et al., 2012). Hi-C data can reveal the global activation state of the chromatin (regions of open or closed chromatin correspond to active or inactive states, respectively). Hubs of activation can then be identified and compared with other data sets to reveal how the genome is rearranged in response to a treatment, pathology, or developmental stage (Belton et al., 2012; Lieberman-Aiden et al., 2009). More recently, Hi-C conformations have been determined in single cells (Nagano et al., 2013; Ramani et al., 2017) and detection of long-range contacts has been considerably improved (Hsieh et al., 2016). A new method has been developed as an offshoot of Hi-C, known as chromatin interaction analysis by paired-end tag (ChIA-PET), which combines ChIP and 3 C to potentially enable detection of all kinds of sites bound by a protein of interest (Davies et al., 2017; Fullwood et al., 2009).

ChIA-PET can be used for the de novo identification of global chromatin interactions. This method is able to detect transcriptional regulation through long-range interaction of chromatin. Using this technique, it is possible to study transcription and epigenetic factors that bind to regulatory DNA elements that are located far away from the promoter of the genes they regulate. Highly specific monoclonal antibodies are used to pull down transcriptional epigenetic proteins of interest. The DNA bound to the protein complexes can then be digested enzymatically or using sonication, and then sequenced to reveal the nature of the protein–DNA interaction. The data output would be a combination of ChIP-seq and Hi-C, making this a strong tool for understanding global gene-regulatory networks inside cells (Fullwood & Ruan, 2009; Fullwood et al., 2009).

The study of methylated cytosine residues in the genome, however, requires a different set of techniques compared to those described above. Cytosine methylation is quantified using sodium bisulfite treatment-based strategies, which convert unmethylated cytosines to uracil (which later on are converted to thymine using PCR amplification). Sodium bisulfite treatment does not affect methylated cytosines, allowing quantification of DNA methylation by the estimation of the cytosine-to-thymine ratio at known CpG sites (Ulahannan & Greally, 2015).

There are currently two approaches to isolating methylated cytosine as part of CpG sites (mCG). The first method involves a targeted approach using chip-based arrays such as the Infinium BeadChip arrays from Illumina. These arrays are designed to capture promoter CpG islands associated with RefSeq genes. The limitation of chip-based methods is that other biologically significant CpG regions on the genome cannot be detected. The second method involves taking a whole-genome bisulfite sequencing (WGBS) approach, or a modified version that allows improved capture of CpG islands, known as reduced representation bisulfite sequencing (RRBS) (Gu et al., 2011). In RRBS, CpG islands are isolated by briefly restriction digesting genomic DNA with the MspI restriction enzyme (Nwankwo & Wilson, 1988), which targets and cleaves CpG-rich regions (Xu et al., 2005). More recently, RRBS has been performed in single cells by reducing the loss of DNA associated with the purification steps. This is achieved by adjusting the buffer system and reaction volumes and combining the whole process into a single tube reaction (Guo et al., 2015).

The main caveat of the chip-based, WGBS-based, or RRBS-based methods is that they are not able to detect non-CpG island-methylated cytosines such as methylated CA sequences (mCA). This can be addressed using another method called MethylC-seq (Urich et al., 2015). In this method, complete genomic DNA is fragmented, processed and ligated to adapter sequences with methylated cytosines. The fragments are then treated with sodium bisulfite, followed by PCR amplification using primers specific for the adapters. The bisulfite-treated DNA fragments are sequenced then base-called against a reference genome, which reveals all of the thymine bases to be either a thymine or an unmethylated cytosine based on their location on the reference genome, while each cytosine base indicates the methylation status within the fragment. This method is more comprehensive than RRBS and DNA methylation arrays; however, it is also more costly, and thus is not a powerful method for studying large numbers of samples (Urich et al., 2015).

Additionally, there are some specialized methods for acquiring in-depth understanding of epigenetic gene regulation. A proteomics-based method known as chromatin affinity purification with mass spectrometry (ChAP-MS) can detect how gene expression regulators collectively assemble at DNA regulatory regions, something that cannot be detected by the ChIP-based methods described above, which can only detect single protein–DNA interactions (Byrum et al., 2012). By using mass spectrometric detection methods, ChAP-MS is also able to identify sequences of chromatin-associated proteins in an unbiased manner. Another method known as assay for

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transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) takes a completely novel approach to epigenomic profiling by studying open chromatin regions. In ATAC-seq, accessible regions of the genome are scanned using a hyperactive Tn5 transposase. These genomic regions can then be sequenced to map regions of regulatory regions, transcription factor activity, and nucleosome positioning (Buenrostro et al., 2013). The main advantage of using ATAC-seq when compared to other epigenomic methods, is that it does not require millions of cells as the starting material, nor complex and time-consuming sample preparation steps. This makes it a powerful tool for studying epigenomics in clinical samples (Buenrostro et al., 2013).

From this section, we can see that epigenetic gene regulation is an extremely complex process that involves several protein and RNA factors. However, several methods have so far been developed to study this phenomenon from different angles. The first major method involves cross-linking DNA-binding proteins with their genomic targets and studying the protein sequence and DNA sequence. This reveals different conformations of epigenetic factors during gene expression regulation. The second major method involves sodium bisulfite treatment of genomic DNA to reveal methylation sites. This is able to reveal gene regulatory sequences across the whole genome. Different variations based on these basic principles balance depth with cost-effectiveness of experiments. These methods, when combined with the iPSC technology discussed in the Section 9.7, can transform our understanding of neurodevelopment.

9.7 ROLE OF IPSC-BASED MODELS IN THE FUTURE OF NEURO-EPIGENOMIC RESEARCH

The genome is a set of instructions that determine the fate of biological organisms. In the case of complex multicellular organisms such as humans, it is a challenge to understand how one set of instructions can yield hundreds of diverse types of cells that interact with each other to form dozens of different types of tissue. However, it is easier to understand that this might be made possible by expressing only a fraction of the genes in each type of tissue: those genes that are relevant to providing the tissue with its unique identity. But how do the cells determine which genes to express, and how is all of this done starting from a single cell – the embryo?

This phenomenon by which genes are selectively switched on or switched off, which are then inherited from one cell generation to the next, such that by studying the developmental stages one can observe the gradual evolution of cell types with evolving functions specific to a developmental stage until they reach their end states, has intrigued scientists for decades. The study of development, known as embryology, has until recently been performed exclusively in laboratory animals under stringent ethical guidelines, as embryos are the inception of all life-forms. But in the past decade there have been groundbreaking inventions now enabling researchers to grow embryo-like tissue in the lab from any individual by collecting skin biopsies, blood, or a few strand of hair. The cells that are "reprogrammed" as a result are known as iPSCs, and they share characteristics with the stem cells that form the embryo.

iPSCs, once generated in the lab, can be artificially induced to form any tissue of the body. This enables in-depth research into the development of different tissues. Nowhere has this method been more relevant than in the study of complex developmental conditions such as autism. During this first wave of research on developmental conditions, many research groups around the world have developed methods to differentiate iPSCs into neurons and three-dimensional brain tissue. Studies have been performed to characterize morphology, physiology, and gene expression in these neurons. These neurons recapitulate many of the characteristics of in vivo neurons. In the future, researchers are aiming to combine the neuronal differentiating methods with the epigenomic methods as described in Section 9.6 to unravel the cellular machinery that conducts the "switching on" and "switching off" of genes as they go through different stages of brain development. In this section, the iPSC technology is discussed in terms of how it is being utilized at present to understand brain development and the promise it holds for studying the epigenomics of development and complex developmental conditions in humans.

9.7.1 Role of the iPSC Technology in Developmental Neuroscience

The method of reprogramming adult cells to iPSCs was first described by Takahashi and Yamanaka in 2006 (Takahashi & Yamanaka, 2006). They first reported this in mouse fibroblasts that were reprogrammed into iPSCs by retroviral transduction using four transcription factors – OCT4, KLF4, c-MYC, and SOX2 – then subsequently applied the technology to human fibroblasts (Takahashi et al., 2007). The pluripotent nature of iPSCs meant that they could be differentiated into neurons by exposing them to the biochemical milieu that is known to push pluripotent cells into cells of neuronal lineage. Early neuronal differentiation studies involved the inhibition of bone morphogenetic protein and SMAD signaling to trigger neural induction (Chambers et al., 2009; Shi et al., 2012). The SMAD inhibition strategy has been used more recently to develop three-dimensional cerebral cortex-like structures of pyramidal neurons, known as human cortical spheroids (Pasca et al., 2015). Another independently developed method that recapitulates various stages of the developing brain as a whole does not use the SMAD inhibition strategy to direct differentiation toward a neuronal lineage. This method is able to generate complex

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heterogeneous tissues by improving growth conditions and providing the environment that is necessary to influence development through time-dependent intrinsic cues associated with the developing brain (Lancaster et al., 2013, 2017).

One of the main driving forces behind developing neural differentiation protocols has been enabling the study of neurodevelopmental conditions. Not surprisingly, syndromic forms of autism, including Fragile X syndrome (Bar-Nur et al., 2012; Sheridan et al., 2011), Rett syndrome (Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Marchetto et al., 2010; Muotri et al., 2010), and Timothy syndrome (Krey et al., 2013; Pasca et al., 2011), were a few of the first neurological conditions to be explored using these methods. An iPSC model of idiopathic autism has also been established during the first wave of studies (DeRosa et al., 2012; Griesi-Oliveira et al., 2015; Marchetto et al., 2017; Mariani et al., 2015), with one of the studies (Mariani et al., 2015) having adopted the cerebral organoid method (Mariani et al., 2015). Genes such as MeCP2, mutations in which are associated with Rett syndrome, and DNMT3A (Sanders et al., 2015) are strongly associated with an autism outcome. MeCP2 and DNMT3A are also DNA methylation factors that play important roles in transgenerational epigenetic gene regulation. DNMT3A is also known to mediate methylation of non-CpG island cytosines as described in Section 9.6. Their mechanisms of action or downstream genes are not well characterized, and how mutations in these epigenetic factors might skew gene expression patterns toward a disease-causing state is not well understood. Whether the presence of mutations in these factors results in atypical responses to environmental insults during brain development is also not known. However, with the development of new epigenomic methods and the falling costs of next-generation sequencing, we could soon use iPSCs to start unraveling some of these mechanisms.

9.7.2 Current Research Shows Atypical Gene Expression Pathways with No Identifiable Genetic Association in Autism

An epigenomic study in autism is still cost prohibitive because autism is a complex condition, and to achieve statistical significance, a large sample size is recommended. Such studies have not been attempted using postmortem brain tissue, as it is a rare resource with highly variable tissue quality. Epigenomic studies using iPSCs have also been avoided, as it was not known whether the epigenetic signature of source tissue is completely reset after reprogramming into iPSCs. However, it has been established that iPSCs are functionally identical to embryonic stem cells, and there is accumulating evidence that their chromatin states are also fully recalibrated to that of a pluripotent cell (Mascetti & Pedersen, 2016; Papp & Plath, 2013; Rouhani et al., 2014).

However, RNA sequencing and microarray-based methods have revealed significant differences in gene expression patterns in convergent pathways associated with autism. This suggests non-genomic factors influencing regulation of gene expression in autism. Voineagu et al. (2011) used autism postmortem brain tissue to undertake gene expression studies using microarrays. They discovered two major dysregulated mechanisms in the autism brain: (1) synaptic function, vesicular transport, and neuronal projection (APMB_asdM12); and (2) immune system and inflammatory, astrocyte, and microglia responses (APMB_asdM16). A 2016 study undertaking RNA-seq using autism postmortem brain tissue by Parikshak et al. (2016) demonstrated attenuated cortical patterning associated with the autism brain (gene modules: ACP_asdM5, ACP_asdM13, and ACP_asdM14). A third study using developing postmortem brain tissue showed enrichment of autism-associated pathways at more advanced stages of development compared to control (gene modules: dev_asdM2, dev_asdM3, dev_asdM13, dev_asdM16, and dev_asdM17) (Parikshak et al., 2013).

Furthermore, one study using autism iPSC-derived organoids identified significant differences in developmental trajectory between autism and control organoids (Mariani et al., 2015). A more recent study using iPSC-derived neurons also identified several autism-associated pathways (Marchetto et al., 2017). A study from our group undertook RNA-seq using iPSC-derived neurons and used similar analysis methods as the above postmortem and iPSC-based studies. We found a very high correlation of gene expression pathways in our iPSC-derived neurons with the gene expression pathways detected in those studies (Figure 9.3) (Adhya et al., 2018). We also found poor correlation of gene expression with exome single-nucleotide polymorphisms found in the participants.

The postmortem brain studies and autism iPSC studies, including one by our group, point toward a global non-genomic gene regulatory mechanism at play in autism, one that is responsible for producing the autism-associated phenotypes irrespective of genetic background. Study of the epigenomics of autism might thus provide crucial insights into why such neurodevelopmental conditions have such heterogeneous genetic backgrounds.

9.7.3 The Significance of Future Clinical Neuro-epigenomic Research Using iPSCs

At present, the epigenetics of neurodevelopment is not well understood. Many of the methods described in Section 9.5 have been developed in the last five years. It has also only been a decade since the Nobel Prize-winning iPSC technology was first published, and most

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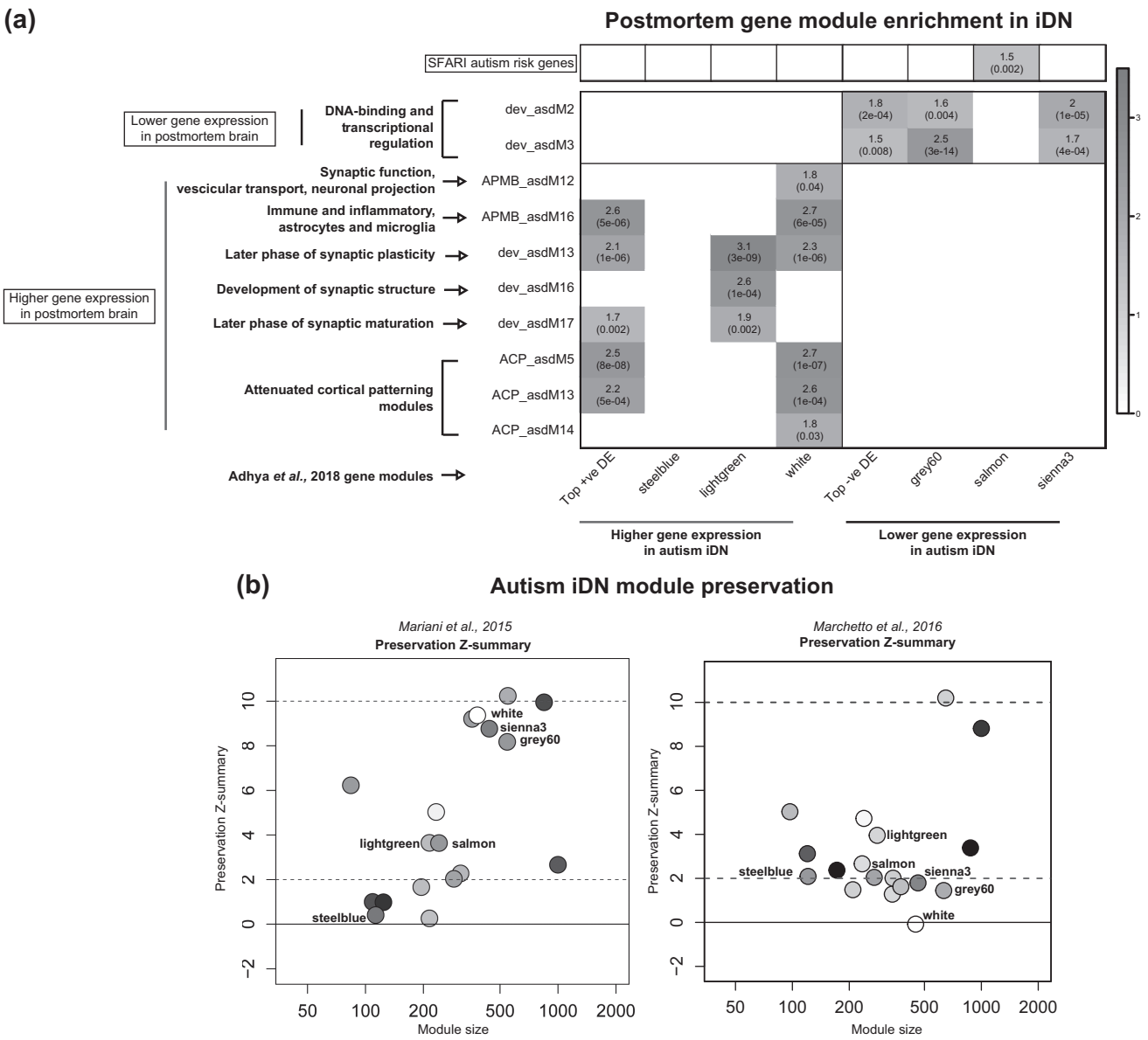

Figure 9.3 Enrichment analysis of autism-associated pathways and modules using induced pluripotent stem cell-derived neurons (iDN). (a) Enrichment of selected autism postmortem gene modules and SFARI autism risk gene list in iDN gene modules. Odds ratios (ORs) after logistic regression have been shown. Only ORs > 1.5 have been shown (*p*-value in parentheses). (b) Module preservation of autism minibrain gene modules in iDN gene modules from this study. DE = differential expression.

neuronal differentiation methods are still under various stages of development. However, as data from iPSC-derived neuronal tissue start accumulating, it is becoming clear that iPSCs are the starting material of choice for characterizing the wide array of phenotypes associated with neurodevelopmental conditions. Large sequencing studies have revealed hundreds of single-nucleotide polymorphisms with varying degrees of penetrance to be associated with autism (Bourgeron, 2015; O'Roak et al., 2011, 2012; Sanders et al., 2015). This has made research into a complex condition such as autism extremely challenging. Subsequent transcriptomic studies have reported quite a different story, and it has now been established that there are a set of convergent gene expression pathways that are associated with autism (Marchetto et al., 2017; Mariani et al., 2015; Parikshak et al., 2013, 2016; Voineagu et al., 2011). These pathways have been repeatedly observed in postmortem brain tissue and iPSC-derived brain tissue, giving rise to additional questions on how genetic mutations interact with cellular gene regulatory systems to manifest a disease state. To learn how these cellular gene regulatory systems work, researchers need to take an in-depth look at the complicated epigenetic machinery of the developing brain. As primary brain tissue from sufferers cannot be obtained, and as postmortem brain tissue is a rare commodity and acquiring it involves many ethical dilemmas, the best solution for researchers is to model

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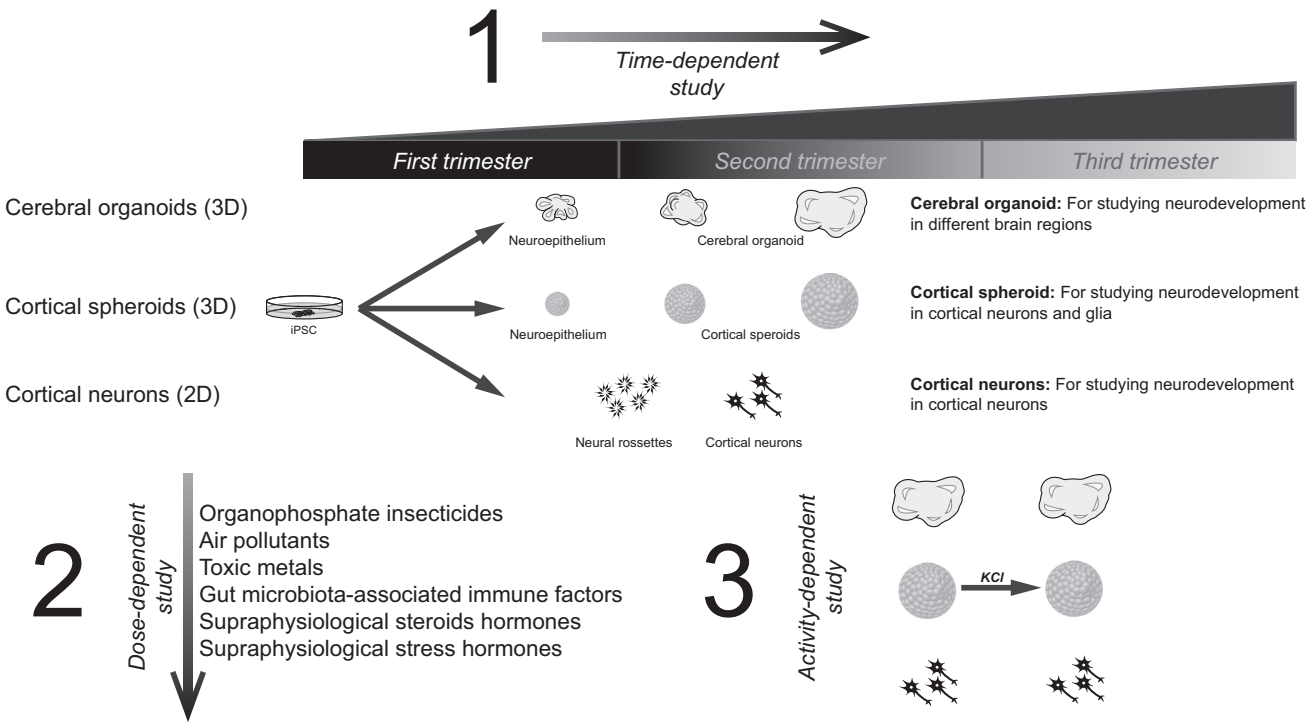

Figure 9.4 Two- and three-dimensional neuronal approaches that can be taken to study neurodevelopment using iPSCs. (1) Time-dependent study, to demonstrate cellular/molecular trajectories during neurodevelopment. (2) Dose-dependent study, to demonstrate effects of environmental agents during stages of neurodevelopment. (3) Activity-dependent study, to mimic the effect of neuronal activity and observe neuronal function during development.

neurodevelopmental conditions such as autism in vitro using iPSCs.

By using two- and three-dimensional neuronal differentiation protocols, early neurodevelopmental stages can easily be recapitulated (Figure 9.4). This allows the study of epigenetics of early stem cell differentiation, when it is most dynamic (Feil & Fraga, 2012). Three approaches can be taken to study neurodevelopment using iPSCs: (1) by undertaking a time-dependent study, one can track epigenetic transitions from one cell state to the next; (2) by adding environmental stimuli associated with autism, one can study epigenetic perturbations at these early stages to track divergences of epigenetic signatures; and (3) by artificially inducing neuronal activity in matured neurons (e.g., by administration of potassium chloride), one can track activity-dependent signaling in autism-associated epigenetic states, and this can mimic cellular differences in adult brain function in autism.

The iPSC technology is able to provide novel insights into human neurodevelopment, something that has not been accurately recapitulated in animal models. By studying epigenetics of transgenerational inheritance during neurodevelopment, we can understand the interaction between the genome and epigenetic factors, and how the environment might be able to cause perturbations to this interaction.

9.8 CONCLUSIONS

Over the last decade, there have been a growing number of studies showing a role of the environment in autism etiology, the development of the iPSC method for studying neurological conditions in vitro, and the development of various epigenomic methods to study transgenerational epigenetic inheritance. The convergence of research in these three fields has opened up a world of research opportunities to study how genes are regulated epigenetically during the progression of neurodevelopmental conditions.

Epigenomic studies in neuropsychiatric conditions have been performed using animals as well as human tissue. Blood and postmortem brain tissue from humans have been used so far. All of these tissue sources have major caveats for studying neuropsychiatric conditions. The animal brain is not able to recapitulate all of the stages of human brain development. Animals also do not share similar behavioral phenotypes with humans. Complex neuropsychiatric conditions such as autism and schizophrenia are difficult to model in animals, as they are heterogeneous conditions with hundreds of associated risk genes involved. Human tissue material such as blood and postmortem brain tissue can be used instead. However, there are questions over the validity of using blood as a biomarker for the brain. Postmortem brain tissue from individuals with neuropsychiatric conditions is a rare resource, and thus all such studies suffer from low sample

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sizes. In addition, it is not possible to study development in neuropsychiatric conditions with postmortem brain tissue, as most of the available brain tissue is from adult brains when it is fully mature. Furthermore, atypical gestational development cannot be studied in prenatal postmortem brain resources, as conditions like autism can only be diagnosed after birth (Bakulski et al., 2016). There have been a number of studies looking at typical epigenetic signatures of the brain and atypical epigenetic signatures in autism using animal models (Kim et al., 2016; McCormick et al., 2017), blood (Hannon et al., 2015; Naumova et al., 2018), and postmortem brain tissue (Nardone et al., 2017; Sun et al., 2016). However, all of these studies share the same caveats as described above.

iPSCs can be used to overcome many of the challenges discussed above. They can be differentiated using various neural differentiation protocols (two- or three-dimensional) to an early or late developmental stage of the brain, then used to investigate epigenetic signatures associated with healthy and diseased states associated with neuropsychiatric conditions. iPSCs also share the same genetic background with the individuals suffering from the particular condition, making it easier to correlate aberrant gene regulation with their genetic predispositions. iPSC-derived neural tissue can also be exposed to environmental toxins to gain insights into how environmental stimuli perturb transgenerational inheritance processes during neural development at very early stages when epigenetic gene regulation is most dynamic.

However, such studies are not without challenges. First, undertaking next-generation sequencing experiments, such as those required for RRBS, and MethylC-seq on a statistically significant sample size can be extremely resource intensive. Cellular reprogramming and neural differentiation also involve significant lab and reagent costs. Second, current neural differentiation methods have some issues that prevent their use as preclinical models of conditions. They can currently be developed until mid-second trimester, although theoretically they can be differentiated for longer. It is also not certain whether neurons developed in this manner show physiological activity similar to in vivo neurons or brain tissue. iPSC-derived three-dimensional neural tissue can also be quite heterogeneous, which compounds issues during analysis. Costs can also increase many times over as a result, as more experiments need to be done to achieve statistically significant observations.

Nevertheless, most issues related to iPSCs are technical in nature and are already being addressed. As the cost of next-generation sequencing falls, methylation sequencing costs will also fall. The scientific community is working closely with biotech companies to develop more efficient reprogramming methods. Neuronal differentiation methods are also improving, and various strategies are being implemented to reduce the heterogeneity of neuronal differentiation. It is understood that one of the main causes of heterogeneity is the health and cell-cycle stage of the starting stem cells. Studies using autism iPSC-derived neural tissue have already demonstrated electrophysiological activity (Marchetto et al., 2017; Mariani et al., 2015), and recent studies in three-dimensional cerebral organoids have shown the ability of rod cells to respond to light stimuli (Quadrato et al., 2017), thus demonstrating physiological activity in neurons derived from iPSCs.

Autism is already associated with environmental risk factors, although we have repeatedly noted throughout this chapter that these await independent replication. Autism is also associated with epigenetic mechanisms. The use of the iPSC technology with epigenomic methods for studying autism is expected to reveal different atypical patterns of transgenerational inheritance associated with autism. It is possible that neuropsychiatric conditions other than autism also have associated environmental risk factors. These kinds of studies would also provide information about the environmental risk factors associated with other neuropsychiatric conditions. Although gene therapy is often discussed as the future of personalized medicine, it is fraught with ethical issues, and it may be the case that a less invasive, lower-risk therapeutic strategy might constitute personalized epigenetic treatments that could recalibrate atypical epigenetic signatures and compensate for dysregulated gene expression without the need to make changes to the genome. Again, there is a need to start to discuss the ethical issues surrounding such approaches, and safety considerations remain of paramount importance.

9.9 ACKNOWLEDGMENTS

The authors were supported by the Autism Research Trust, the Chinese University of Hong Kong (CUHK), and EU-AIMS during the period of this work.

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