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Impact of Early Environment on Children's Mental Health: Lessons From DNA Methylation Studies With Monozygotic Twins

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Over the past decade, epigenetic analyses have made important contributions to our understanding of healthy development and a wide variety of adverse conditions such as cancer and psychopathology. There is increasing evidence that DNA methylation is a mechanism by which environmental factors influence gene transcription and, ultimately, phenotype. However, differentiating the effects of the environment from those of genetics on DNA methylation profiles remains a significant challenge. Monozygotic (MZ) twin study designs are unique in their ability to control for genetic differences because each pair of MZ twins shares essentially the same genetic sequence with the exception of a small number of de novo mutations and copy number variations. Thus, differences within twin pairs in gene expression and phenotype, including behavior, can be attributed in the majority of cases to environmental effects rather than genetic influence. In this article, we review the literature showing how MZ twin designs can be used to study basic epigenetic principles, contributing to understanding the role of early in utero and postnatal environmental factors on the development of psychopathology. We also highlight the importance of initiating longitudinal and experimental studies with MZ twins during pregnancy. This approach is especially important to identify: (1) critical time periods during which the early environment can impact brain and mental health development, and (2) the specific mechanisms through which early environmental effects may be mediated. These studies may inform the optimum timing and design for early preventive interventions aimed at reducing risk for psychopathology.

■ **Keywords:** twins, epigenetics, mental health, child development, birth weight, monozygotic

Numerous studies have gathered evidence that adverse events in utero (e.g., malnutrition during pregnancy) or in the first years of life (e.g., abusive parenting) are associated with a number of physical and mental health problems over the life span (e.g., see Shonkoff et al., 2009). These include, but are not limited to, depression and other mental health problems, diabetes and related metabolic disorders such as obesity, cardiovascular disease, substance abuse, and premature mortality (Bateson et al., 2004; Gale & Martyn, 2004; Gluckman et al., 2008; Heim & Binder, 2012). Although the specific mechanisms through which these well-documented early adverse exposures compromise later health are not yet well-known, there is growing evidence that epigenetic modifications during critical periods of development are one of the mechanisms that mediate the biological response to early adverse environments and health outcome

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(Mill & Petronis, 2008; Szyf, 2011; Tremblay & Szyf, 2010; Booij, Tremblay et al., in press). Indeed, following the demonstration of DNA methylation changes in the hippocampal glucocorticoid receptor (GR) gene promoter following environmental stressors in rats (Weaver et al., 2004) and in postmortem brain tissues (McGowan et al., 2009), numerous studies have reported epigenetic differences between humans with and without exposure to early life adversity (e.g., see Booij et al., 2013; Szyf, 2013).

Although these studies are interesting and useful, the focus on singletons cannot easily determine to what extent epigenetic differences are reflections of the genotype (i.e., differential epigenetic effects of the exposure to environmental risk factors according to the genotype [genotype × environment interactions or genotype influence on the epigenome]) or a marker of environmental exposures). For example, individuals exposed to in utero adversity (e.g., maternal stress/depression, malnutrition) or early adversity (e.g., parental violence) may differ environmentally, but also genetically, from those with low levels of early adversity (Teh et al., 2014).

MZ twins are the ideal genetic controls for studying environmental effects. Although it is technically possible in singleton studies to control for genotypic effects when studying DNA methylation, such studies generally would need a very large sample size. MZ twin study designs are unique in their ability to control for genetic differences because each pair of MZ twins shares essentially the same genetic sequence with the exception of a small number of de novo mutations and copy number variations (Kato et al., 2005). Moreover, if used in a longitudinal design, they can provide indications of the role of epigenetic factors as a mediator between the early environment and developmental outcomes.

Following a brief introduction on the basic principles of DNA methylation, we summarize recent evidence from epigenetic studies in twins and describe how these studies have helped to better understand epigenetic modifications in humans, and provided insight into the specific role of the early environment on development of psychopathology. The importance of longitudinal study designs and directions for future research are also discussed.

DNA Methylation, a Window on the Underlying Mechanism of Environmental Effects on Brain and Behavior

Given our current understanding of the complexity of the genome, it is no surprise that in the early stages of genetic research in relation to child development and psychopathology, scientists were over-optimistic in the power of genetics to provide good risk predictions for complex phenotypes and disease states. With advances in our knowledge of the large number of upstream factors that influence phenotype, it has become clear that understanding genetics alone will not be enough to understand complex disease states. One of such upstream factors that have now been widely accepted as a direct regulator of transcription is epigenetic variation, including DNA methylation.

DNA methylation is an enzymatic process in which DNA methyltransferase enzymes catalyze the addition of a methyl group to cytosines (Wu & Santi, 1985), both during embryonic development, as well as later in life (Razin & Riggs, 1980). Methylation in vertebrates occurs mainly in the dinucleotide sequence CG (Gruenbaum et al., 1981; 1983), but it is clear that cytosine in other sequence contexts can also be methylated, particularly in the brain and in some stem cells (Lister et al., 2013). It has been known for decades that methylated CGs are distributed differently in different cell types, playing a key role in conferring cell type identity (Lister et al., 2009; 2013; Razin & Szyf, 1984). Early studies (Cedar et al., 1983; Stein et al., 1982) that were confirmed by recent genome-wide mapping of DNA methylation in different tissues (Lister et al., 2009; 2013) suggest that DNA methylation in promoter and enhancer regulatory regions that regulate gene activity contribute to gene silencing. Thus, it is well established that DNA methylation is a mechanism that can enable identical genetic sequences to perform different functions according to context. The mechanisms involved in gene silencing by DNA methylation are not fully clear but two mechanisms have been proposed. The first involves blocking the interaction of proteins that activate gene expression to their cognate sequences in DNA (Comb & Goodman, 1990). The second mechanism involves recruitment of methyl-CpG-binding domain proteins (MBDs) to methylated promoters, which then recruit proteins involved in chromatin remodeling and leading to an inaccessible chromatin configuration (Nan et al., 1997).

Perhaps one of the most relevant factors for understanding the importance of DNA methylation in development and disease is that it is generally highly stable over time, but also potentially modifiable in some instances (Ramchandani et al., 1999). This supports a role in long-term programming and reprogramming of gene expression.

In order to guide development during gestation, the human fetus undergoes a series of global methylation and demethylation events (Razin & Cedar, 1993). These global events are then followed by gene and cell-type specific methylation and demethylation (Razin & Cedar, 1993). After this initial programming of the epigenome, this DNA methylation pattern is relatively stable, but also remains vulnerable to alteration by environmental exposures throughout specific periods in gestation and later life (Szyf, 2011). Such differences in DNA methylation could confer inter-individual phenotypic differences without any change in the DNA sequence (Szyf, 2011). Indeed, a number of studies now have shown associations between adverse exposures in utero and DNA methylation patterns later in life. For instance, restriction of certain dietary components such as folic acid and vitamin B12 during gestation is known to affect the DNA methylation pattern in sheep

(Sinclair & Singh, 2007). Moreover, a human study observing siblings, one of which was exposed to a famine during the perinatal period, showed different DNA methylation patterns 60 years later, but only when the famine was present during the first trimester of pregnancy (Heijmans et al., 2008). Moreover, a number of studies have shown associations between measures of in utero growth and patterns of genome-wide or candidate gene methylation (Marsit et al., 2012; Simpkin et al., 2015). In turn, other studies found associations between DNA methylation patterns and adversity during childhood, including parental stress (Essex et al., 2011), low socio-economic status (Borghol et al., 2012), and abuse (Suderman et al., 2014). Given that methylation plays such an important role in development, it is not surprising that it has been heavily associated with various behavioral disease states. These include (but are not limited to) depression, autism, psychosis, bipolar disorder, posttraumatic stress disorders, eating disorders, and Alzheimer's disease (e.g., Booij, Casey et al., 2015; Coppieters et al., 2014; Frieling et al., 2010; Grayson & Guidotti., 2013; Kuratomi et al., 2008; Loke et al., 2015; Mastroeni et al., 2009; Nguyen et al., 2010; Nishioka et al., 2012). However, many of the findings have not been replicated, potentially due to type 1 and type 2 errors associated with insufficient sample size.

From these (and other) studies, it can be concluded that: (1) DNA methylation is shaped and formed largely in utero, (2) these methylation processes are important for early child neurodevelopment, and (3) early adverse environmental exposures, in utero or early in life, affect DNA methylation patterns. Although most studies to date have been cross-sectional and thus cannot provide any insight into cause and effect, research does suggest that early environmental factors ranging from in utero environment to childhood neglect and abuse may act to alter DNA methylation levels, which in turn may affect child development and predispose individuals to develop psychopathology.

It is important to note that much of the research cited above studied the early environment in association with DNA methylation independent of genotypes. There is extensive evidence that genetic variation influences DNA methylation profiles (e.g., Chen et al., 2015; Liu et al., 2014; McRae et al., 2014; Teh et al., 2014). For instance, McRae et al. (2014) compared similarity in DNA methylation levels in 614 individuals from 117 families. They found that approximately 20% of the individual variation in DNA methylation was related to DNA sequence variation that is not located within CpG sites (McRae et al., 2014). It is important to consider the interaction between genetic and environmental effects as well. DNA methylation at a given gene may be more heritable in carriers of a certain allele, but more influenced by environmental factors in carriers of another allele of the same gene. This idea is supported by a study conducted by Yousefi et al. (2013), who found that methylation levels at various sites on the leptin receptor gene were best explained by an interaction between the leptin receptor genotype and maternal smoking behavior. In another study, researchers identified variably methylated regions (VMRs) in a group of neonates and quantified the relative contribution of environmental and genetic factors to methylation at these regions (Teh et al., 2014). They found that 25% of the VMRs were best explained by genotype alone and the other 75% were best explained by the interaction between genotype and environment.

The issue of DNA sequence effects on DNA methylation profiles will be discussed in greater detail in the next sections of this article.

Challenges for Epigenetic Association Studies in Humans Using Singleton Designs

Although correlational studies investigating the relationship between epigenetic mechanisms and various exposures (e.g., maternal nutrition or smoking in pregnancy) or outcomes (e.g., developmental outcomes, diseases) have been informative, they are usually subject to considerable limitations, above those of corresponding genetic analyses that make drawing conclusions difficult. Most notably, the majority are limited in sample size and are therefore inadequately powered to control for the potential confounding influence of genetics that is ensuring the same level of genetic diversity in both groups. This is of particular importance given that variation in the DNA sequence can result in variation in DNA methylation and gene expression levels and thus certain epigenetic changes might be driven by genetic factors and together alter the expression of the gene as described above (Arloth et al., 2015; Quon et al., 2013).

Another issue stemming from the lack of control over genetic factors is that any association between epigenetic modifications and a given functional outcome could actually be a result of underlying genetic profiles leading to both epigenetic variation and functional outcome, independent of any direct relationship between the epigenetic and phenotypic measures. A two-step Mendelian randomization analysis has been proposed as a promising strategy to address such issues (Relton and Davey Smith, 2012). However, there is presently a lack of validity data for genetic proxies for methylation data from tissues other than peripheral blood. Moreover, such two-step Mendelian randomization approach may require very large sample sizes to achieve sufficient power, which is presently not realistic.

Below, we review some of the epigenetic association studies conducted in twins, with an emphasis on MZ twin designs. We will review some of the work that has helped scientists to understand more about the basic principles, followed by reviewing research in relation to child development, with a focus on psychopathology.

Advantages of Epigenetic Research in Humans Using Twin Designs

Distinguishing Genetically from Externally Driven DNA Methylation

One important question that twin studies can help answer is to what extent variation in DNA methylation is driven by the DNA sequence as opposed to environmental or stochastic factors. Research comparing MZ and DZ twins has confirmed that epigenetic heritability varies according to genomic location. That is, certain genomic loci appear to be more influenced by environmental factors while others appear to be more influenced by genetic variation. For example, in comparing variability in DNA methylation as assessed in buccal cells between 46 MZ and 45 DZ twin pairs at 5 and 10 years of age, Wong et al. (2010) found that variation in *DRD4* DNA methylation is mostly due to shared environmental factors, rather than unique environmental factors or genetic factors. This was indicated by high within-pair correlations in both MZ and DZ pairs at age 5 ($r = 0.61$ and $r = 0.43$, respectively) and at age 10 ($r = 0.67$ and $r = 0.64$ respectively), and high within-pair correlations in the change in DNA methylation in both MZ and DZ pairs between age 5 and 10 ($r = 0.52$ and $r = 0.45$, respectively). Further, changes in methylation in the serotonin transporter (*SLC6A4*) gene over time were mainly due to environmental factors unique to each twin, as reflected by low within-pair correlations in both MZ and DZ twins at age 5 ($r = 0.21$ and $r = 0.34$, respectively) and at age 10 ($r = 0.35$ and $r = 0.24$, respectively) and similar within-pair correlations in *SLC6A4* DNA methylation change among MZ and DZ twins ($r = 0.22$ and $r = 0.15$, respectively). Sex-specific differences in twin concordance and change over time was observed for the monoamine oxidase A (*MAOA*) gene. For males, *MAOA* DNA methylation was moderately influenced by genetic factors (within-pair correlation, *r*, was 0.28 for MZ and 0.15 for DZ twins at age 5; and 0.39 for MZ and 0.13 for DZ twins at age 10). By contrast, for females, within-pair correlations at age 5 were -0.11 for MZ and -0.13 for DZ twins; and -0.03 for MZ and 0.1 for DZ twins at age 10, suggesting that DNA methylation of this gene in females is primarily influenced by unique environmental factors. These results suggest that the extent to which DNA methylation is driven by environmental or genetic factors depends on the genomic loci being examined at a given time in development.

The extent to which methylation is heritable also appears to depend on the specific region of the gene being examined. For instance, Kaminsky et al. (2009) found that heritability was highest at CpG sites that were correlated with functional regions and promoter regions. This suggests that there is stronger genetic control over areas where methylation is more functionally relevant. Furthermore, there is evidence that the extent of environmental versus genetic control over methylation varies across tissue. This is supported by studies comparing different cell types. For instance, Ollikainen et al. (2010) found that heritability estimates at methylated regions of the insulin-like growth factor 2 *(IGF2)/H19* locus varied widely between cell types in a sample of 56 MZ and 35 DZ newborn twins, from 14% in granulocytes to 99% in human umbilical vein endothelial cells. Further, Kaminsky and colleagues (2009) studied 57 MZ and 40 DZ adolescent twin pairs to compare epigenetic similarities in MZ versus DZ pairs in white blood cells and buccal cells, using the Human12K CpG island microarrays. It was found that the intraclass correlation (ICC) within pairs was larger for MZ than DZ pairs. Interestingly, the difference in ICC between MZ and DZ pairs was tissue specific; with a larger difference in ICC for buccal cells than for white blood cells (mean ICC_{MZ}-$ICC_{DZ} = 0.35$ for buccal cells; mean ICC_{MZ}-$ICC_{DZ} = 0.0073$ for white blood cells). In addition, in a study in middle-aged female twins (Bell et al., 2012), consisting of 33 MZ and 43 DZ pairs (median age: 57 years), heritability estimate of whole blood genome wide DNA methylation (using the Illumina Infinium HumanMethylation27 BeadChip) was 0.182. McRae et al. (2014) assessed and compared heritability of DNA methylation in peripheral blood leucocytes in 614 individuals from 117 families, consisting of 67 MZ adolescent pairs and 11 adolescent DZ twin pairs, their siblings and their parents. DNA methylation was assessed using the Illumina Infinium HumanMethylation450 BeadChip. The ICC for MZ twins was 0.20, while the ICC for DZ twins was 0.109. Notably, the strength of the ICC observed in DZ twin pairs was comparable with the strength of ICC observed between (non-twin) siblings, mother-offspring ($r = 0.097$) and father-offspring ($r = 0.085$; McRae et al., 2014). Taken together, these findings suggest that DNA methylation status is dependent on both genetic and external factors, with the relative importance of each factor being dependent on the genomic location, cell type, and developmental stage. However, it is important to mention that studies do not only differ in age of the participants and cell type, but they also differ substantially in analytic approach. These methodological differences make it at this stage difficult to compare studies, and to draw any general conclusions about the level of hereditability of DNA methylation levels in the population. Studies comparing levels of hereditability between MZ and DZ twins are summarized in Table 1.

When interpreting heritability estimates for DNA methylation it is important to note that a greater discordance in DZ twins compared to MZ twins may not be due solely to a greater effect of DNA sequence, but may also be due to inherent epigenomic differences present in the zygotes of DZ and MZ embryos and/or heritable epigenetic variation independent of DNA sequence (Kaminsky et al., 2009). More specifically, similarity in the epigenetic profile of blastocysts at the time of splitting may be an important factor accounting for higher similarity of the epigenome in MZ twins compared to DZ twins, who originate from different

TABLE 1

Summary of Studies Reporting Estimates of Hereditability Comparing MZ and DZ Twins

Reference	Sample	Age	Tissue	Method	Hereditability estimates
Wong et al., 2010	46 MZ pairs 45 DZ pairs	Age 5 and age 10	Buccal cells	Sequenom EpiTYPER; *DRD4, SLC6A4; MAOA* genes.	*DRD4*, age 5: MZ: $r = 0.61$, DZ: $r = 0.43$ *DRD4*, age 10: MZ: $r = 0.67$, DZ: $r = 0.64$ *SLC6A4* age 5: MZ: $r = 0.21$, DZ: $r = 0.34$ *SLC6A4* age 10: MZ: $r = 0.35$, DZ: $r = 0.24$ *MAOA*, age 5, male twins: MZ: $r = 0.28$, DZ: $r = 0.15$ *MAOA*, age 10, male twins: MZ: $r = 0.39$, DZ: $r = 0.13$ *MAOA*, age 5, female twins: MZ: $r = -0.11$, DZ: $r = -0.13$ *MAOA*, age 10, female twins: MZ: $r = 0.03$, DZ: $r = 0.1$
Kaminsky et al., 2009	57 MZ pairs 40 DZ pairs	Adolescence (12–15 years old)	White blood cells; buccal cells	Genome wide; human 12K CpgIsland microarrays	Buccal cells: Mean difference in ICC for MZ-DZ twins = 0.35 White Blood cells: Mean difference in ICC for MZ-DZ twins = 0.0073.
Ollikainen et al., 2010	56 MZ pairs 35 DZ pairs	Birth	Cord blood mononuclear cells; HUVECs; buccal epithelial cells; placenta	Sequenom Epityper; 4 differentially methylated regions (DMR) at the *IGF/H19* locus (*H19* promoter DMR; *H19* CTCF6 DMR; *IGF2* ex9 DMR; *IGF2* DMR)	MZ twins: 3–4% absolute within pair methylation difference DZ twins: 4–7% absolute within pair methylation difference. High variability in within pair differences for each of the DMRs. Absolute within pair difference in methylation levels in MZ twins: 4–8% for HUVEC; 3–9% in placenta; 2–5% for cord blood and buccal cells.
Bell et al., 2012	33 MZ pairs 43 DZ pairs	Median age: 57 years	Whole blood	Genome wide; Illumina Infinium HumanMethylation27 BeadChip	MZ: ICC = 0.306 DZ: ICC = 0.2145 Heritability estimate: 0.182
Gordon et al., 2012	22 MZ pairs; 12 DZ pairs	Birth	Cord blood mononuclear cells; HUVECs; placenta	Genome wide; Illumina Infinium HumanMethylation27 BeadChip	Cord blood: $h^2 = 0.12$ HUVEC: $h^2 = 0.07$ Placenta: $h^2 = 0.05$
McRae et al., 2014	67 MZ pairs 111 DZ pairs	Adolescent twins	Blood Leucocytes	Genome wide; Illumina Infinium HumanMethylation450 BeadChip	MZ twins, $r = 0.20$ DZ twins, $r = 0.109$
Boks et al., 2009	23 MZ pairs 23 DZ pairs	Adults (mean age for twins = 40 years)	Peripheral blood	Illumina GoldenGate methylation assay	96/431 CpG sites (23%) yielded significant heritability

Note: MZ = monozygotic; DZ = dizygotic; *DRD4* = dopamine receptor D4; *SLC6A4* = serotonin transporter; *MAOA* = monoamine oxidase A; ICC = intraclass coefficient; HUVEC = human umbilical vein endothelial cells.

zygotes. Albeit potential zygotic effects may be small (McRae et al., 2014), these effects may have implications for the heritability of disease states and the contribution of DNA methylation to the transmission of various phenotypes.

Assessing Stability Throughout the Lifespan

Another important question is whether DNA methylation alterations induced by the environment are stable over time. If DNA methylation is to act as a mediator of gene-environment interactions, it must be shown that there is a degree of instability in the epigenome where the environment can have an effect.

Zhang et al. (2015) examined stability of methylation using blood collected from 10 pairs of adult MZ twins and 8 unrelated adults sampled every 3 months for 9 months. DNA methylation was assessed using the Illumina Infinium HumanMethylation450 K BeadChip. In the MZ twin pairs, it was found that 0.087–1.53% of the CpG sites showed differential methylation within pairs at a false discover rate-adjusted p value $< .05$. Furthermore, it was found that while DNA methylation at most genomic loci is stable for at least 9 months, a subset of loci vary in DNA methylation over the 9-month period (Zhang et al., 2015). Methylation of genes relevant to environmental interactions may also be more sensitive to environmentally mediated methylation variation. More specifically, Gordon et al. (2011) found that most variably expressed genes in 14 MZ newborn twin pairs were genes involved in the response to stress, chemicals and xenobiotics, as well as genes that play a role in immune system processes. This included genes such as chemokine ligands (*CCLs*) and interferon-inducible proteins (*IPs*), as well as interleukin-1 beta (*IL1B*), interleukin-9 (*IL9*), lysozyme (*LYZ*), and *CD14*. Together, these genes were classified as genes involved in 'response to external signals'. In this study, variability in expression was taken to be a proxy for measuring epigenetic discordance. These results provide support for epigenetics as a mediator of gene × environment interactions and provide supporting evidence of a dynamic interaction between environmental factors and gene expression, which epigenetic factors are thought to underlie.

Our research group previously studied DNA methylation variation as assessed in saliva in a sample of 15-year-old MZ twins in adolescence (Levesque et al., 2014). We assessed genome-wide DNA methylation using the Infinium HumanMethylation450 BeadChip in a sample of 37 MZ twin pairs. Notably, we found 96–99% overlap in twin methylation profiles. Interestingly, within-pair variability was observed in genes relevant to development, cellular mechanisms, tissue, cell morphology and various disorders. Further, by repeating DNA methylation assessments in the adolescent twins 3–6 months later, we found that methylation at some genes was stable over 3–6 month, while methylation at other genes was not. Interestingly, we found that at the *HLA-DQB1* gene (a gene important for immune system functioning), certain sites were variable over time while others were stable. Together, we interpreted these results as suggesting that variability in these genes may underlie phenotypic differences between twins and that certain genes and gene pathways may be more stable while others may be more variable and responsive to environmental factors. It will be important in future twin studies to determine the extent of drift over time and any periods in life which may be associated with dramatic change in the epigenome.

In sum, comparisons between MZ and DZ twins have taught us that while methylation levels have the potential to be stable over time, they can also vary in response to environmental stimuli and as a result of stochastic drift. They also highlight the importance of studying interactions with genotypes. Further, increasing exposure to different environmental factors as twin's age is thought to underlie an age dependent increase in methylation discordance in MZ twins over time. This increasing change in methylation may be a steady change over time, but may also be pronounced at specific developmental periods such as adolescence when drastic phenotypic changes occur.

Insight Into Early Developmental Origins of Psychopathology — The Example of Birth weight

An important obstacle in studying prenatal or early postnatal influences on development of mental disease is the way the environment is assessed. Retrospective measures of early life exposures generally rely on mothers' memory, and thus may not be accurate and are potentially subject to confounding by current mood or disease state. Second, since it is impossible to randomize exposure to early life stress, interpretation is often limited in that it is impossible to tell whether any difference would be due to an early or later environmental factor or genetic effect. As mentioned above, MZ twins are ideal to control for the genetic factors in such circumstances. By using well-documented longitudinal cohorts, MZ twin studies can better discriminate the early environmental factors by controlling for postnatal confounding factors and genotype.

A common method to index adversity at the level of the individual fetus in MZ twins is birth weight (BW). In both singletons and twins, BW is one of the best indices of in utero adversity and predictors of cognitive, behavioral and cardiovascular outcome, even when BW is within the normal range (Barker, 2004; Kramer, 1987; Newcombe et al., 2007; van Os et al., 2001). In addition to having the same genetic sequence, MZ twins also have many prenatal environmental characteristics in common (e.g., gestational age, maternal factors). Thus, differences in BW must be related to differences in non-genetic factors (often referred to as non-shared environment) acting in utero, such as differences in placental function. Theoretically, such differences could have a direct impact on brain development; they may also interact with the environment and lead to differential susceptibility to environmental stressors. Investigation of within pair methylation differences among MZ twins

provides a unique natural experiment for measuring the contribution of adverse early life environment factors on child development, independent of genetic effects. Thus, to the extent that BW is associated with outcome (e.g., DNA methylation) and within-pair BW discordance often predicts later life discordance for a range of phenotypes, unique prenatal characteristics may be a potential underlying pathway accounting for this relationship. Indeed, two studies have shown a relationship between BW discordance and measures of adolescents' brain morphology in twins (Levesque et al., 2015; Raznahan et al., 2012).

To our knowledge only a few reports examining BW-related variations in epigenome-wide DNA methylation in MZ twins have been published (Gordon et al., 2011; Souren et al., 2013; Tan et al., 2014). Souren and colleagues (2013) studied adult twins with methylation data from saliva samples. They did not find any associations between BW discordance and DNA methylation that survived multiple testing corrections. A second study of blood samples from adult MZ twins also found no association between BW discordance and differences in methylation (Tan et al., 2014). The third twin study that tested the association between BW and DNA methylation examined human umbilical vein endothelial cells from newborn MZ twins (Gordon et al, 2012) and found evidence for an association at only one CpG site in the *APOLD1* gene (gc02813863). Taking into account the observed association between BW and DNA methylation in singletons (see above), and the well-demonstrated role of BW discordance on cognitive and brain morphology outcome (Levesque et al., 2015; Newcombe et al., 2007; Raznahan et al., 2012), one possible explanation for this lack of associations in twin studies is that variability in DNA methylation and BW within MZ twin pairs is not sufficient to consistently show robust associations in epigenome-wide analyses. In addition, the epigenetic associations observed in singletons may additionally be driven largely by genetic factors influencing DNA methylation levels, whereas in MZ twins other factors, such as non-coding RNA or post-translational modifications, might be driving the differences in BW. Moreover, these studies are limited in that DNA methylation was assessed at a single point in time and that DNA methylation was not measured in brain but in a peripheral tissue. Future studies with MZ twins need to use pairs with higher levels of BW discordance and with repeated DNA methylation and other epigenetic markers assessments over time to further investigate these differences.

Epigenetic Twin Studies and Psychopathology

Applying epigenetic analyses to the discordant MZ twin design, comparing methylation patterns between the affected twin and the unaffected co-twin has shown promise in the field of psychopathology, an area where, perhaps more than other disciplines, genetic research has failed to live up to its original promise. To date, the most studied disorders have been depression and psychotic disorders.

Using a genome wide analysis, Dempster et al. (2014) identified differentially methylated regions (DMR; using the Illumina Infinium HumanMethylation450 BeadChip) in buccal cells of 18 adolescent twin pairs discordant for depression. The top ranked DMR was located in *STK32C*, a gene that codes for serine/threonine kinase for which the exact function is not known. They were also able to validate the findings in postmortem cerebellum samples from 14 adults with major depressive disorder (age 46.9 years) and 15 controls (age: 48.1 years). Interestingly, Cordova-Palomera et al. (2015), using the Illumina Infinium HumanMethylation450 BeadChip, built on previous findings of increased variability in DNA methylation in affected co-twins of 34 adult twins (17 MZ pairs) discordant for depression. They found a differentially DNA methylated probe in blood in the *WDR26* gene, a gene that has also been associated with major depression in genome-wide association studies and whose expression in blood has been proposed to be a useful biomarker for depression. Further, Zhao et al. (2013) found in a sample of 84 MZ twin pairs an association between discordance in DNA methylation (as assessed in blood using pyrosequencing) and discordance in depressive symptoms, with increased differences in DNA methylation predicting increased differences in depression symptom scores. In a genome-wide study (12 MZ pairs discordant for depression, 12 pairs concordant for no depression and low neuroticism, age between 31–63 years), Byrne et al. (2013) found that overall DNA methylation levels (using the Illumina Infinium HumanMethylation450 BeadChip) in blood were lower in depressed twins compared to control twins; however, this was only observed in females. They also found that for both sexes there was greater variance in DNA methylation between the depressed twins compared to control twins (Byrne et al., 2013).

Using a sample of 22 MZ twin pairs (age 20.9 years) discordant for major psychosis, Dempster et al. (2011) used the Illumina Infinium HumanMethylation27 BeadChip to identify a number of epigenetic loci at which variability in blood cells was associated with bipolar disorder and schizophrenia. Furthermore, these loci were associated with biological networks involved in neurodevelopment and implicated in psychopathology. Castellani et al. (2015), using data from two families comprising MZ female twin pairs (age 43 years and age 53 years) discordant for schizophrenia, and their parents, identified variability in DNA methylation of genes (as assessed in blood cells using the NimbleGen Human DNA Methylation Promoter Plus CpG Island 720 k Array) involved in two specific networks related to cell death and survival, cellular movement and immune cell trafficking, both of which have been suspected to be involved in the origin of schizophrenia. Finally, in a genome-wide analysis (using methylation-sensitive representational difference analyses to lymphoblastoid cells) of a 49-year-old male MZ

twin pair discordant for bipolar disorder, Kuratomi et al. (2008) found significantly lower DNA methylation of the *peptidylprolyl isomerase E-like* (*PPIEL*) gene, which was confirmed in a case control study in 23 bipolar patients and 18 controls using pyrosequencing. However, the role of this gene and its functional implications are not yet clear (Kuratomi et al., 2008).

Although these studies provide insight into the association between the role of environmentally driven DNA methylation and health/disease, it is of interest that the DNA methylated genes identified in epigenome-wide research go beyond the 'traditional' monoaminergic or hypothalamic–pituitary–adrenal axis regulating genes that have frequently been associated with psychopathology. The specific role of identified differentially-methylated genes in the disease is generally not known. It is also important to note that these studies have all been cross-sectional, and thus temporal relationships between early environment, development and brain and behavioral outcome cannot be determined. Moreover, these studies were generally conducted in peripheral tissues and thus the relationship between these peripheral DNA methylation changes and DNA methylation changes in particular brain regions are unknown. Lastly, there is a general lack of replication across studies, which probably reflects different analytical approaches, tissues, ages and insufficient sample sizes.

Suggestions for Future Research

Although the MZ twin method is likely the most powerful method available for understanding the impact of environment on child development and mental health in humans, it is imperative that studies are well designed and confounds are adequately controlled for in order to make valid inferences. To understand mediating/predictive factors, temporal contiguity is required, with the cause preceding the effect. Thus, longitudinal studies are needed to test if an environmental factor *preceded* the outcome. Experimental studies with twins can also address that problem, but they would be still more powerful because they could test causal effects in a relatively small sample size compared to equivalent singleton studies (Tremblay et al., in press).

The current literature suggests that DNA methylation may act as a mechanism by which environmental factors can impact behavioral outcomes, and that this is likely achieved by altering neural systems involved in brain development. Although a limitation of DNA methylation research in humans is that DNA methylation cannot be assessed directly in the living human brain since DNA methylation patterns are tissue specific (Booij et al., 2013; Szyf, 2009), there is emerging evidence that the information obtained in peripheral measures is informative of the human brain (see Booij, Tremblay et al., 2013; 2015). Specifically, a number of studies now show correlations between DNA methylation, functional and structural frontal-limbic brain function, as well as with in vivo measures of monoamine function (Booij, Szyf et al., 2015; Dannlowski et al., 2014; Frodl et al., 2015; Wang et al., 2012). Further, although peripheral methylation would not necessarily reflect the state of methylation of the same gene in the brain (Booij et al., 2013), both saliva and blood DNA methylation levels have been associated with levels in brain tissue (Provencal et al., 2012), and DNA methylation in saliva has been shown in fact to be more similar to brain methylation than methylation in blood (Smith et al., 2015). It is critical to explore the exact nature of this relationship between peripheral DNA methylation, early environment and brain development using twin designs. Indeed, preliminary results of a study in our twin cohort suggest that DNA methylation may mediate the association between BW discordance and brain morphology discordance (Casey et al., 2015).

Experimental and longitudinal twin designs with repeated measures of emotional and biological development from birth onwards are needed to establish the *developmental interactions* between environment, DNA methylation, and human brain development, while controlling for DNA sequence. These studies will enhance our understanding of *how, why,* and *when* alterations in the human brain and risk for psychopathology develop.

Randomized controlled trials are especially needed to further understand causality. Twin designs provide a unique opportunity to study potential efficacy of preventative treatments, while controlling for genetic factors. For instance, as previously discussed, low BW is a predictor of poorer physical health and psychopathology, and discordance in birth weight is very common. Treatment studies can therefore be devised that attempt to prevent the development of negative health outcomes by using intervention strategies. The intervention can be applied to the at-risk twin, with the healthy twin serving as an ideal control. For example, in regard to the negative impact of discordance in BW, the low BW twin could receive enriched care while the normal BW twin serves as a control. Since the quality of the environment is important for healthy development, one would predict that the smaller twin with enriched care would become more similar to its co-twin in terms of physical and mental health (Tremblay et al., in press).

Overall, targeting twins for a RCT is a powerful way of determining the effectiveness of an intervention strategy. DNA methylation discordance at genes relevant for development could also be examined before and after intervention to give insight into potential underlying mechanisms linking enriched environment to neural development.

Conclusions

To date, studies aimed at understanding basic epigenetic mechanisms and their role in both normal and pathological development have been highly informative and led to promising new lines of research. Only the twin design offers

the genetic control necessary to confidently draw conclusions about the molecular mechanisms underlying the environmental role in disease in a cost effective manner using sample sizes compatible with current technologies.

Moving forward will involve the use of longitudinal and experimental twin designs from pregnancy onwards to produce integrated knowledge on the coordinated development of DNA methylation, the brain, behavior and health. The outcome of these studies — identification of critical time periods during which the environment affects the brain and disease risk, as well as the mechanisms through which environmental effects occur — should inform the optimum timing and design of early preventive interventions to reduce risk for physical health problems and psychopathology.

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