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REVIEW

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Neuroimaging Epigenetics: Challenges and Recommendations for Best Practices

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Abstract—Neuroimaging epigenetics is an interdisciplinary application of epigenetics to cognitive neuroscience that seeks to identify molecular and neural predictors of human behavior. This approach can be sensitive to the dynamic interaction between biological predisposition and environmental influences, and is potentially more informative than an approach using static genetic code. Recent work in this field has generated considerable enthusiasm, yet caution is warranted since any novel cross-disciplinary approach lacks a set of established conventions or standards. In this paper we review existing research in the field of imaging epigenetics, outline important caveats and considerations, and suggest a set of guidelines for researchers conducting this work.

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Key words: neuroimaging epigenetics, cognitive neuroscience, neuroimaging, fMRI, behavioral epigenetics, epigenetics.

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INTRODUCTION

The ultimate goal of psychological research is to explain behavior in a way that it can be predicted and manipulated, and recent technological advancements have enabled the use of complex and fine-grained predictors. Behavior can now be explained at multiple levels of analysis, including the identification of molecular variables which contribute to stable individual differences, such as a person's genetic code. When genes produce individual differences in observable physical or psychological attributes, we refer to these as phenotypic outcomes, or phenotypes.

Since there are multiple steps in the biological pathway between genes and behavior, an intermediate phenotype – such as neural activity – is known as an *endophenotype* (Gottesman and Gould, 2003). Because they are further up the biological pathway, endophenotypes can provide valuable mechanistic information about the development of and susceptibility to behavioral phenotypes like psychopathology. Endophenotypes of psychological variables are investigated within the multidisciplinary field of *(neuro)imaging genetics*, where

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researchers use neuroimaging to identify structural or functional variability of the brain that presumably mediates the relationship between genes and behavior.

There are limitations to this imaging genetics approach, however. While genes are able to explain some psychological attributes, there is considerable scientific interest in molecular predictors that are sensitive to environmental influences. Epigenetic modifications – chemical changes made to DNA and histone proteins that affect how genes are expressed – are appealing candidates since they can reflect the direct influence of the environment on one's body. Further, epigenetic changes such as DNA methylation are measured as continuous variables and therefore do not suffer from loss of statistical power due to grouping participants by genotype, which is common in genetics research. Consequently the use of neural endophenotypes to bridge the link between epigenetic variability and behavior has developed into the rapidly growing subfield of neuroimaging *epi*genetics (Nikolova and Hariri, 2015; Nikolova et al., 2016; Wiers, 2012).

The neuroimaging epigenetics approach is quickly gaining in popularity, but it is accompanied by a set of unique challenges. As with any interdisciplinary work, the marriage of two methods requires twice the expertise and a careful understanding of the subtleties, vagaries, and pitfalls of both methods, as well as a consideration of any issues created by their use in conjunction. The aim of this paper is to define these challenges, and provide a framework through which researchers in this field can produce work of the highest quality.

While we intend the target audience of this paper to be cognitive neuroscientists interested in adopting a neuroimaging epigenetics approach, many of the issues discussed are germane to behavioral epigenetics more broadly and may be of interest to a diverse audience.

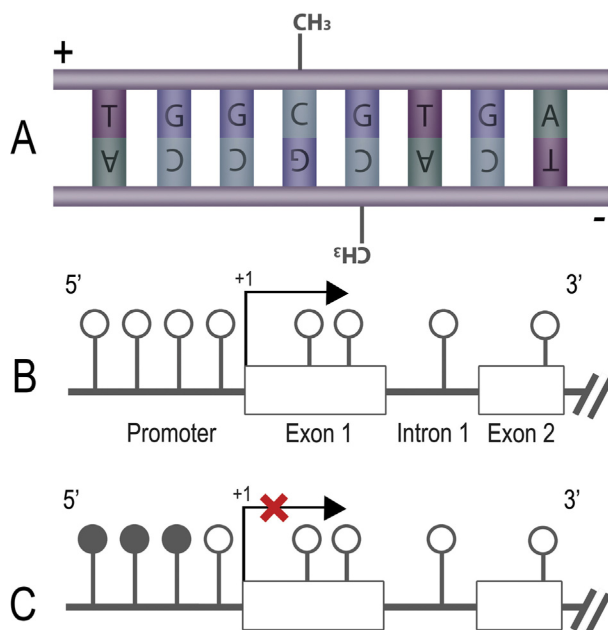

Fig. 1. DNA methylation affects gene transcription. A. Double-stranded DNA displaying the covalent bond between a cytosine (+strand, 4th position from left; −strand, 4th position from right) and a methyl group (CH_3). The CpG site on the +strand comprise the cytosine and guanine in the 4th and 5th positions from the left (−strand: 4th and 5th positions from the right). B. Gene schematic demonstrating position of CpG sites (empty circles). +1 = transcription start site. C. Schematic demonstrating a methylated promoter region (filled circles), which downregulates gene transcription.

An epigenetic primer

DNA methylation and histone modifications involving the tails of histone proteins (e.g., histone acetylation) comprise two of the main forms of epigenetic modifications. These epigenetic marks influence gene expression through a variety of mechanisms, including manipulating access of gene transcription factors through chromatin compaction or blocking transcription factor binding sites (Watt and Molloy, 1988) and/or recruiting transcriptionally repressive methyl-CpG binding proteins (Jones et al., 1998; Nan et al., 1998). While both of these processes are integral in regulating gene expression and often work in concert, this paper will focus on DNA methylation since it is the epigenetic mark most studied at the intersection of neuroimaging and epigenetics.

DNA methylation occurs when a methyl group forms a covalent bond with a cytosine base, most often at the site of a CpG (cytosine-guanine) dinucleotide (Fig. 1). Enzymes called DNA methyltransferases (DNMTs) catalyze the addition of the methyl group to the cytosine, where it becomes 5-methylcytosine. This enzymatic addition to DNA modulates the cell's transcriptional machinery, and when CpG methylation occurs within a gene promoter or enhancer this is typically associated with transcriptional silencing (Jones, 2012). Once established, DNA methylation is mitotically heritable, meaning the epigenetic mark is transmitted to each of its daughter cells through replication (Smith and Meissner, 2013). Thus, depending on the stage of development in which it was established, methylation can be conserved across tissues (methylated during early embryonic development before cell differentiation), or differ between different tissue types (methylated during later development).

Unlike a person's genotype, which is stable across time, the epigenome is dynamic and temporally labile: methylation can be actively or passively demethylated. Methylation patterns can be heritable, stochastic, or arise through environmental exposures such as stress or smoking. The epigenetic sensitivity to external influences has been called "experience-dependent individuation of the genome" (West and Orlando, 2014), and explains, for instance, how monozygotic twins can share a genome yet be phenotypically dissimilar.

While DNA methylation is an integral part of normal human development and is vital for processes such as cell differentiation, aberrant methylation can occur and negatively impact gene transcription and downstream processes. Functional consequences of aberrant methylation range from disease (such as cancer) to non-pathological phenotypic variability.

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BEST PRACTICES FOR NEUROIMAGING EPIGENETICS

Issue 1: choice of target site

A cognitive neuroscientist searching for an epigenetic modification to predict phenotypic variability at the neural level can be said to be on the ''hunt for the epiallele" (Finer et al., 2011). For the purpose of this review, we define **epiallele** as a locus on the genome whose epigenetic state varies between individuals and accounts for different phenotypes. In the context of DNA methylation, this could reflect a single CpG site (also known as a **methylation variable position**; Rakyan et al., 2011) or an entire genomic region of up to 1000 base pairs in length (also known as a **variably methylated region**).

However, with 28 million CpG sites in the human haploid genome (Fazzari and Greally, 2004), how does one choose a locus to test for a functional association with neural phenotypes? Once a candidate gene has been identified, one needs to narrow the focus on sites which may be of functional significance. For instance, the *OXTR* gene, which codes for the oxytocin receptor, has 392 CpG sites (429 if you include a promoter 3000 base pairs upstream of the transcription start site). However, not all of these sites will be associated with phenotypes. Here we describe a variety of considerations for choosing candidate epialleles for use in explaining neural endophenotypes, including interindividual variability, sensitivity to environmental influences, temporal stability, and interactions with genotype.

Interindividual variability

Choosing loci for epigenetic modifications which are highly variable between people is crucial for achieving the needed statistical variance for probing associations with neural endophenotypes. For example, when DNA methylation levels at single CpG sites are assessed using pyrosequencing, methylation levels of 1–2% are typically within the error range of the assay. Thus, comparing individuals using extremely hypomethylated loci (e.g., a mean of 1.5% methylation) is problematic both pragmatically (restriction of range) and theoretically (questionable validity). Nonetheless, this has been common practice: historically, the search for epialleles has focused on CpG islands – clusters of CpG sites which occur in frequencies greater than what would be expected by chance (Gardiner-Garden and Frommer, 1987). However, CpG islands tend to be hypomethylated compared to other genomic regions, meaning that they contain far less methylation than would be expected. Interest has now shifted toward genomic regions up- and downstream from CpG islands called CpG island ''shores" (Irizarry et al., 2009). These island shores tend to be variably methylated between people, impact gene expression (Irizarry et al., 2009), and can be sensitive to environmental influences such as early life stress (Bockmühl et al., 2015). Because a given CpG site may or may not be variable between individuals, it is *imperative* for interpretation of putative epialleles that researchers include within scientific reports the summary statistics of central tendency and dispersion for methylation levels of reported epialleles, and identify any significant outliers.

Temporal stability

DNA methylation is thought to be the most stable epigenetic modification (Cedar and Bergman, 2009). Because of its role in cell differentiation and genomic imprinting, most DNA methylation occurs during embryonic development and is maintained through cell division (Kafri et al., 1992; Monk et al., 1987; Ushijima, 2003). However, there are many locations within an individual's genome where methylation is not stable; CpG sites can be actively methylated and demethylated over time, either stochastically or through environmental exposures (Wolffe et al., 1999).

Loci that are both variably methylated between people and whose epigenetic state remains unchanged over meaningful timespans are known as **temporally stable epialleles** (Beyan et al., 2012). Exactly what constitutes a meaningful timespan is dependent on the specific scientific question, but temporal stability of DNA methylation has been reported, for instance, between 3 and 6 months (Lévesque et al., 2014), 2 years (Gemma et al., 2013), 5 years (Wong et al., 2010), and between 11 and 20 years (Talens et al., 2010). In a recent study (Flanagan et al., 2015), DNA methylation was assessed over 6 years and 17% of the tested sites were determined to be temporally stable epialleles; other studies have shown that temporally stable epialleles were already apparent at birth and remained stable through the first three years of development (Beyan et al., 2012). However, because of the lability of DNA methylation, temporally stable epialleles could occur at any point in development, meaning that later occurring epigenetic marks would vary across tissue types.

By contrast, **metastable epialleles** are a form of temporally stable epiallele that occur during early embryonic development, and thus are conserved across tissue types (Harris et al., 2013). They are thought to be quite rare: a recent attempt to quantify metastable epialleles revealed that $< 0.01\%$ of the tested regions showed metastability that was not attributable to genetic variation (Waterland et al., 2010). Because of the early acquisition and temporal persistence of these modifications**,** metastable epialleles are of particular relevance to researchers studying early prenatal exposures (e.g., nutrition, chemical exposure, maternal stress) or those with prenatal models for emergence of psychiatric disorders or other individual differences (Warner and Ozanne, 2010). For instance, studying seasonal variation in the diets of Gambian women revealed that nutrition at the time of conception was predictive of methylation levels at metastable epialleles in their children (Dominguez-Salas et al., 2014). Because of their early acquisition, metastable epialleles would not be appropriate to study if the *genesis* of the methylation was presumed to occur later in life, for instance, if methylation of the glucocorticoid receptor gene (*NR3C1*) was thought to mediate dysregulated neural processing of emotion in soldiers after experiencing combat-related trauma. In this example, methylation of

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NR3C1 would be expected to be a consequence of trauma experience in adulthood, which precludes the early acquisition of methylation inherent in metastable epialleles.

Temporal stability is thus an important consideration in identifying candidate epialleles, and determining the lability of an epiallele will be an important step in interpreting the causal relationship between epigenetics and neural endophenotypes. The researcher might be interested in a more (e.g., ‘‘trait-like”) or less (‘‘state-like”) stable epiallele (Lévesque et al., 2014); however, since there are no conventions dictating meaningful time-frames for stability, the onus is on the researcher to decide and defend the form of stability that is most relevant to the research question being assessed. Temporal stability likely depends on a number of factors, including the genomic location and the tissue type being assessed, and thus we strongly recommend the use of pilot studies to establish the timeframe of stability for the candidate epiallele.

Sensitivity to external influences. Individual variability in epigenetic modifications can be genetically determined, environmentally determined, or stochastic. If the relevant research question posits an environmental influence on epigenetic variation, then it may be advantageous for researchers to choose loci that have been previously associated with life stress or other environmental influences; certain target sites may be particularly sensitive to external influences while others are not. For instance, differential methylation between neonatal twin pairs (both MZ and DZ) is quite low at CpG islands and increases with distance from islands, suggesting that islands are less susceptible to prenatal environmental influences (Gordon et al., 2012). DNA methylation of specific genes has also been studied longitudinally in twin pairs, showing for instance, that methylation of the dopamine receptor D4 gene (*DRD4*) and serotonin transporter gene (*SLC6A4*) varies between twins as a function of unshared environmental exposures (Wong et al., 2010). Twin designs are therefore useful for identifying non-heritable methylation that may be sensitive to the environment.

Importantly, DNA methylation at certain loci seems to be malleable only during sensitive periods: in a seminal behavioral epigenetics study using rats, Weaver and colleagues showed that the level of maternal care affects the offspring's methylation levels of *NR3C1* within 6 days postpartum (Weaver et al., 2004). These methylation patterns and their associated dysregulated HPA axis responses persist into adulthood, suggesting that the first week of life is a sensitive period for epigenetic programing of the rodent stress response. In humans, early-life trauma contributes to demethylation of *FKBP5*, a gene whose product is important for glucocorticoid receptor function (Klengel et al., 2013). Importantly, childhood but not adult trauma predicted demethylation of *FKBP5* – this was supported by in vitro experiments demonstrating that a glucocorticoid challenge to hippocampal progenitor cells resulted in demethylation of *FKBP5, but only when the challenge was conducted prior to cell differentiation.* Methylation did not respond to a later (post-differentiation) challenge, suggesting that these effects are also specific to sensitive periods of development.

Interactions between genotype and epigenotype. Some proportion of variation in DNA methylation can be explained by genotype (Gertz et al., 2011; Shoemaker et al., 2010), a phenomenon known as **allele-specific methylation** (Rakyan et al., 2011). A person's genotype influences methylation at proximal CpG sites, typically within 1000 base pairs, and as the distance between a genetic polymorphism and a target CpG site decreases, the influence of that polymorphism on methylation increases (Gibbs et al., 2010).

Adding further complexity, certain types of genetic variants will create an additional CpG site in allele carriers versus non-allele carriers, known as an **obligatory epiallele** (Richards, 2006). This creates an additional level of variability between people since each person can carry zero, one, or two variably methylated epialleles. For instance, in rs4680 on the human COMT gene, a G > A mutation produces a methionine amino acid (Met) instead of a valine (Val). Because this mutation changes a G that normally follows a C base, Met alleles lack a CpG site present in Val alleles (see Fig. 2). Due to the differences in protein structure, Val allele-carriers have greater COMT enzymatic activity, which catabolizes the prefrontal cortical dopamine that is crucial for executive function. For this reason, Met homozygotes have been observed to have greater prefrontal efficiency and behavioral accuracy in working memory tasks (Egan et al., 2001). However, methylation of the obligatory epiallele can interfere with transcriptional machinery, reducing the level of COMT and boosting dopamine in the prefrontal cortex. Recently, Ursini and colleagues (Ursini et al., 2011) observed that Val homozygotes whose obligatory epialleles were highly methylated had lower levels of COMT transcript, greater prefrontal efficiency and response accuracy in a working memory task, suggesting that highly methylated Val homozygotes' dopamine profile was similar to Met homozygotes. In this way, methylation of the obligatory epiallele can be viewed as a compensatory mechanism: despite their genotypic differences and structure/efficiency of their COMT enzyme, methylated Val homozygotes were phenotypically similar in working memory to Met homozygotes.

Issue 2. Target site and gene expression

Depending on the genomic location of the candidate epiallele, epigenetic modifications may have different effects on gene transcription, and thus it is critically important to establish that the association occurs in the predicted direction. As mentioned above, DNA methylation at gene promoters is nearly universally associated with transcriptional silencing (though there have been exceptions to this; Lam et al., 2012). While it is common practice to assess DNA methylation in gene promoters, there is often empirical motivation for selecting target loci in exonic or even non-coding intronic regions; for instance, certain intragenic sites may be locations for

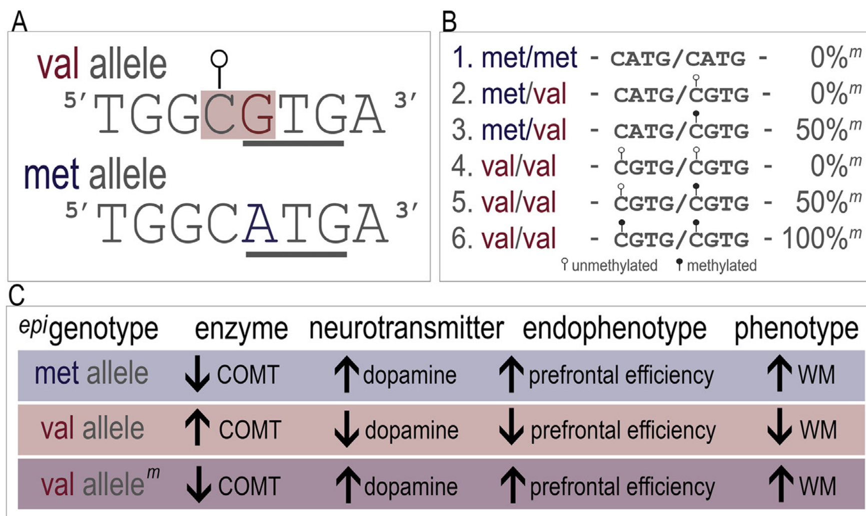

Fig. 2. *COMT* rs4680 creates an obligatory epiallele whose methylation predicts behavioral and neural phenotypes. A. DNA sequence demonstrating Val and Met alleles: the G > A mutation is depicted in color and the resulting valine and methionine codons are underlined. The shaded area highlights the location of the CpG site which forms the obligatory epiallele on the Val allele B. There exist 6 possible combinations of genotype and methylation for Val158Met: the middle column depicts the relevant DNA sequence and its methylation state. For each cell sampled, % methylation be 0, 50, or 100% methylated depending on zygosity and presence of methylation. Total% methylation is computed by averaging across all cells sampled in order to provide a continuous measurement ranging from 0-100%. C. Methylation of Val alleles can produce phenotypes that are similar to Met allele neural and behavioral phenotypes (e.g., more prefrontal efficiency and improved working memory performance). Methylation of the Val allele reduces transcription of COMT; thus, while the COMT form it produces is still highly active, the lower transcript levels from the methylated allele rescues prefrontal dopamine in these individuals. WM = working memory.

alternative promoters whose efficiency can be affected by epigenetic modification (Maunakea et al., 2010). It is less well established that methylation at these sites is associated with transcriptional silencing and must be independently verified. For instance, some studies have shown that gene-body methylation levels tend to *positively* correlate with gene expression (Rakyan et al., 2008; Singer et al., 2015), but may depend on the type of tissue (e.g., proliferative; Aran et al., 2011).

Manipulating gene expression: reporter genes. In humans, the functional effects of methylation on gene expression are difficult to experimentally manipulate in vivo, but we can use human cells lines and non-human animal models for (*in vitro* and *in vivo*) experimentation. One direct manipulation is the use of reporter genes, which involves inserting a section of DNA (e.g., promoter region from a human gene) into a DNA molecule called a reporter plasmid, and transfecting (introducing) this DNA construct into a host cell to create a model for gene expression. These reporter genes can encode, for instance, luminescent proteins which can be easily visualized. A popular model in epigenetics research is a CpG-free luciferase reporter gene, whose DNA insert (e.g., promoter) can be methylated in vitro without affecting the overall methylation state of the reporter gene (Wang et al., 2012). Changes in luciferase expression due to the de novo methylation are hypothesized to reflect how methylation of the inserted DNA sequence should affect expression in vivo in humans. For instance, Kusui and colleagues used this method to identify a critical regulatory region within *OXTR* that downregulates expression of the gene when methylated (Kusui et al., 2001). Cognitive neuroscientists wanting to study a candidate epiallele whose influence on gene expression has not yet been established by reporter gene should consider conducting this experiment to validate the functional effects of their site of interest.

Measuring gene expression. Additional evidence that DNA methylation affects gene expression in the predicted direction can be gained by obtaining human post-mortem tissue and correlating methylation levels with gene expression. Both transcription (RNA levels) and translation (protein levels) can be used in this context. For example, Labonte and colleagues (Labonte et al., 2012) used post-mortem hippocampal tissue from suicide victims with and without a history of abuse, and from control subjects to examine whether early life stress was related to methylation at sites in *NR3C1*. They found that early life stress from abuse was indeed related to differential methylation of *NR3C1*, and further that methylation at specific CpG sites predicted levels of mRNA within the same hippocampal tissue. It is also possible for researchers to use existing databases to investigate relationships between epigenetic modifications and gene expression: using, for example, DNA methylation and RNA-Seq data from the same tissue samples in the NIH Roadmap Epigenomics Project (http://www.roadmapepigenomics.org) could be helpful in generating or refining hypotheses about the functional effects of a candidate epiallele.

Issue 3: choice of tissue to sample

Peripheral tissue proxy. Since DNA methylation is a mechanism through which cells differentiate during early development, a characteristic of the methylome is variability across tissue types. While we are interested in methylation-related gene expression specifically in the brain, it is not yet possible to assess methylation levels of human neural tissue in vivo. For this reason, scientists studying humans typically use peripheral tissue methylation levels as a proxy for neural methylation levels. Popular choices for peripheral methylation level assessment include any nucleated cells that are inexpensive and non-invasive to acquire, such as PBMCs, saliva, and buccal swabs. Nikolova et al. (2014) used a combination of these tissues across a *discovery* cohort and an independent *replication* cohort to confirm that target CpG sites in both tissues were

similarly methylated, and that each group's methylation levels were predictive of neural activity within their respective cohorts. Crucially, because of their underlying differences, one can never *compare* methylation of two individuals or groups using samples derived from different tissues, nor can these data be aggregated.

Tissue specificity and composition. Because tissues are often comprised from several cell types, methylation patterns may also differ within tissues. PBMCs, for instance, are comprised of several types of white blood cells (T cells, B cells, NK cells, monocytes and dendritic cells) and methylation patterns may vary according to cell type (Philibert et al., 2011). Thus, interindividual variability in methylation levels might be confounded by different proportions of cell types. Covarying for cell counts within each sample would be one solution for this; another method would be to verify that all cell types within the sample were similarly methylated at the loci being investigated (Brennan and Flanagan, 2012). Researchers have also recently noted differences in DNA methylation levels across the brain in a genome-wide analysis (Gibbs et al., 2010); while methylation patterns were generally similar between the four tissue types sampled (frontal cortex, temporal cortex, pons, and cerebellum), the researchers noted that frontal and temporal cortices showed the greatest similarity to each other and the cerebellum was the most dissimilar of the tissues. Presumably, the greater the heterogeneity of the cells comprising the tissues, the more dissimilar their methylation patterns. While it has previously been difficult to separate neuronal tissue in order to profile cell-type-specific methylation patterns, a recently developed procedure enabled researchers to distinguish between three types of neurons from mouse neocortex: excitatory pyramidal neurons, parvalbumin-expressing fast-spiking interneurons, and vasoactive intestinal peptide-expressing interneurons (Mo et al., 2015). These researchers observed significant epigenomic diversity between each type of neuron, noting that certain characteristics of methylation profiles depended on whether the neurons were excitatory or inhibitory, and suggesting epigenetic heterogeneity that reflects the neuron's role in cellular signaling.

Further complicating this matter are the experience-dependent modifications which occur in postmitotic neurons and reflect cognitive processes such as learning and memory (Sweatt, 2009). Researchers have observed that neuronal depolarization – presumably as a result of cognitions such as memory formation – will dynamically alter neuronal methylation patterns which in turn affects gene expression in regions of the brain critical to memory processes such as hippocampus (Day et al., 2013; Miller et al., 2008; Miller and Sweatt, 2007). Given that not all cells within a neural subregion participate equally in a cognitive process – indeed it has been proposed that epigenetic modifications in hippocampal neurons may specifically track memory engrams (Ripoli, 2017) – there is likely to be significant heterogeneity in overall methylation patterns in cells comprising neural tissue. While these epigenetic modifications have been shown to occur primarily in genes contributing to synaptic plasticity, more research using cell-type-specific analyses will be necessary to establish the homogeneity of methylation across cells at candidate epialleles. Until this can be done, caution is warranted in making inferences about the underlying populations of cells contributing to an overall methylation profile.

Metastable epiallele. As mentioned previously, metastable epialleles have been defined as epigenetic modifications that occur early in embryogenesis, and thus their methylation profiles should be similar across tissue types. This suggests that peripheral tissues are reliable and readily available proxies for assessing methylation status of neural tissue. However, the ease of peripheral proxies comes at a price; these methylation levels are not sensitive to ongoing environmental influences (e.g., life stress), but only reflect environmental and stochastic influences during the first few weeks post-conception, since most de novo methylation occurs between embryonic implantation and gastrulation. Thus, a metastable epiallele can be treated more like a genetic polymorphism (though a continuous one) rather than a mark of environmental influences. This does not mean to say that environment does not interact with metastable epialleles (see Interpretation section), simply that the genesis of their methylation occurs earlier in development than other forms of temporally stable epialleles.

Justifying association between peripheral and neural tissue methylation. Researchers can support their use of peripheral tissue methylation as a proxy for methylation levels in neural tissue by employing similar methods for verifying effects of methylation on gene expression. One can use animal experimentation to harvest both peripheral and neural tissues from the same animal and verify that methylation levels in the two tissues are significantly related. This is useful insofar that genes and CpG sites are conserved across animal and human species. We can also test the relationship between peripheral and neural tissue methylation using post-mortem human tissue. While these samples are often small and not representative of the general population (e.g., disease, aging, or suicide), they provide a useful confirmation that the chosen peripheral tissue's methylation can serve as a proxy for the neural tissue of interest.

No relationship between methylation of peripheral and neural tissues. Importantly, several authors have made the argument that peripheral tissue methylation does not need to correspond directly to neural tissue methylation in order for it to be of importance for psychological research (Booij et al., 2013). Aberg and colleagues have elucidated two models for the relevance of peripheral tissue methylation: the mirror site model and the signature model (Aberg et al., 2013). When peripheral and neural tissues have similar methylation patterns, peripheral tissue methylation is associated with phenotypes because this relationship is mediated by neural tissue methylation: this exemplifies the mirror site model. When peripheral

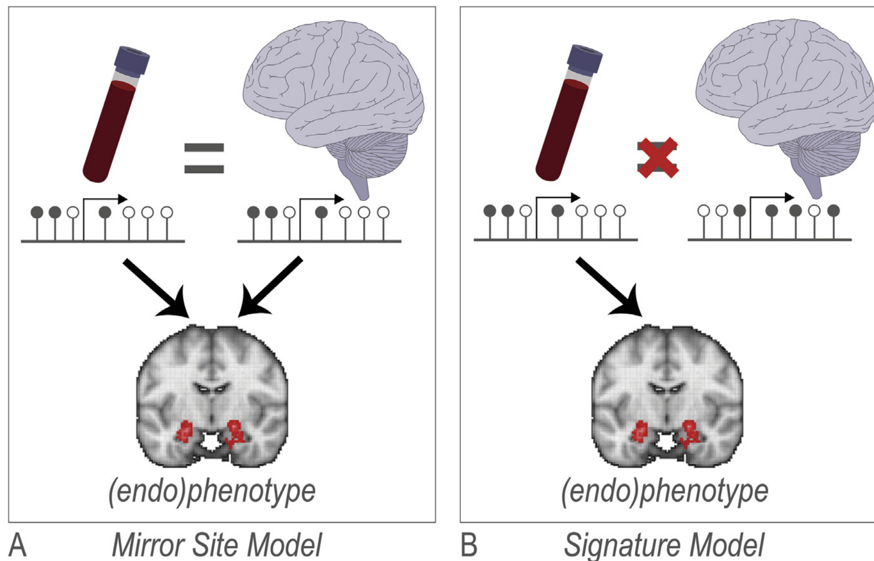

Fig. 3. The Aberg mirror site and signature models for the relationship between peripheral and neural tissue methylation. In the signature model, peripheral blood methylation may be important – regardless of its relationship to neural tissue methylation – if it predicts phenotypes.

and neural tissues have different methylation patterns, but peripheral tissue methylation is still associated with phenotypes: this exemplifies the signature model (Fig. 3). In this case, peripheral tissue methylation may be a biomarker or "signature" of an environmental stressor which contributed to the emergence of a phenotype without affecting methylation of neural tissue. For instance, an environmental exposure might trigger a somatic immune response that dysregulates the SAM and HPA-axis, affects neural development (e.g. altered functional connectivity), and precedes the emergence of an anxiety-related phenotype without directly affecting neural tissue methylation levels (or resulting in a different pattern of neural tissue methylation). In this example, PBMCs might show an altered methylation pattern that corresponds with a more anxious neural endophenotype (e.g., amygdala reactivity to stress) and behavioral phenotype (e.g., anxiety disorder), *regardless* of the relationship between PBMC methylation and amygdala tissue methylation. This would, of course, depend heavily on the gene being studied, how it is expressed somatically, centrally, and how it interacts with other complex biological systems. Importantly, *evidence has been found in support of* ***both*** *the mirror site and the signature models*, suggesting that peripheral methylation can be a useful biomarker insofar that it correlates with phenotypes (Aberg et al., 2013). Although the mechanistic pathway linking peripheral methylation with phenotypes is less clear in the signature model, it would still have prognostic value as it was altered by the same event which evoked the ultimate phenotype.

Issue 4: neuroimaging considerations

Neuroimaging epigenetics affords considerable advantages over traditional imaging genetics in predicting endophenotypes from molecular differences. In lieu of bifurcating data by genotype, we can model epigenetic modifications as continuous predictors of neural endophenotypes; this provides us with the statistical power to measure individual differences in neural endophenotypes. However, conceptually and practically, there are several considerations for neuroimagers in pursuing this line of research in order to maximize an experiment's validity, interpretability, and explanatory potential.

Interpreting neural endophenotypes. In neuroimaging epigenetics, neural endophenotypes can be structural (Liu et al., 2015; Rubin et al., 2015) or functional neural outcomes, as in BOLD response (Frodl et al., 2015; Moser et al., 2015; Ziegler et al., 2015) or ERP components. When using functional neuroimaging, our strongest piece of advice for the cognitive neuroscientist incorporating epigenetics into their research is to *use a clearly interpretable neuroimaging paradigm where it is well-established which patterns of neural activity represent a deficient or pathological phenotype.* Working with neural endophenotypes is difficult since neuroimaging data are open to interpretation: for example, what does it mean to have greater BOLD activity in a given region in a given task? Depending on the task and population, more recruitment of prefrontal regions could reflect less efficient processing, or could reflect greater allocation of attention. For this reason, we suggest using well-validated imaging paradigms, ideally those that have been used in patient populations to establish what a pattern of disordered response should look like. For example, greater amygdala activity in response to threatening faces is generally considered a "threat response" and has been validated as a neural endophenotype of anxiety (Stein et al., 2007). Two recent neuroimaging epigenetic studies used this task to demonstrate that methylation of sites on *OXTR* (Puglia et al., 2015) and *SLC6A4* (Nikolova et al., 2014) were associated with anxiety-related neural phenotypes, i.e., increased amygdala reactivity. Because of difficulty in interpreting their effects, novel functional neuroimaging paradigms would not be as appropriate here. By contrast, established resting-state networks could potentially be used insofar that they have been validated to predict clinically relevant traits or other phenotypes of interest (Menon, 2011). It may also be advantageous for researchers to use a functional imaging paradigm that maximizes *interindividual variability*. If a paradigm has strict demands on attention or is calibrated to an individual's level of performance, variability between participants will be more limited than in a paradigm with relaxed task demands (Yarkoni and Braver, 2010). For example, a working memory paradigm in which participants are

trained ahead of time, and task difficulty is calibrated to each participant's working memory capacity will improve the mean task signal by reducing individual variability; the result will be a robust aggregate signal. This is ideal for case–control studies since reducing sources of variability that are unrelated to case status will improve one's power to detect group differences. By contrast, a paradigm with relaxed task demands (such as passive viewing of photos or videos) where participants might engage different attentional or computational strategies, will increase variability in neural response and facilitate the detection of endophenotypes. We have had success using an fMRI paradigm in which participants passively view videos adapted from Heider and Simmel (1944), where geometric shapes interact in a way that is spontaneously anthropomorphized in healthy adults. With relaxed task demands, participants are free to attend to any aspects of the stimuli, and evidence suggests that the underlying neural processes may change as a function of social cognitive ability (as in autism; Castelli et al., 2002, and endogenous oxytocin levels; Lancaster et al., 2015). Consequently, we expected to find differences in neural endophenotypes as a function of methylation of *OXTR*, which has been shown to be disrupted in autism (Gregory et al., 2009) and may contribute to individual differences in social perceptual ability, even in healthy participants. Indeed, we were able to show that methylation of *OXTR* predicted neural activity in this paradigm (Jack et al., 2012).

Issue 5: statistical considerations

When considering a strategy for analyzing neuroimaging epigenetic data, the concerns are threefold. One must decide how to analyze the epigenetic data, how to analyze the neuroimaging data, and how to use one to predict the other. Many of the considerations here mirror those apparent in imaging genetics research, and many of the sophisticated analytic strategies that have been adopted to identify neural endophenotypes (e.g. multivariate analysis of fMRI data; Liu and Calhoun, 2014), can similarly be applied here. However, given that DNA methylation is a continuous variable that can be assessed in a variety of ways (Laird, 2010), a number of additional considerations must be made.

Resolution. Depending on the method of acquisition and specific hypotheses, methylation values can be drawn from single CpG sites (e.g., bisulfite pyrosequencing, Illumina Infinium HumanMethylation BeadChip, and in many cases EpiTYPER), or from a cluster of CpG sites across a region of a gene (e.g., other forms of DNA microarrays). Each form of measurement has a distinct resolution: many microarrays will provide a single methylation estimate for a large cluster of proximal CpG sites, whereas single CpG site assays will obtain discrete methylation values for every individual site. While DNA microarrays are common in behavioral epigenetics, the majority of the extant work in neuroimaging epigenetics uses single CpG site assays. This latter form of measurement can be focal or widespread: pyrosequencing can target a few proximal CpG sites in any genomic location, whereas the Illumina array will capture methylation at CpG sites across the genome (although this coverage is limited to the sites on the array, which comprise ~1.7% of all CpG sites in the case of the 450 K array). Additionally, technological advances in next generation sequencing are enabling researchers to achieve greater coverage at high resolutions cheaper and more efficiently than ever before; however, each method has its particular caveats which should be considered (for a comprehensive review of the challenges of genome-wide methylation analysis and in-depth discussion of various methods' strengths and weaknesses, we recommend Laird, 2010).

Data reduction. Adjacent CpGs vary in their level of interrelation: if they fall within the same functional region of a gene (e.g., promoter region), proximal CpG sites may have similar effects on gene expression (e.g., downregulation) and thus it may be appropriate to average across them to create a single predictor variable. Alternatively, given their variable intercorrelations, a more informative strategy for data reduction would be to identify patterns of interrelation using a data-driven method like principal component analysis (PCA). Nikolova and colleagues investigated 20 CpG sites upstream of the SLC6A4 transcription start site and probed associations between methylation levels and amygdala reactivity to threat: they successfully used PCA to reduce the number of CpGs to a handful of orthogonal factors and predict amygdala response (Nikolova et al., 2014). Data reduction is key in guarding against the inflation of type 1 errors, which is a concern for imaging genetics analysis (Meyer-Lindenberg et al., 2008). While research has shown that imaging genetics has good control for false-positives at normal-to-conservative statistical thresholds (Meyer-Lindenberg et al., 2008; Silver et al., 2011), the number of DNA methylation predictors is variable and will likely result in a greater number of statistical tests. Where data reduction is not possible, corrections for multiple comparisons will need to be applied. There are currently no conventions for data reduction or multiple comparison corrections in neuroimaging epigenetics research, but these will need to be developed, particularly as large-scale epigenetic datasets become easier to produce.

One caveat to data-driven approaches involves the functional significance of CpG sites. Previously when describing a candidate epiallele approach, we advocated for choosing a target epiallele based on identified characteristics (Issue 1) and verifying its role in gene expression (Issue 2). However, data-driven approaches are incognizant of these characteristics; thus, if candidate epialleles are statistically derived, one should prioritize assessing their functional significance on gene expression. Otherwise, the mechanism linking CpG methylation with neural activity would be difficult to interpret.

Replicability. The number of candidate CpG sites available for analysis raises similar issues to those associated with replicability of imaging genetics studies.

Since the number of CpG sites in the genome is so great (~28 million), replication of effects at individual CpG sites should be an important consideration as it has been for replicating SNP effects within imaging genetics for the last several years (Corral-Frias et al., 2015), and in the scientific enterprise more broadly (Nosek et al., 2012). One route to improving replicability is to ensure that one has sufficient statistical power with which to detect effects.

Power. A priori power analyses for fMRI research are challenging to compute since they rely on many factors: false-positive rate, first-level contrast, first-level design matrix, group-level contrast, group-level design matrix, effect size, within-subject variance, temporal autocorrelation matrix, and between-subject variability (Mumford and Nichols, 2008). Thus, researchers can compute power analyses using existing fMRI and epigenetic data (obtained from past or pilot studies), though this would only generalize to the same task and a similar population. One point of consideration for neuroimaging epigenetics studies concerns the fact that they are correlational designs which attempt to model interindividual differences in brain activity and thus require greater sample sizes than the one-sample t-test designs which formed the basis of original power recommendations in fMRI research (between 12 and 25 subjects; Desmond and Glover, 2002). Whereas a simple one-sample t-test would require 32 subjects to detect a moderate effect size at an alpha level of 5%, a simple correlational design would require 85 subjects to achieve the same power. Much like individual differences research in fMRI, neuroimaging epigenetics work will require large sample sizes for adequate power (Yarkoni and Braver, 2010).

Issue 6: sample selection

Sample homogeneity. Population stratification exists when subgroups within a population have different allele-frequencies, often a consequence of racial ancestry. Because genotype exerts a considerable influence on the methylome, population stratification is a major issue in DNA methylation work. Indeed, methylation of several hundred CpG sites has been shown to differ among racial groups (Fraser et al., 2012; Heyn et al., 2013). Analytic approaches have been proposed to control for stratification confounds (Barfield et al., 2014), but the most straightforward strategy is to study racially homogeneous participant samples.

Other variables, such as age, also influence methylation. In longitudinal samples, aging is associated with increased variability and site- and tissue-specific increases and decreases of methylation (Talens et al., 2010). This phenomenon is known as epigenetic drift (Egger et al., 2004). Age-related changes are stable enough that methylation from a number of sites can reliably be shown to predict a person's chronological age (Horvath, 2013). For these reasons, using homogeneous participant samples will be important for establishing the initial body of neuroimaging epigenetics work; these effects can later be replicated and extended using heterogeneous samples.

Issue 7: interpretation

A final word of caution concerns the researcher's interpretation of epigenetic marks and their relationship with neural endophenotypes. Because of the lability of epigenetic modifications, one of the unique challenges of behavioral epigenetics is in distinguishing *causal* from *consequential* phenotype-related epigenetic variation in cross-sectional designs. Particularly when trying to establish the etiology of disease states, it is tempting to assume that the chain of causality always follows from environmental impacts → epigenetic variation → disease phenotype. However, epigenetic variation is not always an effect of the environment on one's body; methylation is often stochastically determined and may precede any environmental influences that are known to trigger disease states. When sampling epigenetic measurements, environmental exposures, and neural endophenotypes simultaneously in a cross-sectional design, it is difficult to speculate on the time course over which these variables emerged.

An alternative model for the convergence of environment, epigenetics, and phenotype is an epigenetic-environment interaction. Similar to gene-environment interactions, epigenetic-environment interactions suggest that a stable, pre-existing epigenetic modification (for instance, a metastable epiallele) confers susceptibility to environmental exposures and may result in different disease states. In this way, epigenetic variation is not a *consequence* of environmental exposure, but is still causally related to phenotypes through exposures. In a recent study of emotional memory and PTSD, DNA methylation of *NR3C1* was assessed both in survivors of the Rwandan genocide and in a sample of Swiss adults who also underwent functional neuroimaging (Vukojevic et al., 2014). Methylation levels of *NR3C1* predicted risk for PTSD, and impact of intrusive memories in Rwandan males. In the Swiss neuroimaging study, methylation predicted memory performance and response during retrieval of valenced stimuli in males. Overall, the results suggest that DNA methylation of *NR3C1* might contribute to memory processes supporting risk for PTSD. Importantly, the overall methylation levels did not differ between Rwandan and Swiss groups: because of the difference in trauma experienced between the groups, it is unlikely that variability in methylation came about through exposure to stress. Instead, methylation patterns may have pre-existed any traumatic exposure *but enabled a greater sensitivity to stress* which led to PTSD in the Rwandan genocide group. This may be an example of an epigenetic-environment interaction where the sensitivity conferred by the methylation patterns interacted with stress to produce a disease phenotype. Further, given that the Swiss sample was free of psychiatric illness and presumably had not experienced stress to the degree experienced by the Rwandan sample, the BOLD differences predicted by methylation might reflect a neural

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endophenotype that represents the differential sensitivity to stress. Further research would be needed to disentangle causality, but this example illustrates the significance of considering the relationship between epigenetics and the environment for interpreting how each contributes to the development of a phenotype.

CONCLUSION

The application of epigenetics to understanding individual differences is nascent, with the use of epigenetics to identify neural endophenotypes being younger still. While this quickly growing field of neuroimaging epigenetics is exciting for its potential to identify the molecular mechanisms underlying behavior, there are boundary conditions for its successful implementation. Here we have described the field's major pitfalls and limitations, and suggested best practices for conducting and interpreting its work. Our hope is that this set of guidelines will enable others to perform research of the highest quality and innovation. While we have proposed a number of ideal standards with which to adhere in conducting neuroimaging epigenetics research, it is unlikely that all researchers will be able to apply all standards within each set of experiments. It is impractical, for instance, to expect a cognitive neuroscientist to have access to post mortem neural tissue, or to have the resources to develop a murine or cell-line methylation model. For these reasons, we emphatically recommend fostering cross-disciplinary collaboration. A fine-grained understanding of human behavior necessitates experimentation at several levels of analysis, from behavioral to molecular, and requires experts and resources spanning multiple disciplines. With the establishment of large collaborations and research consortiums, we have no doubt that research in neuroimaging epigenetics will continue to grow and contribute to our understanding of the molecular basis of human behavior.

SUMMARY BOX: KEY RECOMMENDATIONS

- Choose locus with high individual variability.
- Establish temporal stability of the methylation.
- Consider genetic influences on methylation.
- Verify how methylation affects gene expression.
- Justify your use of peripheral tissue (are you assuming mirror or signature model?)
- Choose an appropriate neuroimaging paradigm.
- Ensure adequate power and control false positives.
- Select a homogeneous sample.
- Carefully interpret causation.

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