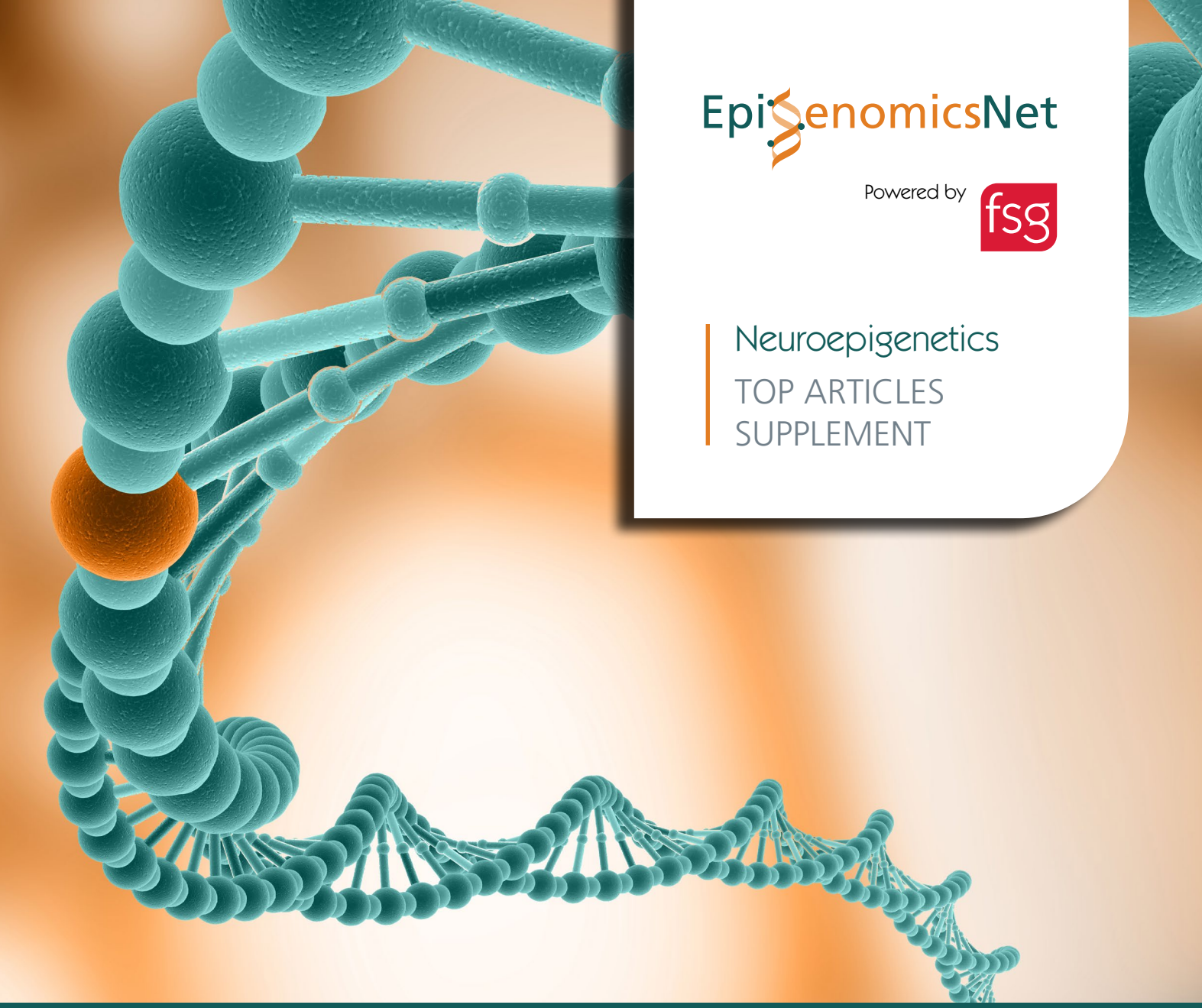

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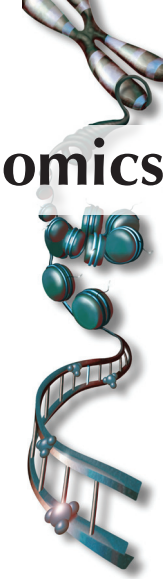

DNA methylation signatures of mood stabilizers and antipsychotics in bipolar disorder

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Aim: In view of the potential effects of psychiatric drugs on DNA methylation, we investigated whether medication use in bipolar disorder is associated with DNA methylation signatures. **Experimental procedures:** Blood-based DNA methylation patterns of six frequently used psychotropic drugs (lithium,quetiapine,olanzapine, lamotrigine, carbamazepine, and valproic acid) were examined in 172 bipolar disorder patients. After adjustment for cell type composition, we investigated gene networks, principal components, hypothesis-driven genes and epigenome-wide individual loci. **Results:** Valproic acid and quetiapine were significantly associated with altered methylation signatures after adjustment for drug-related changes on celltype composition. **Conclusion:** Psychiatric drugs influence DNA methylation patterns over and above cell type composition in bipolar disorder. Drug-related changes in DNA methylation are therefore not only an important confounder in psychiatric epigenetics but may also inform on the biological mechanisms underlying drug efficacy.

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Keywords: antidepressant • antipsychotics • bipolar • cell type • DNA methylation • medication • mood stabilizer

Epigenetic mechanisms are important in the development of the CNS and brain plasticity throughout life by influencing gene expression [1,2]. A growing body of evidence suggests that the epigenome also contributes to the pathogenesis of several psychiatric disorders [3–5]. Among numerous epigenetic mechanisms, DNA methylation is the most frequently studied epigenetic mark [6,7]. In this process a methyl group is attached to 5′-cytosine residues at cytosine–guanine sequences (CpG) in the DNA [8]. The majority of the CpG sequences are heavily methylated, resulting in a relatively stable repression of gene activity [9]. However, the CpG sequences that cluster at promoter regions of genes, called CpG islands, generally display relatively low levels of DNA methylation [10]. Although a large proportion of DNA methylation programing is stable and genetically regulated [11–14], environmental factors such as nutrition and medication can influence this process [15,16]. For instance prenatal exposure to famine is associated with hypomethylation at the *IGF-2* gene in humans [17]. Such changes in methylation status of promoter CpGs can occur across the life span in a small but significant part of the genome [16,18]. In cancer the possibility to influence DNA methylation has already lead to therapeutic pharmaceutical applications [19]. With regard to psychotropic medication, compelling evidence emerges from several preclinical and *in vitro* studies indicating that a variety of psychotropic medication show epigenetic effects as well including alterations in DNA methylation (for review see Boks *et al.* [20]). Psychotropic medication can affect DNA methylation by altering activity of DNA methyltransferases

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(DNMTs) that are essential in initiating and maintaining DNA methylation [10,21] during development and in adulthood [22].

In order to investigate the epigenetic effects of psychotropic medication, bipolar disorder (BD) patients are of particular interest because the treatment of both mood and psychotic symptoms require a wide variety of pharmaceutical compounds, including antipsychotics and mood stabilizers [23,24]. This in contrast to schizophrenia patients who are generally all on the same class of drugs. An example of a drug that we are able to study in BD patients is valproic acid; a mood stabilizer that acts as an histone deacetylase inhibitor [25] and indirectly counteracts hypermethylation of GABA promoters by inhibiting DNMT1 in prenatal stressed mice [26,27]. In human studies valproic acid use is associated with altered DNMT1 expression in the frontal cortex of patients with schizophrenia (SCZ) and BD with psychosis [28,29]. There are many more examples of psychotropic drugs that alter epigenetic marks in candidate gene studies in mice as well as postmortem brains of schizophrenia patients [30–34] (for review see Boks *et al.* [20]).

Even though several preclinical and *in vitro* studies have reported medication-related changes in DNA methylation, it is unknown whether such changes are truly present in psychiatric patients. Therefore we examined the DNA methylation signatures of psychotropic medication in the blood of 172 BD patients. These patients used a variety of mood stabilizing and antipsychotic drugs [23,24] that allowed the study of DNA methylation signatures of the mood stabilizers lithium, valproic acid, carbamazepine and lamotrigine, as well as the antipsychotics olanzapine and quetiapine. Because of the known association between cell type composition and medication, this study carefully addressed possible confounding effects of cell type composition [35,36].

Experimental procedures

Participants

Participants were eligible for participation if they had three or more Dutch grandparents and met criteria for diagnosis of BD. Data were collected in two waves at the Utrecht Medical Center; the first wave from 2009 to 2011 included 122 participants whereas in the second wave 50 participants were included between December 2011 and May 2013. The study was approved by the Utrecht Medical Center ethical review board and performed according to the ICH guidelines for Good Clinical Practice and the latest amendments of the Declaration of Helsinki. All participants gave their written informed consent prior to their inclusion in the study and were financially compensated.

Procedures

General

Participants were invited to the UMC Utrecht for the assessment that included a blood draw and interview. The interview was conducted by at least one well-trained independent rater. Clinical characteristics including mood and psychotic symptoms, comorbid psychiatric diagnosis, number of manic and depressive episodes, and age of disease onset were established with the Comprehensive Assessment of Symptoms and History (CASH) [37]. Participants of the second wave were assessed with the Structured Clinical Interview for DSM-IV (SCID) [38]. Current use of psychoactive substances was determined with the CASH in the first wave and a self-report questionnaire in the second wave. All participants reporting psychiatric medication use (antidepressants, benzodiazepines, anticonvulsants and antipsychotics), were on a stable (at least 1 month) dosing schedule. If participants smoked daily, they were defined as a smoker.

DNA methylation analyses

Whole blood DNA was extracted using Gentra Puregene Kit (Qiagen, CA, USA). DNA concentration was assessed using riboGreen, and integrity using BioAnalyser. Bisulphite conversion was conducted using Zimo kits (ZYMO Research, CA, USA) using standard procedures. Genome-wide DNA methylation levels were assessed using Illumina Infinium HumanMethylation27K BeadChip (Illumina) arrays in the first wave (n = 122) and Illumina Infinium HumanMethylation450K BeadChip (Illumina) arrays in the second wave (n = 50). Samples were equally distributed over the 24 arrays balancing gender and age on each of them to reduce any batch effects to the minimum. Intensity read outs, quality control parameters and methylation measures were obtained from the genome studio software. DNA methylation measures were excluded based on a detection p-value larger than 0.001. Probes with failed detection in more than 1% of the participants or less than five beads in 5% of samples were excluded as were samples with more than 1% of probes failed [39]. X chromosome, Y chromosome or nonspecific probes were removed [40]. Probes with SNPs of Minor Allele Frequency larger than 5% within 1 base pairs of the primer were also removed after constructing ancestry estimates based on their PCs as proposed by Barfield *et al.* [41]. After this step the 27 and 450 k data were combined, selecting the probes surviving quality control and present on both arrays (22,988 probes). The combined set was subsequently quantile normalized using the lumi package to remove technical variation between individuals.

Batch effects were analyzed by investigating the association of the PC of the methylation levels with plate, sentrix array and position using correlation and visual inspection of heat maps (see Supplementary Material 1, Figure 1). The Combat procedure as implemented in the sva package was used to remove batch effects for sentrix array. In this way we also remove any differences that may have occurred by using the two types of arrays in different experiments. After this procedure no batches for array, plate or sentrix were apparent [42] (see Supplementary Material 1, Figure 2). Finally cell type composition was calculated based on the methylation values for the 27 k data using the Houseman algorithm [43], while the calculation for the 450 k data was based on relating the methylation values to data derived from FACS sorted methylation data using the Houseman algorithm as implemented in a minfi based procedure [44]. In short, we used DNA methylation data from the arrays to analyze several differentially methylated regions that are markers of immune cell identity. To estimate sample-specific cell proportion in our whole blood samples, we applied a statistical algorithm [43] based on cell specific methylation profiles of an independent reference dataset of flow sorted cell types (monocytes, $CD4^+$ and $CD8^+$ T cells, granulocytes, B cells, natural killer cells) [45]. Cell type composition was investigated as a potential confounder (see Figure 1, Supplementary Material 1, Figure 2 and the results section cell type composition).

Statistical analyses

All statistical analyses were carried out using R version 3.1.2 [46]. For regression modeling, the Limma package was used [47]. Outliers were detected using Cook's Distance with a cut-off value of 1. If more than ten participants reported taking a specific type of medication, this medication type was added as a covariate to the linear model (see Table 1 & Supplementary Material 1, Table 1). Following this criterion olanzapine, quetiapine, lithium, carbamazepine, valproic acid and lamotrigine were included as specific medication types in all models. For methylation, β values were used for graphical display, but analyses were carried out using M-values (log2 ratio of methylation probe intensity) which has better statistical validity [48]. Because methylation may vary with age, sex and smoking [11], these were included as covariates in all analyses. To investigate population stratification, ancestry was estimated from methylation-based PCs as proposed in the Barfield study [41]. Population stratification did not play a role (see Supplementary Material 1, Figures 1–3) and methylation-based population PCs were not included in the models. First, the potential confounding effects of cell type composition was investigated by analyzing the association of medication with cell type composition. To account for confounding due to cell type composition, the cell count variances were regressed out while protecting for the association between medication and methylation all other analyses (see Supplementary Material 1, Figure 3) as implemented in the sva package [42]. Finally, in accordance with WGCNA default preprocessing steps [49], we checked for any obvious outliers in our sample with an average linkage hierarchical cluster analysis of the DNA methylation levels as implemented in the hclust function of the stat package in R [46,50]. No outliers were identified and all analyses were performed on 172 subjects.

Cell type composition of whole blood

We investigated whether the differences in DNA methylation between medication groups were due to differences in cell type composition of the samples (i.e., whether changes in cell counts were a mediator of the relationship between medication, global methylation levels and blood cell counts). First, to determine for which medication types there was an association with cell type composition a multivariate analysis of variance was performed with the five cell types (natural killer [NK], Bcell, CD8T, CD4T and monocytes) as outcome and the six medication types (olanzapine, quetiapine, lithium, carbamazepine, valproic acid and lamotrigine), sex, age and smoking status as determinants. Then PCs for methylation were calculated as a measure for global methylation and mediator analyses were performed with the mediation package in R [51]. In the mediator analyses the first five PCs were the outcome measures, the biggest cell fraction the possible mediator and the dependent measure was the medication type that had the highest correlation with the selected cell type in the multivariate analysis. The other cell types, sex, age and smoking were added to the model as covariates.

Network analysis

Weighted gene co-expression network analysis was performed with the WGCNA package in R to identify and characterize methylation clusters [49,50] based on their relationship with medication, the PCs and biological processes (using GO-term analysis). The association of the medication types with the identified methylation clusters was investigated using a linear model including age, sex and smoking status as covariates. Results were reported only for models with a good fit (p-value < 0.05).

The PCs were calculated for all 22,988 loci and based on the scree plot we used the first five PCs for analyses (see Supplementary Material 1, Figure 10, proportion explained variance per PC was: PC1 = 0.055;

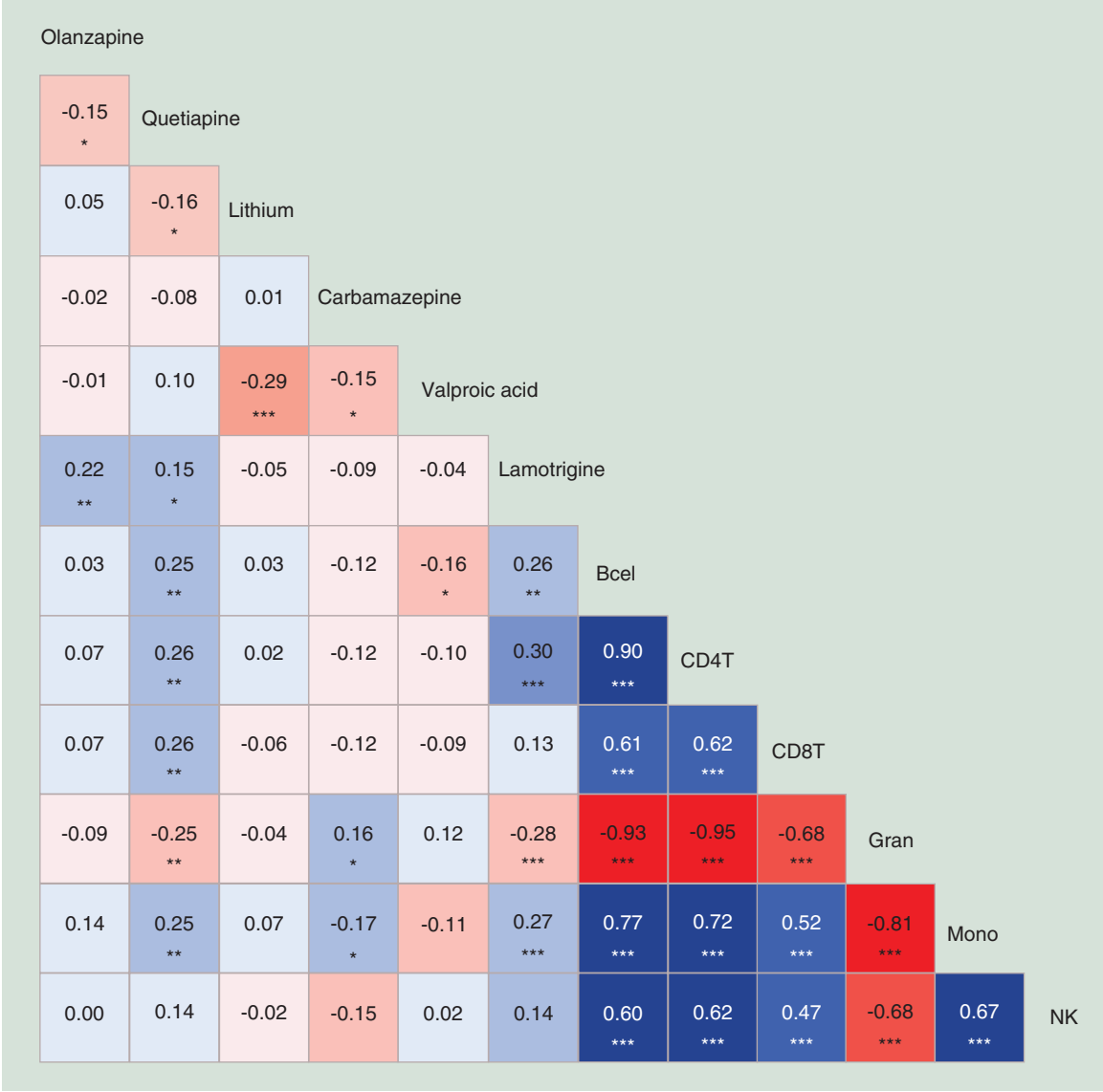

Figure 1. Heatmap depicting the correlation between medication and cell counts.
Significant values are denoted by * $p < 0.05$, ** $p < 0.001$, ***$p < 0.0001$.
CD8T: CD8 T cell; CD4T: CD4 T cell; Gran: Granulocyte; Mono: Monocyte; NK: Natural killer cell.

PC2 = 0.046; PC3 = 0.028; PC4 = 0.024; PC5 = 0.018, cumulative proportion = 0.17). First, the association between each PC and the selected medication types was tested in a linear regression model with smoking status, age and sex as covariates. Second, the correlation between the identified methylation clusters and PCs was explored by correlating the WGCNA methylation cluster module score to the PC scores. Finally, to investigate enrichment for biological processes with the GOstat package [52], we tested the GO-terms of the probes in the identified methylation clusters against all GO-terms of the probes surviving quality control. We only reported biological enrichment if the GO-term is significant ($p < 0.05$) after applying bonferroni correction for all GO-terms tested.

Epigenome-wide association study

The association between all 22,988 loci and the six selected medication types was tested in one overall linear model with age, sex and smoking status as covariates. From this model coefficients per medication type (adjusted for the other medication types) were extracted and the distribution of p-values was investigated by QQ-plotting and calculation of the genomic inflation factor. Only if the genomic inflation factor and visual inspection of the QQ plot indicated an acceptable distribution of p-values (see Supplementary Material 1, Figures 4–9), did we include the analysis results for the top 1000 probes in Supplementary Material 2. Epigenome-wide significant results were loci with a p-value lower than 0.05 after applying false discovery rate (FDR) correction.

Detailed analysis of candidate genes

Based on previous DNA methylation studies we selected the following candidate genes: *RELN* [31,53,54], *SLC1A2* [55], *MTNR1A* [56], *IGF2* [57,58], *H19* [57,58], *BDNF* [34,59], *SLC6A4* [55] and *GAD1* [31]. We interrogated all the probes on these selected candidate genes for their association with our six selected medication types in one overall linear model with age, sex and smoking status as covariates. Per medication type the p-values were adjusted for multiple testing by applying FDR correction ($\alpha = 0.05$).

Results

Baseline characteristics

A summary of the sample characteristics can be found in Table 1. Six medication types were used by more than ten patients, in order of number of users lithium (65%), followed by valproic acid (19%), quetiapine (17%), olanzapine (16%), carbamazepine (9%) and lamotrigine (8%). All other medication types were randomly distributed over these six main medication types (see Supplementary Material 1, Table 1). Diagnoses were: 169 patients with Bipolar type I disorder and three patients with Bipolar type II disorder.

Association between cell type composition & medication

There was a significant association between the five cell types and quetiapine (see Figure 1 Pillai's trace = 0.13; F(5, 158) = 4.9; p = 0.0003) and valproic acid (Pillai's trace = 0.07; F(5, 158) = 2.4; p = 0.04), but not for lamotrigine (Pillai's trace = 0.06; F(5, 158) = 2.3; p = 0.05), olanzapine (Pillai's trace = 0.05; F(5, 158) = 1.9; p = 0.10), lithium (Pillai's trace = 0.03; F(5, 158) = 1.0; p = 0.41) and carbamazepine (Pillai's trace = 0.02; F(5, 158) = 0.9; p = 0.50) (also see Figure 1 for correlation plot). The biggest cell fraction in our sample is CD4T and in the follow-up ANOVA the strongest association with CD4T was present in quetiapine users (F(1, 162) = 16.7; p = 6.7×10^{-05}). However, we found no evidence that the effect of quetiapine on global methylation, expressed as PCs one till five, was mediated by CD4T (proportion mediated for PC1 = 0.007; p = 0.78; PC2 = 0.003; p = 0.85; PC3 = -0.008; p = 0.89; PC4 = -0.009; p = 0.91; PC5 = 0.004; p = 0.81). To correct for any possible confounding due to cell type composition all other analyses were performed on methylation data with the cell type composition effects regressed out while conserving the association with DNA methylation.

Association between medication & network analysis of the methylation levels

We investigated DNA methylation levels represented in WGCNA modules and PCs. We derived seven modules based on the intercorrelation patterns among probes of which the blue (F[9, 162] = 2.74; p = 0.005), the red (F[9, 162] = 3.06; p = 0.002), the yellow (F[9, 162] = 3.56; p < 0.001) and, the green (F[9, 162] = 5.97; p < 0.001) modules showed a good (significant) fit. The gray module contained 14,208 remaining probes that were not correlated to any of the six modules. Figure 2 shows that several of the medication types were related to a WGCNA module. The strongest finding is the association of the blue module (containing 2103 probes) with valproic acid use (B = 0.040; p = 0.009), this module was related to the response to wounding GO-term GO:0009611 (see Supplementary Material 3). In the yellow module (1450 probes, enriched for stimulus and detection-related GO-terms see Supplementary Material 3) with valproic acid (B = -0.032; p = 0.028) and lamotrigine (B = 0.045; p = 0.038). In the red module (254 probes, enriched for immune-related GO-

Table 1. Sample characteristics (n=172).

Variable	n (%) or mean (range)
Age, years; mean (range)	43 (19–77)
Female sex, n (%)	94 (55)
Smoking, n (%)	74 (43)
Age at onset, years; mean (range)	26 (7–60)
Number of episodes; mean (range)	9.3 (1–27)
Lithium, n (%)	112 (65)
Olanzapine, n (%)	27 (16)
Quetiapine, n (%)	29 (17)
Valproic acid, n (%)	33 (19)
Carbamazepine, n (%)	15 (9)
Lamotrigine, n (%)	14 (8)

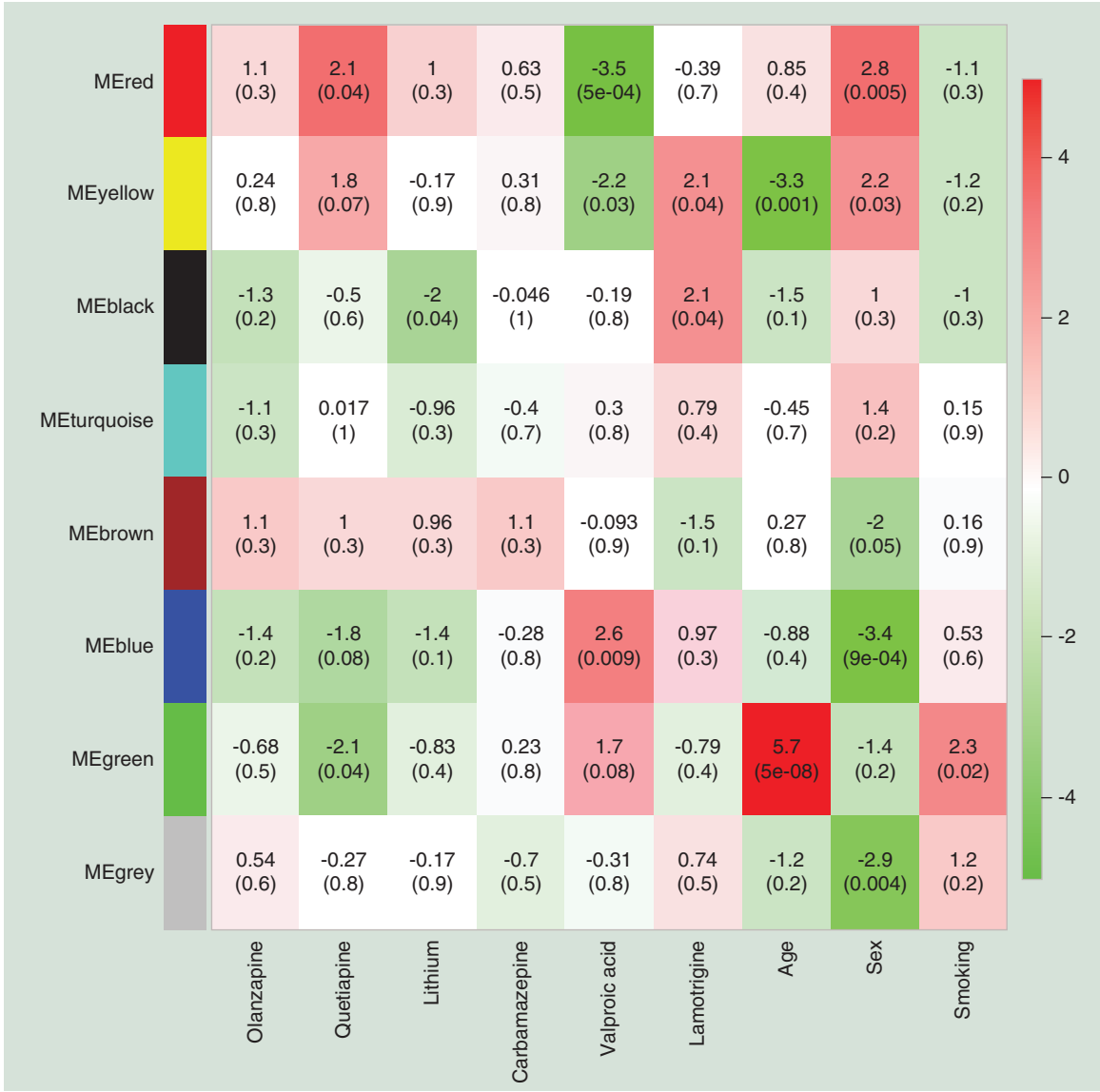

Figure 2. Heatmap for the relationship between the different WGCNA modules and the six selected medication types. In each cell the top value corresponds to the model t-value, whereas the bottom value between brackets denotes the p-value for this particular covariate.

terms see Supplementary Material 3) with quetiapine (B = 0.033; p = 0.040) and valproic acid (B = -0.053; p = 0.0005). Finally the green module (974 probes, enriched for neurogenesis, embryonic and regulatory GO-terms see Supplementary Material 3) was associated with quetiapine (B = -0.031; p = 0.036). Supplementary Material 3 shows the full results of the modules and their enrichment.

Valproic acid was also significantly related to higher values of the second PC of methylation levels, while olanzapine and lithium were associated with lower values on this PC (see Figure 3 and correlation plot in Supplementary Material 1 Figure 3) (model fit: F(9, 162) = 2.28; p = 0.02; olanzapine B = -0.43; t = -2.0; p = 0.05; lithium B = -0.33; t = -2.0; p = 0.05; valproic acid B = 0.47; t = 2.3; p = 0.02). Consistently; the blue module (related to valproic acid use), was highly correlated to PC2 (Blue: r = -0.81; p = 3.1×10^{-42}). Furthermore, lamotrigine was related to lower values of the fourth PC (model fit: F(9, 162) = 7.83; p < 0.001, lamotrigine B = -0.67, t = -2.6; p = 0.009). Finally, lamotrigine users scored higher, whereas quetiapine users scored lower on the fifth PC (model fit: F(9, 162) = 2.08; p = 0.03, lamotrigine B = -0.43; t = -2.0; p = 0.05; quetiapine B = -0.43; t = -2.0; p = 0.04).

Genome-wide association between medication & DNA methylation levels

The distribution of p-values was acceptable for olanzapine, lithium and carbamazepine (respective

genomic inflation factors: 1.011, 1.075 and 0.974, see Supplementary Material 1, Figures 5–10 for qq plots and Supplementary Material 2 for the top 1000 probes). None of the associations between probes and these three medication types were significant after FDR correction, but for carbamazepine the highest ranking probe (cg24523000) is located on the *GABRA1* gene (logFC = 0.18; p = 0.205).

Association between medication & methylation on candidate genes

The results for the methylation probes for specific candidate genes (*RELN, SLC1A2, MTNR1A, IGF2, H19, BDNF, SLC6A4* and *GAD1*) are presented in Supplementary Material 1, Table 2, but overall the association between specific medication types and methylation status of the loci did not provide any replication for these candidate genes after FDR correction.

Discussion

This study explored the influence of six psychotropic drugs on blood-based DNA methylation levels by analyzing networks, PCs, hypothesis driven candidate genes and epigenome-wide association in BD patients. The network and PCs analyses study global DNA methylation changes, whereas the candidate gene and epigenome-wide techniques evaluate individual methylation sites. The main findings of this study suggest that, after adjustment for cell type composition in whole blood, psychotropic medication use remains associated with alterations in DNA methylation levels at least in methylation networks and potentially at individual loci. Our study shows that DNA methylation based co-expression networks and PCs are linked to several medication types. The network modules indicate that immune and neurogenesis-related processes are involved. In the candidate- and epigenome-wide analysis no specific

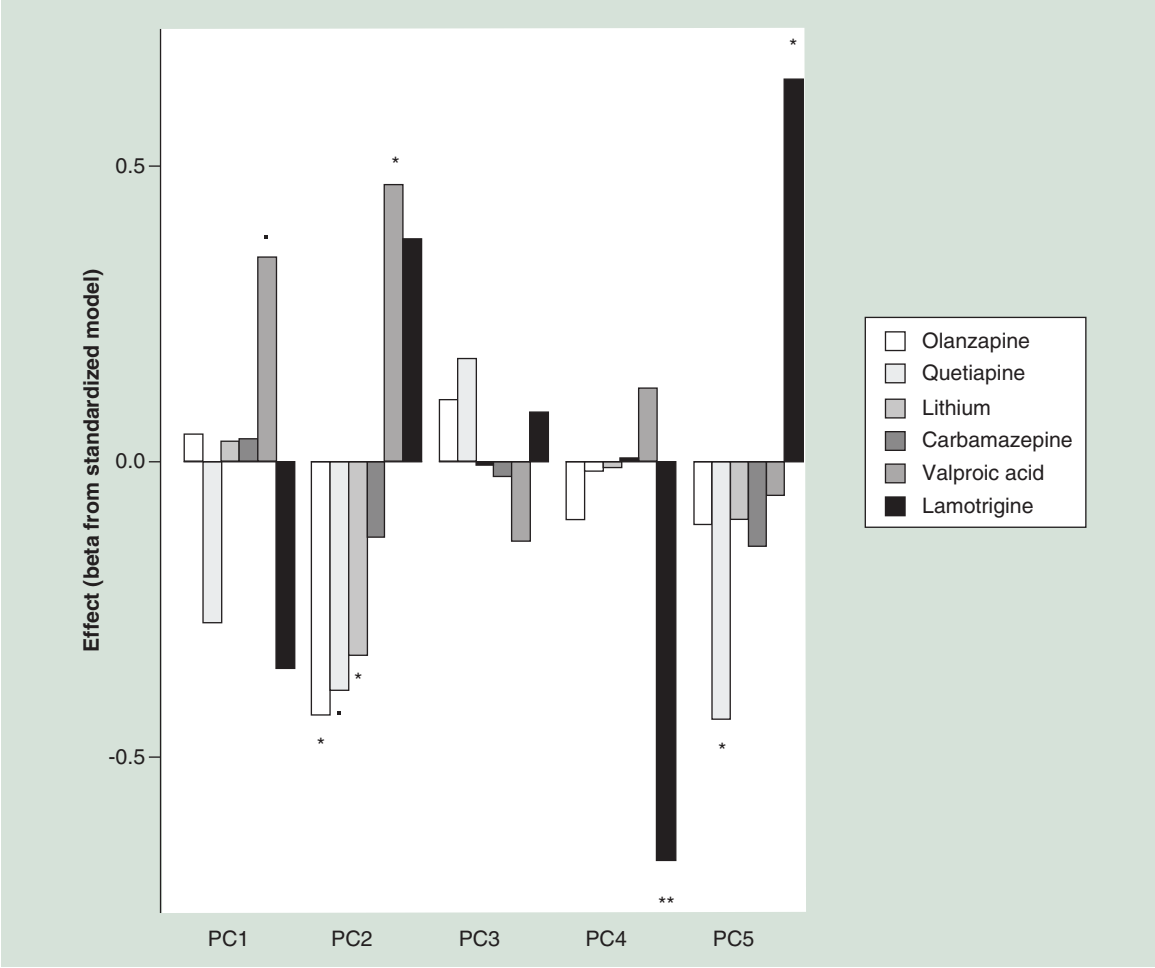

Figure 3. Barplot depicting the association between the six selected medication types and global DNA methylation measures principal component one till five. To enable comparison of the impact of the association between the five PCs and the six selected medication types the β from a standardized model are used in this graph.
• p < 0.10; *p < 0.05; **p < 0.01; ***p < 0.001.
PC: Principle component.

differentially methylated CpG site survived multiple testing correction, but qq plot analysis and trend level results suggest that this is most likely the result of limited power. Overall both network and single locus analyses implicate biologically plausible mechanisms for future epigenetic studies of psychotropic drug action. However, the cross-sectional design of the current study prevents a direct causal inference from the methylation differences and should be interpreted with caution.

The gene weighted correlation network analysis is an unbiased and data-driven method which has a high stability across tissue [12]. Four methylation networks were related to the use of valproic acid, quetiapine or lamotrigine. Valproic acid showed the strongest associations and was linked to three different co-expression modules, consistent with valproic acid's documented relation with DNA methylation and neurotrophic actions such as promoting neurite growth and cell survival enhancing neuronal function [60,61]. In terms of methylation co-expression networks, valproic acid as well as lamotrigine use were associated to a network characterized by the GO term detection stimuli, but the strongest connection between any medication type and a methylation network was for valproic acid and an immune-related methylation network. Altered immune system responses and increased inflammation are frequently linked to psychiatric disorders (for review see [62]). Interestingly valproic acid can reduce immune cell signaling by inactivating several enzymes involved in inflammation [63]. Quetiapine use was also related to the same immune-related methylation network and anti-inflammatory properties [64,65]. Thus, the immune-related methylation network could reflect the inflammation-reducing properties of valproic acid and quetiapine [63–65]. More importantly quetiapine use was linked to a methylation network with the highest enrichment for neurogenesis, which could correspond with the neurogenesis enhancing properties of quetiapine [66]. Reinstating adult neurogenesis is another potential treatment target for psychiatric disorders [67,68], and the current findings could provide new leads to study the mechanism of action of psychotropic drugs such as quetiapine.

Another reflection of the relevance of medication use for DNA methylation is the association of the PCs of methylation with medication. Particularly, the second PC of DNA methylation was associated with several medication types including valproic acid, olanzapine and lithium use (see Figure 3). Although the explained variance of this second PC was modest (4.6%), it could indicate that different types of medication may affect similar methylation in a similar way.

One approach to explore DNA methylation levels per individual locus was to perform a genome wide association study of all six medication types. Although after adjustment for multiple testing the associations rendered nonsignificant, the highest ranking probe for carbamazepine was on the *GABRA1* gene, which encodes for one of the subunits of GABA-A receptor in the GABA neurotransmitter system. Interestingly, the *GABRA1* gene has been proposed in the literature as a possible candidate gene for BD [69,70]. These findings suggest potential local effects on methylation of specific genes by psychotropic medications. Although the shapes of the QQ plots suggest a signal, limited sample size may have led to insufficient power to provide evidence. Limited power may also explain the inability to replicate several hypothesis-driven analyses of previously associated candidate genes.

All analyses performed, were adjusted for whole blood cell type composition (for review see [71]). That such adjustments are important is underscored by the profound influence of psychotropic medication on cell count of a variety of cell types reported here. Psychotropic medication in almost all classes has been reported to cause changes in cell type composition. Mechanisms include direct toxic effects upon the bone marrow, the formation of antibodies against hematopoietic precursors or involve peripheral destruction of cells [72–76]. Valproic acid may exhibit cell type composition alterations through immunosuppressive effects by activating apoptosis of activated lymphocytes and by weakening the cytotoxic effects of NK cells as well as the function of macrophages and monocytes but the underlying mechanisms need further investigation [77]. In our data particularly quetiapine and valproic acid use exerted a notable influence on cell type composition reaffirming this known effect of medication on cell type composition and underscore the need to adjust for this confounding effect in studies of whole blood. However, the reported DNA methylation differences were not mediated by the cell type differences and DNA methylation differences remained after elaborate adjustment for cell type and using network analysis that are more robust to tissue type influences.

Caution is required when interpreting results of this explorative cross-sectional DNA methylation study. The main limitations lie in the cross-sectional observational study design. In absence of randomization, blinding, placebo control groups and a longitudinal set up, there remains a risk of selection bias, confounding by indication and the inability to infer causality. Inherent to the study design is the presence of potential residual confounding, such as genotype, nutrition, other medical conditions or concomitant nonpsychotropic medication use. Finally since participants often use several medication types at the same time, it is not possible to fully disentangle selective effects of each medication type. Regarding the effects of polypharmacy (i.e., patients taking other types of medications), in our population the use of other psychotropic medication is low and ran-

domly distributed across the six main medication types. Even though we cannot completely exclude the influence of other medication types on our results, this suggests that psychiatric polypharmacy is probably not of large influence. The use of blood also poses a limitation considering that most effects of psychotropic medication are in the brain. Several studies have now pointed out that although there are vast differences between tissue types, particularly blood and brain [78,79], the differences between exposed and nonexposed individuals are often reflected in multiple tissues, with larger effect sizes for the differences between individuals than for differences between tissues [78,80]. Moreover because blood cells are also exposed to these drugs and many of the lymphocytes, such as B, T and NK cells, express similar receptors (e.g., BDNF, dopamine, GABA) as neuronal cells [81] the results are likely to be of use. For instance haloperidol administration in mice is associated with correlated changes in blood and brain methylation in more than 65% of the affected methylation sites [82].

Overall the current study found a profound influence of psychotropic medication on cell counts, but also presents evidence for an association between psychotropic medication and DNA methylation levels over and above altered cell type composition. Nevertheless, the precise nature of this association remains to be established in longitudinal studies.

Future perspective

Our understanding of the interaction between environmental exposure, such as psychotropic medication, and DNA methylation is in its early stages. Studies in cancer have succeeded in developing compounds that are essentially epigenetic drugs.

Considering the importance of epigenetic mechanisms in brain development and plasticity, manipulation of these epigenetic mechanisms may be a new target for treatment of psychiatric disorders. Indeed our study underscores the potential of psychiatric drugs to alter DNA methylation signatures and therefore highlights the need to further investigate and develop epigenetic treatments of psychiatric disorders. A challenge remains to extend the current study of the epigenome by including other relevant epigenetic mechanisms. Subsequently the molecular relevance of such epigenetic changes needs to be established. Ultimately the goal should be to establish clinical epigenetic therapy for psychiatric disorders in the future.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi.15.98

Financial & competing interests disclosure

This study was made possible by a NARSAD Young Investigator Grant to MPM Boks. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Aims & methods

- Cross-sectional observational study of methylation signatures of psychotropic medication in whole blood DNA of 172 patients with bipolar disorder focusing on:
  - Weighted gene co-expression networks
  - Principal component analysis
  - Epigenome-wide association analysis
  - Hypothesis-driven gene analysis

Conclusion

- Psychotropic medication has a profound influence on blood cell type composition.
- Over and above altered cell type composition this study provides evidence that psychotropic medication exerts an effect on DNA methylation levels of individual loci and networks.

Recommendations

- The influence of psychotropic medication is currently underestimated in epigenetic research and should be taken into account as an important confounder.
- Further exploration of the epigenetic effects of psychotropic medication can inform about potential drug mechanisms and facilitate the development of epigenetic drugs for psychiatric disorders.

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Perspective

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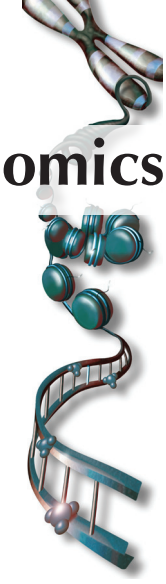
Epigenomics



DNA methylation in psychosis: insights into etiology and treatment

Evidence for involvement of DNA methylation in psychosis forms the focus of this perspective. Of interest are results from two independent sets of experiments including rats treated with antipsychotic drugs and monozygotic twins discordant for schizophrenia. The results show that DNA methylation is increased in rats treated with antipsychotic drugs, reflecting the global effect of the drugs. Some of these changes are also seen in affected schizophrenic twins that were treated with antipsychotics. The genes and pathways identified in the unrelated experiments are relevant to neurodevelopment and psychiatric disorders. The common cause is hypothesized to be aberrations resulting from medication use. However, this needs to be established by future studies that address the origin of methylation changes in psychosis.

Keywords: antipsychotics • DNA methylation • environment • epigenomics • exposure • mental disorders • monozygotic twins • olanzapine • psychosis

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It is now apparent that the manifestation of the genetic code into psychiatric phenotypes including mental disorders is not determined solely by DNA sequence [1,2]. The causation of psychiatric disorders involves complex interactions involving chromatin, where epigenetic signals superimpose a regulatory role. In fact, it has been suggested that the missing heritability seen in neuropsychiatric disorders could be due in part to the effect of epigenetic patterning [1,2]. This perspective suggests that the epigenome is in a dynamic state influenced by both deterministic as well as stochastic processes. This complexity also makes it difficult to tease apart the underlying factors that contribute to its state at any given time [3]. It represents a major challenge for future studies. For now, and for a variety of reasons, this research is accentuated by studies on epigenetic processes involving DNA methylation. DNA methylation in mammals involves the modification of cytosine to methylated cytosine (or its equivalent) in the genome. The phenomenon is sequence specific and needed for the proper functioning of the genome. DNA methylation provides regulatory roles in cellular functioning via regulation of gene transcription [4], genomic imprinting [5], gene splicing [6] and chromatin structure and stability [7]. Indeed, any aberration from normal patterns of methylation may cause abnormal cellular functioning including disease phenotypes [8]. Potentially, DNA methylation profiles can be altered by various factors including seasonal, social and environmental factors as well as chemicals and drugs [9,10]. This dynamic property may help to further the understanding of disease processes including mechanisms of actions of drugs that are used to treat disease. For example, it remains unknown how antipsychotic drugs control emotional and behavioral symptoms. The most accepted explanation is that antipsychotics have their own receptor-binding profiles, pharmacologic profiles and mechanisms of action [11–14]. Often, the treatment protocol involves 'testing and trying' toward finding a suitable drug and its appropriate dose for each patient. Some patients fail to respond to one antipsychotic but subsequently show a robust response to a different drug despite the fact that both

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block the D2 receptor, which is believed to be their mechanism of action [15]. Also, adverse effects of antipsychotic drugs vary greatly across patients [16]. Further, the delayed response of antipsychotics [17] and the associated variable metabolic side effects remain poorly understood [18–20]. It is however understood that drugs used to treat psychiatric disorders may cause epigenetic changes in the genome [21,22]. These changes have been identified in patients with psychosis that were on antipsychotic medication as compared with controls who were not on any medication. In fact, there have been a number of reports on epigenetic profile differences in psychosis patients, but the role of epigenetic changes in the causation of psychosis remains poorly understood [23]. The questions remain, whether these changes are the causes or the effects of the disease process and what is the role, if any, of drugs used by patients? In this perspective, we will discuss the role of DNA methylation in the etiology, pathophysiology and treatment of psychosis.

The etiology of psychiatric disorders involves DNA methylation

Psychiatric disorders, such as schizophrenia, may run in families as well as result from the response to a variety of drugs and chemicals. Schizophrenia has a high heritability (~80%) but monozygotic twins show a high discordance rate (~50%) [24]. The most extensive study performed on schizophrenia to date, has identified 108 loci, most of them with additive effects, that may contribute to this disease [25]. Also, some of the associated markers may contribute to the disease by affecting gene expression. These results argue for the potential involvement of regulatory mechanisms, particularly DNA methylation, in the development of the disease [26]. These mechanisms may underlie aberrations in neurodevelopment known to exist in a number of mental disorders [27–29]. The direct involvement of DNA methylation in schizophrenia has been assessed using a variety of inventive approaches, such as post-mortem human brains, familial relatedness including monozygotic twins discordant for the disease and animal models. In this perspective, we will use selected results to make the point that the epigenome is involved in the development of psychiatric disorders in general and in particular in schizophrenia, as summarized below.

First, studies on brains from patients with schizophrenia and matched controls have identified differences in DNA methylation [30]. The results are comparable to similar studies on blood samples from schizophrenia patients [30]. The questions of both tissue specificity and the effect of drugs are critical and present concerns in studies on methylation involving human brain disorders. Indeed, a recent study on medication free patients [31] suggests that the methylation effect is indeed a part of the complexity of epigenetic studies on schizophrenia. Also, some nonbrain tissues may serve as markers for abnormalities in the brain [32]. Further, the genes affected in patients are related to a number of pathways particularly the glutamatergic and GABAergic neurotransmission pathways, which have been previously implicated in psychosis [33,34].

Second, results on DNA methylation analysis of blood DNA from monozygotic twins discordant for schizophrenia further support the involvement of DNA methylation in psychosis [35]. Methylation of genomic DNA and promoter methylation of specific genes in blood samples of twins discordant for schizophrenia showed hypermethylation and hypomethylation of several genes [34]. These findings are consistent with the global increase and decrease in methylation of promoter regions of several genes in brain tissues in a rat model [36]. These findings suggest that a common epigenetic regulation mechanism may be applicable both in the brain and in peripheral tissues of schizophrenia patients [37]. Also, observed changes in methylation in all these studies report epigenetic changes that may have resulted in the disorder and also changes induced by the drugs administered to treat the patients. In addition, studies involving medication-free schizophrenia patients suggests that altered DNA methylation could be involved in the pathophysiology of schizophrenia [31]. Differences in methylation between identical twins have been identified as early as in newborn twin pairs [38]. Also, these differences change over time, supporting the potential for neurodevelopmental programing and reprograming in the causation of this disease [35]. Additional contributions to the discordance of monozygotic twins may involve *de novo* mutations [39–41] and epimutations [42], strengthening the case for dynamic processes including DNA methylation in psychosis. These processes are likely directed by genetic as well as random and environmental contributors over the lifetime [43]. We assessed the blood DNA methylation in two pairs of unrelated monozygotic twins discordant for schizophrenia using Methylated DNA Immunoprecipitation (MeDIP) [Castellani *et al.*, Unpublished Data]. The genomic DNA was processed at ArrayStar Inc (MD, USA); this included the MeDIP, sample labeling and hybridization to the NimbleGen Human DNA Methylation Promoter Plus CpG Island 720k Array. DNA was extracted from whole blood samples and the arrays were analyzed using Partek Genomics Suite® version 6.6 (MO, USA), Partek Pathways (Fishers Exact Test) and Ingenuity Pathway Analysis (Ingenuity Systems Inc., CA, USA). The results show that the monozygotic twins differ in DNA methylation. Interestingly, differentially methylated genes affect common pathways in

the schizophrenic twins [Castellani CA *et al.*, Unpublished Data]. Specific pathways identified include cell death and survival, cellular movement and immune cell trafficking network. In addition, Ingenuity Pathway Analysis (IPA) has identified protein kinase A signaling (p = 3.09E-04), granzyme A signaling (p = 6.83E-03), G protein signaling (p = 1.24E-02), serotonin receptor signaling (p = 1.72E-02) and UVB-induced MAPK signaling (p = 2.12E-02) as canonical pathways affected in this disease. Additionally, the top physiological system functions identified were nervous system development and function, immune cell trafficking and behavior. These results on blood DNA argue that DNA methylation in schizophrenia is common. Its pattern predominates the pathways that are compatible with their manifestation [Castellani CA *et al.*, Unpublished Data].

Third, evidence is emerging from human as well as animal models that antipsychotic drugs may function via their effect on DNA methylation. For example, a significant increase in DNA methylation (60.5 vs 37.6%) has been observed in schizophrenic patients who were treated with antipsychotic medication versus those that were not [34]. Similar results have been found in twins with major depressive disorder and on medication as compared with their unaffected co-twin not exposed to medication [44]. The authors also suggest that this difference could be due to antidepressants rather than as a cause or result of the disease alone [44]. It argues that methylation changes are not only involved in the etiology of psychosis, but also play a role in the response to antipsychotics [45,46]. Interestingly, antipsychotics were reported to have improved the efficacy of histone deacetylase (HDAC) inhibitors when administered in combination, through downregulation of genes such as *mGlu2* [47]. Also, previous reports suggested that antipsychotic drugs impacted DNA methylating enzymes only when the HDAC inhibitor, valproate, was administered concurrently [48]. This suggests that the impact of antipsychotics on DNA methylation may involve indirect mechanisms [47,48]. However, the specific mechanisms of actions of antipsychotics are not yet fully understood. Further study is required to investigate the possible mechanisms by which DNA methylation functions in regulating chromatin structure, stability and thereby gene expression.

We used a rat model to evaluate the effect of an antipsychotic drug (olanzapine) on genome-wide DNA methylation using methylation chips [36]. It offers for the first time, a published assessment of methylation differences between brain regions (hippocampus and cerebellum) and liver as a nonbrain reference in the same individual. The results show that olanzapine causes differential methylation that is tissue specific [36]. This response is similar but not identical between hippocampus and cerebellum and very different in the liver. Explicitly, olanzapine caused methylation changes in genes encoding for members of the dopamine pathways. They include *Drd1, Drd2, Drd5*, *Slc18a2*, *Ddc8* and *Comt*. Most of these genes (17/19) showed an increase in methylation in their promoter regions with *in silico* analysis [49]. These results strongly indicate the potential to suppress transcription, particularly in brain regions [49,50]. The findings support the dopamine hypothesis of psychosis and argue that antipsychotic drugs may mediate disease symptoms by their effect on the methylation of genes of critical pathways that include dopamine. Interestingly, the observed methylation alterations in the liver are compatible with the adverse effects of olanzapine that include metabolic syndrome and increased body weight. We conclude that DNA methylation may play an important role in the efficacy, as well as side effects, of the drugs used to treat psychosis.

Methylation changes in psychiatric disorders affect multiple pathways

Psychiatric disorders are highly heterogeneous in manifestation and causation. They involve multiple mechanisms including a large number of genes affecting relevant pathways. In fact, schizophrenia is associated with > 108 genes that participate in a number of pathways [25]. Most of these pathways are consistent with leading pathophysiological hypotheses. Some of these pathways are rare while others may be relatively common, such as the dopamine pathway. Such conclusions drawn from genome-wide associations are also compatible with methylation studies that have reported hypermethylation of the serotonin transporter gene promoter particularly in schizophrenia patients [50]. Some pathways are shared by a number of manifestations. As an example, *CDK5* and *CREB* signaling is often reported in schizophrenia [51], in excessive anxiety induced by stress [52], as well as in depressive-like behavior [53]. Interestingly, altered DNA methylation of genes involved in the *CREB* pathway has been reported in a recent study [54]. We feel that this complexity is complemented by differences in methylation across relevant tissues as well as by differential responses to different drugs. For example, the effect of a second-generation antipsychotic, blonanserin, causes hypermethylation in *DRD2* and *HTR2A* in human neuroblastoma cells [55]. Also, methylation at a transcription factor-binding site of the *5-HT1A* gene was reported to be associated with the treatment response to negative symptoms in schizophrenia patients [56]. In addition, a number of genes including *Drd2* and *Drd3* were downregulated in the nucleus

accumbens and prefrontal cortex of rat brains due to antipsychotic treatment, while *GLRA1* was found to have a hypermethylated promoter region [57]. Further, *DRD2, DRD4* and *DRD5* promoters were significantly methylated in schizophrenia patients as compared with healthy controls [58], suggesting that the dopamine network is actively involved in an increased risk for psychosis. We conclude that alterations in DNA methylation are in fact important to the etiology of psychosis. Also, alterations in DNA methylation may mediate the efficacy and side effects of antipsychotic drugs. The results also argue that not all pathways involved may have an equal contribution to psychosis. We will address this issue under two separate headings using results generated from our rat model [36,49].

DNA methylation & dopaminergic pathways in psychosis

Olanzapine-induced DNA methylation changes in rats included hypermethylation of genes of dopamine synthesis, receptors, transporters and metabolism [49]. It follows a report on olanzapine-induced methylation changes in the promoter regions of genes involved in the dopaminergic pathways in humans [59] arguing that transcriptional repression of those genes may be critical [60]. A similar conclusion might be applicable for other genes of this pathway including *DRD5* [61-64] and *COMT* [65]. Interestingly, the observed methylation peaks often overlapped with genomic regions containing CTCF binding sites, which are frequently associated with gene promoters and involved in genome organization [66]. The efficacy of antipsychotic drugs may represent an indirect effect via alterations in DNA methylation, which may take time to act. These results from different experimental setups suggest that the dopaminergic pathway is likely to serve as an essential framework in the etiology as well as treatment of psychosis.

DNA methylation & nondopaminergic pathways in psychosis

Results on olanzapine-induced methylation changes in our rat model identified six of the 18 genes to be involved in the GABAergic, glutamatergic and cholinergic pathways [36]. Among them, three (*Gls, Psd* and *Psd2*) were affected in hippocampus: one (*Nr1*) was affected in cerebellum, and two (*Nr2b* and *Glud1*) in the liver. Interestingly, a deficit of brain γ-aminobutyric acid (GABA)ergic function in schizophrenia patients has been linked to the downregulation of GABAergic genes [67]. The cadherin pathway was also affected by antipsychotic-induced DNA methylation in a rat model [36]. These adhesion molecules that constitute a super-family of transmembrane receptors mediate Ca^{2+}-dependent cell-to-cell communication [68]. The organization of these genes allows differential expression including gene-specific DNA methylation and differential splicing to facilitate expression of specific cadherin(s) in different cells and cell types [69]. Taken together, the results offer novel insights into the role of DNA methylation in altering expression of genes involved in nondopamine pathways potentially affecting the pathophysiology of psychosis. It is likely that additional pathways critical in psychosis will be identified in the future. There is every reason to hypothesize that at least some of these genes will involve alterations regulated by DNA methylation.

Conclusion

DNA methylation may play an important role in psychiatric disorders. Emerging evidence shows that antipsychotic drugs that are used to treat such disorders may also involve alterations in DNA methylation. This conclusion is based on the results from two distinct, yet overlapping, projects from our lab. First, our study on olanzapine treated rats showed that olanzapine-induced DNA methylation affects psychosis relevant pathways, including the dopamine, ephrin, GABAergic, cholinergic and cadherin pathways. However, an increase in promoter DNA methylation and GABAergic gene expression downregulation has been detected in the post-mortem brain of psychosis patients [70,71]. Also, Reelin promoter hypermethylation and its reduced mRNA expression has been reported [72]. This argues that antipsychotic-induced methylation (whether direct or indirect) may underlie the amelioration of psychotic symptoms as well as account for certain adverse effects including metabolic syndrome through upregulation or downregulation of relevant genes and thus have the potential to lead to an increase or decrease in impairment. As expected, the observed methylation changes were cell type specific and offered novel insight into the mechanism of action of antipsychotics [36]. However, the study did not assess DNA hydroxymethylation, which would be of future research interest. Second, the study on monozygotic twins discordant for schizophrenia, which included patients exposed to antipsychotic drugs, has uncovered many of the same pathways and networks, particularly relating to the dopamine pathway and GABAergic functions. These network functions have previously been linked to schizophrenia and were found both in our olanzapine-treated rats and in schizophrenia-affected twins when compared with their unaffected co-twins. Overall, the collective understanding of DNA methylation in psychosis demonstrates that methylation changes in the genome likely play a role in disease etiology. In addition, antipsychotic-induced changes in DNA methylation suggest that antipsychotic use could result

in methylation changes as a consequence of treatment. Thus, it remains the challenge of future research to tease apart the specific roles of methylation in the etiology of the disease, in response to drugs, and also in developing effective therapeutic strategies involving reversal of methylation. The results have provided a reminder that clinical research should no longer be about nature versus nurture, but instead about the complex interplay of nature and nurture. Also, the implications of these findings need to be carefully examined. They may help develop early diagnosis as well as methylation based grouping of patients to overcome heterogeneity. We note that DNA methylation is reversible and that this may allow for targeted methylation changes in the treatment of neurodevelopmental disorders.

Future perspective

Future epigenetic studies are sure to contribute a wave of Epigenome-Wide Association Studies (EWAS) results. It is critical to pair any EWAS data with a better understanding of the theoretical backdrop of epigenetic changes. This will include a better understanding of epigenetic response to treatment and other influences including environmental factors. Moving forward, a database of epigenetic profiles in healthy controls, in patients on antipsychotics and in patients without medication will allow researchers to begin to unravel some of these questions. For example, recent studies have begun to unravel involvement of aberrant DNA methylation changes involving medication-free [31] and drug naïve patients [50]. Methylome-wide studies also suggest that the methylation status of blood reflects those in the brain, supporting the use of blood as a surrogate tissue [73]. The timing of epigenetic change is crucial to ascertaining the role of epigenetics in complex traits such as psychosis, which may need further attention.

The inherent dynamic and responsive nature of the methylation landscape makes it both a candidate for the explanation of disease discordance in monozygotic twins for a number of phenotypes yet also presents an experimental challenge given its complexity and the vast number of factors that are now known to affect such changes. The question moving forward remains: What epigenetic changes being identified in cases are causal toward disease? Technological advances, increased sample sizes, model organism studies and an increased understanding of the timing and origin of epigenomic mutations will help us uncover the answers to some of these questions, which in turn will allow for a better understanding of the complex phenomenon of methylopathy. Finally, molecules will have to be identified that will help correct such aberrations and ameliorate the disorder.

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Executive summary

The etiology of psychiatric disorders involves DNA methylation

- Studies on brain and blood from patients with schizophrenia show differences in DNA methylation. This includes differences in monozygotic twins discordant for schizophrenia.
- We argue that methylation plays a critical role during neurodevelopment. Any aberration may contribute to the development of psychosis.
- Antipsychotic drugs that are often used to treat psychosis may function via their effect on DNA methylation.

Methylation changes in psychosis affect multiple pathways

- Alterations in DNA methylation may mediate the efficacy as well as side effects of antipsychotic drugs accomplished by a variety of unrelated pathways.
- The dopamine pathway and genes involved in glutamatergic neurotransmission are hypothesized to play a major role in psychosis related disorders.
- The dopaminergic pathway may serve as a prominent framework for the treatment of psychosis.
- Nondopaminergic pathways such as GABAergic, glutamatergic and cholinergic pathways likely also play a significant role.
- Other pathways, some still unknown, may be important in selected patients including families.

Future perspective

- There is a need to fully understand the involvement of DNA methylation in neurodevelopmental disorders.
- This understanding will be critical in the development of novel corrective measures currently not available in psychosis, which remain a societal burden.

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• of interest; •• of considerable interest

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Perspective

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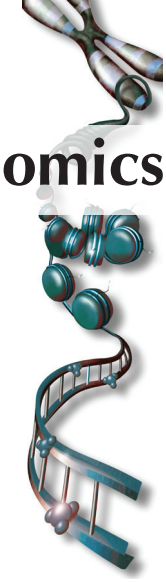
Epigenomics



Merging data from genetic and epigenetic approaches to better understand autistic spectrum disorder

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Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder that is characterized by a wide range of cognitive and behavioral abnormalities. Genetic research has identified large numbers of genes that contribute to ASD phenotypes. There is compelling evidence that environmental factors contribute to ASD through influences that differentially impact the brain through epigenetic mechanisms. Both genetic mutations and epigenetic influences alter gene expression in different cell types of the brain. Mutations impact the expression of large numbers of genes and also have downstream consequences depending on specific pathways associated with the mutation. Environmental factors impact the expression of sets of genes by altering methylation/hydroxymethylation patterns, local histone modification patterns and chromatin remodeling. Herein, we discuss recent developments in the research of ASD with a focus on epigenetic pathways as a complement to current genetic screening.

Keywords: common final pathway • DNA hydroxymethylation • DNA methylation • genetics • prenatal environment • transcriptome

The Center for Disease Control estimates that approximately one in 88 children are diagnosed with autism spectrum disorder (ASD). ASD is a neurodevelopmental disorder characterized by symptoms that include impaired social and contextual interactions, difficulties with communication skills and restrictive or repetitive patterns of behavior. ASD is considered to be genetically complex and inherited ASD can be caused by either single-gene defects or chromosomal abnormalities. It is also thought that multiple alleles of small effect that are present in high frequency in the population contribute to ASD heritability. ASD is symptomatically heterogeneous with many of the diagnostic symptoms showing considerable variability in severity. Females with ASD exhibit greater deficits in social communication, lower cognitive ability, reduced levels of restricted interests, weaker adaptive skills, as well as, greater difficulties with externalizing problems when compared with males [1]. Children with ASD exhibit a broad range of sensory differences both in terms of severity and with respect to sensory subtype focus [2].

The broad symptomatic profile of patients with ASD has hampered the search for diagnostic biomarkers of the disorder. Idiopathic (nonsyndromic) ASD, for which an underlying cause has not been identified, represent the majority of cases. Individuals with ASD traced to either single-gene mutations or defined chromosomal/cytogenetic abnormalities (syndromic autism) exhibit characteristic features that are often accompanied by additional comorbidities [3,4]. Genetically defined syndromic disorders which phenotypically overlap with ASD include Rett, Fragile X, Tuberous sclerosis, Asperger's, Smith–Lemli–Opitz syndromes, 22q11.2del, etc. [5]. Adding to these complexities is the observation that 70% of children diagnosed with idiopathic ASD exhibit comorbid medical, developmental or psychiatric conditions [3,6], including intellectual disability [7]. According to one review, over 100 genes have been linked to syndromic forms of autism [8]. In addition, there

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are over 400 high-confidence, nonsyndromic autism related genes that have been implicated by genetic approaches [9]. Interestingly, not all children with a predisposing genotype develop ASD [10]. This suggests that additional environmental factors likely interact with the genome in producing ASD. We discuss current findings related to molecular mechanisms of ASD and propose that the fetal environment interacts with predisposing genotypes to alter the developmental trajectory of cellular transcription in the brain.

Individuals diagnosed with ASD are often associated with alterations in the expression of specific genes depending on the presence of specific polymorphisms in the genotype. For example, null alleles in MET produce a frameshift and premature stop codon which results in little if any protein arising from the affected allele [11]. The MET proto-oncogene is a candidate ASD susceptibility gene. In addition to the genotype, the prenatal environment induces factors that modify the expression of distinct and likely overlapping gene sets. It seems plausible that the transcriptome of affected individuals reflects contributions from both the genetic background (genotype) and from environmentally mediated changes to the developmental transcription program. In other words, even though ASD is considered a complex genetic disorder, the complexity lies not only in the genetics, but rather in how the environment interacts at the level of the genotype to influence gene expression relative to neurotypical development. Ultimately, gene–environment interactions determine the specific ASD phenotypes associated with each patient. Increased risk of ASD is associated with mutations in genes that overlap with chromatin remodeling proteins, transcriptional regulators and synapse-associated proteins [12]. Interestingly, these genes are also targets of environmentally induced changes in gene expression.

ASD genetics: background

During the last decade, it has become increasingly clear that in addition to SNPs, copy number variants (CNVs) account for a significant percentage of ASD disease burden. During the last decade, both *de novo* and inherited gene mutations have been shown to contribute to increased risk of psychiatric disorders, including ASD. Whole exome sequencing studies have led to the identification of *de novo* CNVs in sporadic ASD patients, although specific variants are often rare and overlap with other psychiatric disorders [12,13]. The use of larger patient and control sample sizes has increased the confidence in the risk associated with specific CNVs. A recent analysis of extended ASD pedigrees suggests that the etiology of ASD is complex and does not necessarily involve the same genetic mechanisms across even closely related cases [14]. Recent estimates suggest that the numbers of genes genetically associated with ASD are greater than 1000 [12]. At the same time, these loci account for only a small fraction of the estimated heritability. While it seems clear that advances in our understanding of the genetic causes of ASD have been made by recent findings, additional work is now needed to further our understanding of the underlying biology of how various risk genes interact and the role of the environment in shaping these interactions.

In spite of the recent identification of large numbers of ASD risk genes and associated pathways, current estimates indicate that syndromic ASD accounts for less than 10% of total diagnoses [15]. Current estimates of heritability vary between 38 and 90%. A recent study of Swedish children was used to determine the relative recurrent risk of ASD [16]. Results show that the individual risk of ASD increased with genetic relatedness. Moreover, the heritability of ASD was estimated at 50% indicating that half of the risk is likely due to environmental factors that may either be shared or not shared [16].

Many of the mutations that have been implicated in ASD by genetic studies reside in genes that encode proteins associated with synaptic function, transcriptional regulation and chromatin-remodeling [12]. The functional diversity of genes implicated in ASD suggests the possibility that the etiology of ASD may be as heterogeneous as is the variable symptomatology [8]. There is also considerable overlap in genes associated with ASD and intellectual disability, as well as between ASD and epilepsy [8]. This suggests that the genetic origins of ASD may not only be difficult to tease apart but that the etiological heterogeneity of ASD may be just as complex. However, reducing phenotypic heterogeneity (by subphenotyping) did not increase the statistical power of a recent association analysis, suggesting the possibility that phenotypic heterogeneity is not simply the consequence of genetic variation [17]. Collectively, the large numbers of syndromic ASD-like disorders and the associated genetic variability indicate that ASD may represent a common end point for a collection of neurodevelopmental disorders that arise from a finite set of genes associated with brain function. While we are a long way from appreciating how genetic background influences global gene expression profiles, it seems plausible that the ultimate impact of genetic mutation lies in the downstream consequences of altered gene expression and function. The goal for the foreseeable future is to provide a better understanding of how specific genes function to disrupt specific biological pathways and whether these pathways are amenable to pharmacotherapeutic interventions.

Transcriptome profiles in ASD brain

As noted above, the symptomatic profile of individual patients is heterogenous. This observation when coupled with the concept that the etiological heterogeneity of ASD is complex leads to the suggestion that biochemical studies of ASD brain may not prove informative. Instead, however, what is emerging from ongoing mechanistic studies across the world is that the pathophysiology of ASD may converge on common biological pathways [18–20]. It seems likely that the observed symptom heterogeneity that is typical of ASD clinical presentation arises as a result of common pathophysiological mechanisms that interfere at different times (different trimesters) or with distinct brain structures (different cortical areas, hippocampus, etc.) during development. In addition, it also seems plausible that the variable ASD symptomatology associated with idiopathic ASD arises due to the consequences of epigenetic mechanisms acting on multiple, distinct genetic locations at different times and brain regions during development.

Many of the genes enriched in lists of genetic variants associated with increased ASD risk are linked to neuronal function. Analysis of gene expression relationships in specific human brain regions allows for the identification of modules of interconnected genes that correspond to distinct cell types. The modules, constructed by Weighted Gene Co-Expression Network Analysis (WGCNA) of genome-wide transcriptome data [21], are designed to provide information regarding coexpression networks that correspond to neurons, oligodendrocytes, astrocytes and microglia [22,23]. Moreover, they provide information regarding specific functionalities associated with the transcriptional programs of coexpressed genes. These relationships are useful in defining genes that are differentially expressed across multiple neuropsychiatric diseases [24]. Transcriptome studies of postmortem ASD brain show that modules of genes associated with brain function are dysregulated in ASD cortex. Comparisons of coexpressed genes in different brain structures show that regional variations in gene expression that highlight differences between frontal and temporal cortex are attenuated in ASD brain [25]. This finding supports the idea that gene expression related to cortical patterning is likely disrupted. WGCNA of the genes downregulated in ASD brain regions illustrate that one of the modules consists of genes related to both interneuron and synaptic function. Many of the synaptic function mRNAs have also been identified as ASD-susceptibility or risk genes. In addition to the decreased expression of genes associated with neuronal and synaptic function, the expression of genes enriched in astrocytes and microglia are increased in ASD cortex [25]. The involvement of genes associated with synaptic function and immune responses suggests the possibility that neuroinflammation or altered immune function in the brain may be responsible for the disrupted neuronal/synaptic function observed in ASD [26].

A recent transcriptome analysis of multiple ASD brain regions confirms these findings and also implicates genes associated with the activation of microglia and the immune response [20]. The microglial cell state module, which negatively correlates with the synaptic transmission module, includes genes associated with the type I IFN pathway, viral response genes and cytokine-mediated signaling pathway genes. Microglia have been shown to be important to the process of synaptic pruning by engulfing material in the brain through the complement receptor pathway [27,28]. Synaptic pruning prevents the overaccumulation of neuronal connections that accumulate during neurodevelopment and is linked to specific genetic mutations common in ASD patients [29]. The observed microglial/immune system dysfunction also raises the possibility that transcription profiles of ASD brain mirror the consequences of environmental insults such as prenatal infection.

DNA methylation writers

The downstream consequences of environmental factors such as maternal stress or infection reside in how cells of the brain respond to these prenatal or early life events. Transcriptionally active chromatin is open and characterized by the presence of transcription factors and coactivators that bind DNA in nucleosome-free regions. In closed chromatin, the DNA is condensed and bound by large numbers of repressor proteins. The transitions between open and closed chromatin states are facilitated, in part, by changes in the methylation status of the DNA. DNA methylation is a cell-type specific epigenetic modification that impacts chromatin architecture by influencing nucleosome positioning and the binding of various methylation readers to discrete regions of the genome. DNA methylation is critical during development of the brain and plays a key role in diverse processes from synaptic plasticity [30], learning and memory [31], to cognitive decline [32]. DNA methylation consists of the enzymatic addition of a methyl group to the C5 position of cytosine using *S*-adenosyl methionine and DNA as substrates. DNA methylation is a stable epigenetic mark that when located proximal to regulatory elements associated with transcription start sites, including promoters, most often facilitates the formation of a repressed chromatin state (reviewed in [33,34]). Approximately 70% of CpGs genome-wide are symmetrically methylated [35]. Regions of high CpG density, called CpG islands, are generally undermethylated as compared with other

regions of the genome with moderate-to-low CpG density. Stretches of DNA flanking the CpG islands, called CpG island shores, are thought to contain sites corresponding to cell-type specific differential methylation [36]. Genome-wide mapping of DNA methylation in the frontal cortex of humans has shown that in addition to traditional CpG methylation, non-CpG methylation is prominent in brain [37–39]. Interestingly, the positions of CpH are remarkably well preserved between neurons of different individuals [40]. Non-CpG methylation (or CpH methylation) is enriched at regions of low CpG density, in the vicinity of protein-DNA binding sites and, like CpG methylation, inversely correlates with gene expression [38,40]. Moreover, methylation of CpH occurs *de novo* during neuronal maturation and represents the majority of cell-type specific DNA methylation that promotes repressive chromatin and inhibits transcription [39]. In addition, determination of genome-wide methylation shows that megabase-sized regions of DNA exist that are devoid of mCpH, and these so-called mCpH deserts occur in genes such as the immunoglobulin VH locus which encodes variable domains of the immunoglobulin heavy chain and in olfactory receptor gene clusters [40]. Comparisons of mCpH/CpH and chromatin accessibility indicate that low-accessibility regions of chromatin tend to contain minimal amounts of mCpH. The more-accessible genomic regions exhibit a proportional relationship between accessibility and mCpH content, while mCpG levels do not show this proportionality [40]. These data support the idea that CpG and CpH methylation are independently regulated.

DNA methylation is initiated and maintained by members of the DNMT family (DNMT1, DNMT3A, DNMT3B and DNMT3L) of DNA writers which methylate cytosine to form 5-methylcytosine (5mC) (see Figure 1). DNMT1 is involved in maintaining the methylation status of CpGs during replication and is referred to as the maintenance methyltransferase. DNMTs 3A and 3B, so-called *de novo* methyltransferases, initiate new methylation in most cell types [33]. In addition to methylating DNA, DNMT family members bind to DNA in association with additional proteins, such as MeCP2, forming stable repressor complexes [41,42]. Genome-wide mapping of the localization of DNMT proteins to DNA in human differentiated and undifferentiated cells show that each DNMT binds to discrete and overlapping intragenic regions depending on cellular differentiation status [41]. Interestingly, comparisons between the genome-wide locations of DNA methylation and DNMT binding suggest that DNMTs exert methylation-independent regulatory roles particularly in the vicinity of promoters and transcription start sites [41].

Cell-type specific patterns of methylation have been observed in different cortical brain regions and cerebellum using DNA isolated from postmortem human brain [43,44]. A large portion of differentially methylated regions (DMRs) are detected at or near genes that are selectively expressed across different brain regions [43]. Interestingly, it was noted in this study that 5mC is under-represented at classic CpG island containing promoters and enriched at intragenic CpG islands and promoters containing low levels of CpGs [43]. A more recent comparison of the DNA methylation profiles of four cortical regions, cerebellum, thalamus, hippocampus and pons showed that, with the exception of the cerebellum, DNA methylation patterns are far more homogeneous between different regions of the same individual, than they are for a single brain region between different individuals [45]. These studies show that while the mRNA profiles differ markedly in different brain regions, the methylation landscape does not. This study suggests that DNA methylation may not determine patterns of cell-specific gene expression as previously thought. By contrast, in the cerebellum, a strong inverse correlation was observed between gene expression profiles and DNA methylation data [45]. These studies present contrasting points of view regarding the role of DNA methylation in regulating the cellular phenotype. The differing conclusions could be related to the approaches used in producing the results of the respective studies. That is, one group used human DNA methylation microarrays [43,44] while the second group used methyl-CpG-binding domain (MBD) affinity purification followed by deep-sequencing [45]. In both cases, only a fraction of the total sites in the genome that can be methylated are assayed. Moreover, both approaches do not provide adequate coverage of mCpH sites in the genome.

DNA methylation readers

Proteins that read methylated (5mC) and hydroxymethylated (5hmC) DNA bind these modified sequences through their evolutionarily conserved DNA-binding domains. Of the family of DNA methylation readers that bind methylated and/or hydroxymethylated DNA, MeCP2 is by far the most well studied. *MeCP2* is X-linked and is subject to X chromosome inactivation. It is genetically linked to the autism-related disorder Rett syndrome [46]. The purification, sequencing and cloning of *MeCP2* from rat brain was first reported some 25 years ago [47,48]. The single polypeptide chain contains a methyl-binding domain (MBD) and a transcriptional repression domain [49–51]. Structural comparisons with other MBD proteins, including MBDs 1–4, support the idea that the MBD is common to proteins that recognize and bind methylated cytosines in DNA [52].

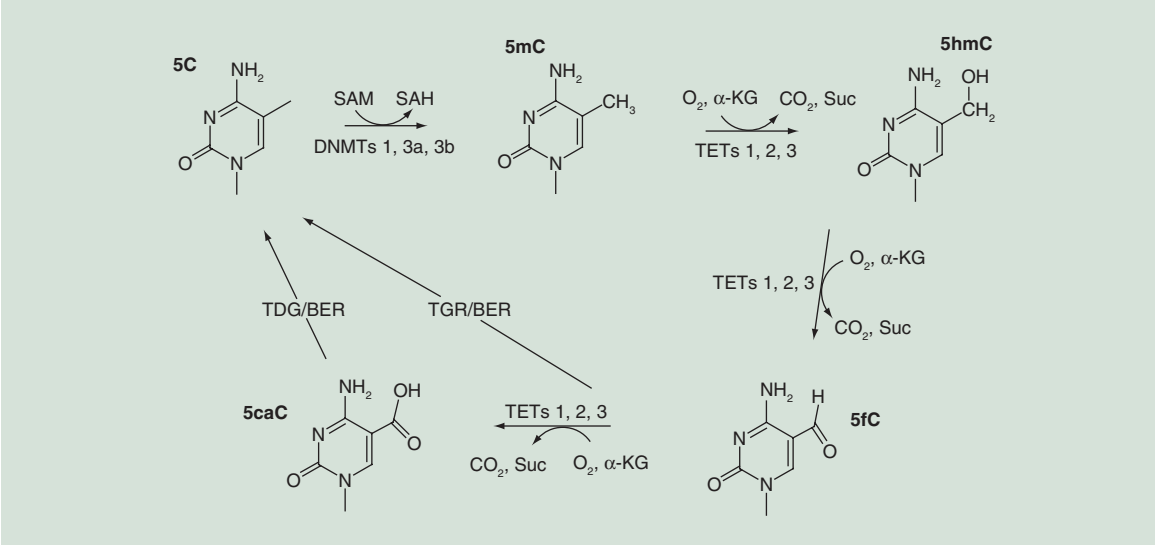

Figure 1. DNA demethylation pathway. DNA methylation, which utilizes the universal methyl donor SAM, is catalyzed by members of the DNMT family of proteins (DNMT 1, 3a and 3b). 5mC is a repressive chromatin mark that negatively correlates with gene expression. The demethylation of DNA, which re-activates transcription, occurs through several steps that involve the hydroxylation of the methylated cytosine. DNA hydroxymethylation, the first step in this cascade, is catalyzed by members of the TET (TETs 1–3) family of Fe(II)/α-ketoglutarate-dependent dioxygenases. These enzymes further oxidize the hydroxymethyl group to form 5fC and 5caC. Both 5fC and 5caC can be excised by TDG generating an abasic site which is subsequently replaced by BER enzymes regenerating the unmodified C. The intermediates in DNA demethylation (5hmC, 5fC and 5caC) accumulate to different extents in cells indicating that each epigenetic DNA mark is stable and likely serves a distinct function. This is thought to occur by the interaction of each modified base with a variety of DNA readers [86,87]. For example, unmodified cytosines (5C) are recognized and bound by proteins such as DNMT1 and TET1 that contain a -CXXC- motif in their zinc finger DNA-binding domain [42]. Methyl-binding domain proteins, like MeCP2 and other MBDs, bind to 5mC.
BER: Base excision repair; SAM: *S*-adenosylmethionine.

For many years (as indicated above), methylation was thought to occur predominantly at CG dinucleotides, although, as noted above, 5mC had been found in CA, CT, CC (i.e., CH, where H = A, T or C) sequences as well [53,54]. Recent estimates of non-CG methylation in mouse and human indicate that levels of 5mCH in the brain are at least as high as 5mCG if not more so [39,40]. In addition, the TET family of proteins oxidize 5mC to form 5hmC (see Figure 1), which acts as a stable epigenetic mark in neurons [55]. These findings raise the possibility that MeCP2 and other methyl-CpG binding domain proteins bind to multiple methylated or hydroxymethylated dinucleotides other than mCpG. But while MeCP2 has a high affinity for single symmetrically methylated CG dinucleotides, recent studies show that the MBD of MeCP2 also binds mCH with a strong bias for mCA [39,56]. In addition, it was also shown that the affinity of MeCP2 for 5hmC is considerably reduced relative to 5mC. This result contrasts with previous findings that identified MeCP2 as the major 5hmC-binding protein in the nervous system [57]. The recombinantly-expressed MBD of MeCP2 binds instead the rare hmCA modified dinucleotide [56]. Because of the low abundance of this modification in the neuronal genome, the significance of this finding is currently not clear. These findings challenge the concept that the MBD of MeCP2 binds both 5mCG and 5hmCG. However, MeCP2 may not strictly mimic the recombinantly expressed MBD and may exhibit altered binding properties when present as part of the native molecule.

As indicated above, *MeCP2* is defective in a majority of Rett syndrome patients [46]. There is significant symptomatic overlap between Rett syndrome and ASD, including deficits in verbalization skills and loss of purposeful hand movements. While many Rett syndrome patients were initially diagnosed with ASD prior to genetic analysis, most do not meet DSM-5 diagnostic criteria for ASD [58]. Nevertheless, posmortem brains from both disorders show cellular abnormalities that include reductions in dendritic branching and cell soma size and reductions in the numbers of dendritic spines [59–61]. While the function of MeCP2 is primarily associated with neurons and astrocytes [62], the distribution of MeCP2 has been reported in a large number of cell types [63]. MeCP2 associates preferentially with methylated DNA and next-generation sequencing shows that its genome-wide binding

tracks methyl-CpG density [64]. The MeCP2 primary transcript is alternatively spliced producing multiple (MeCP2E1 and MeCP2E2) isoforms. While both isoforms localize to neurons, astrocytes and oligodendrocytes, MeCP2E1 is relatively uniformly distributed in different brain regions, whereas MeCP2E2 shows differential enrichment in distinct brain regions [63]. Moreover, MeCP2 is differentially phosphorylated in an activity-dependent manner that is dependent on calcium [65,66]. Site specific phosphorylation of MeCP2 is known to impact both its binding to DNA and protein interaction partners such as CREB1, SIN3A, HP1 and SMC3 [67]. Moreover, activity-dependent phosphorylation alters MeCP2 function so that it orchestrates a genome-wide response to neuronal stimulation during nervous system development [68]. In addition to phosphorylation, MeCP2 has been shown to undergo additional post-translational modifications including acetylation, ubiquination and sumoylation (reviewed in [69]).

Mice deficient in MeCP2 in GABAergic neurons develop features reminiscent of ASD including stereotyped behaviors, deficits in social behavior, motor function, cognition and sensorimotor gating [70]. MeCP2 functions in cooperation with multiple chromatin repressors in binding to 5mC, including the Sin3A repressor protein and HDACs [50,71]. Chromatin immunoprecipitation assays demonstrate that MeCP2, DNMT1, DNMT3a and HDAC2 bind to the same region of DNA of the GAD1 and RELN promoters in NT2 cells [72]. MeCP2 has a substantial role in modifying chromatin architecture. In addition to repressing gene transcription, gene dosage studies show that MeCP2 positively regulates the expression of a wide range of genes [73]. By associating with the transcriptional activator CREB1 and binding to cAMP response element-binding (CREB) sites at the promoters of activated targets [73]. In addition to Rett syndrome, MeCP2-mediated regulation of gene expression has been implicated in a wide number of neurodevelopmental disorders, including, as discussed below, ASD [74].

DNA hydroxymethylation

In addition to 5mC, 5hmC accumulates in the genome in mammalian cells (see Figure 1). The identification of 5hmC in cerebellar Purkinje and granule neurons of the cerebellum [75], and the demonstration that knockdown of ten-eleven translocase methylcytosine dioxygenases (TETs) reduce the amounts of cellular 5hmC levels [76] established a mechanism by which DNA methylation might be reversed. For many years, it was thought that the stability of the methyl group on the cytosine ring made DNA demethylation thermodynamically unfavorable. 5hmC is formed from 5mC by the enzymatic oxidation of the methyl group which is catalyzed by members of the TET (1–3) protein family. 5hmC is both an intermediate in DNA demethylation and a stable epigenetic mark that plays a role in various processes in the brain [77]. Moreover, 5hmC accumulates at promoters, gene bodies and distal regulatory elements [78,79]. It is thought that once converted to 5fC and 5caC, the modified base is committed to demethylation by the action of thymine deglycosylase (TDG) [80]. Recent genome-wide maps of 5hmC in the brain have led researchers to attribute distinct functions to this modification depending on genic localization and local 5hmC density. That is, the function of 5hmC at distal regulatory elements called enhancers is different from its role within gene bodies or its role at mRNA splice sites. 5hmC is thought to impact transcription positively because it is located along the gene bodies of actively transcribed genes and it often also correlates with gene expression [81]. This is supported by data showing that the genome-wide location of 5hmC also negatively correlates with the location of two repressive chromatin marks, namely H3K27me3 and H3K4me3 [82]. Having said this, it appears that the role of 5hmC may be even more complex [83]. Base resolution maps of 5mC, 5hmC, 5fC and 5caC show that there is a Tet-mediated gradient of 5mC oxidation state that correlates with enhancer activity [80]. This suggests that one role of the TET proteins is to facilitate enhancer activity during development from the fetus to the adult [81].

As indicated in Figure 1, the TET proteins further oxidize 5hmC to form 5-fC and 5-caC, both of which are also stable epigenetic marks that accumulate in the brain across the genome [84,85]. 5hmC interacts with a large number of distinct nuclear proteins that recognize and read this epigenetic mark both in embryonic stem cells and in adult brain [57,86,87]. The 5hmC oxidation product, 5fC, is also read by a diverse set of proteins that include transcriptional regulators, DNA repair factors and chromatin regulators [87]. Both 5fC and 5caC (Figure 1) are specifically bound by TDG, which catalyzes the formation of abasic sites that are subsequently repaired by enzymes of the base excision repair (BER) pathway producing unmodified cytosine [88–90]. DNA demethylation via TET-mediated oxidation occurs in the brain in response to depolarization. Some 3000 CpG sites undergo active DNA demethylation in dentate granule neurons following synchronous neuronal activation [91]. The demethylated CpGs are located at or proximal to neuronal-specific genes implicated in neuronal plasticity [91]. The simultaneous activation of multiple genes by DNA demethylation in response to depolarization allows for an orchestrated transcrip-

tional response as an adaptation or plasticity program of the neuron. While many studies of TET proteins in the CNS have focused on TET1, TET3 was recently shown to act as a sensor of synaptic activity in the brain and to regulate surface levels of GluR1 in response to synaptic activity [92]. It is now recognized that all three TET proteins serve distinct functions in the brain and that neuronal differentiation is accompanied by increased levels of all three TETs.

DNA methylation in ASD

Female patients heterozygous for mutations in the X-linked methyl DNA-binding protein MECP2 are highly likely to exhibit symptoms of Rett syndrome. Because of the symptomatic overlap between Rett syndrome and ASD, DNA methylation would seem to be a likely mechanism associated with the pathogenesis of ASD. In addition, recent studies support the concept that epigenetic factors converge on DNA methylation as a consequence of environmental factors that are relevant to ASD. Studies of single ASD gene candidates provide evidence of altered methylation at multiple genes including *BCL2* [93], *EN2* [94], *MeCP2* [95,96], *OXTR* [97], *RORA* [93], *SHANK3* [98] and *UBE3A* [99]. While these candidate gene studies support a role for methylation in ASD, it is as yet unclear as to what may be causing these changes and why these particular genes are targeted.

In contrast, data from genome-wide DNA methylation studies have produced mixed results. A genome-wide methylation analysis of DNA from cerebella and cortices (Brodmann area 9 [BA9]) of ASD subjects versus matched typical developing controls found no significant differences in DNA methylation between groups [100]. This study was performed using the Illumina HumanMethylation27 BeadChip, which measures upwards of 27,000 probes across the genome. A second study independently reported results from multiple brain regions (dorsolateral prefrontal cortex, temporal cortex and cerebellum) of 20 ASD and typically developing controls using the updated Illumina HumanMethylation450 BeadChip. They identified multiple DMRs [101]. Positive results within each tissue were subsequently validated. The DMRs in the temporal cortex were proximal to the following genes: *PRRT1, TSPAN32/C11orf21* and *ZFP57*. In cerebellum *SDHAP3* was identified [101]. The DMRs are interesting and biologically diverse. PRRT1 is thought to be expressed primarily in the hippocampus and is a component of the outer core of the AMPAR complex. This DMR may be important in regulating the expression of the corresponding gene [102]. A second DMR identified in this study is located in a region that overlaps *TSPAN32* and *C11orf21*. While the function of the C11orf21 protein is not known, TSPAN32 appears to be a scaffolding protein important for cellular immunity [103,104]. ZFP57 is important for DNA imprinting marks during development [105,106] and recruits DNMTs [107]. Finally, the DMR identified in cerebellum is located on a CTCF binding site and active regulatory element associated with a noncoding RNA and a small coding RNA. It is also an alternative promoter of *SDHAP3* which is involved in succinate metabolism. Analysis of whole blood DNA from MZ twins discordant for ASD identified numerous significant DMRs, including many in genes previously implicated in ASD. These DMRs are widespread and correlate with autistic trait scores [108]. The specific sites that have been identified thus far support a role for aberrant DNA methylation in ASD brain and blood and provide insight into novel genes dysregulated in ASD.

The most recent methylation array analysis of ASD compared two cortical regions, BA24 and BA10, and also used the HumanMethylation450 BeadChip [109]. Like the previous two studies [100,101], these authors report data using a small set of postmortem ASD and control brain. In BA10, over 5329 CpG sites were detected as differentially methylated and 10745 CpGs were identified as differentially methylated in BA24 [109]. Interestingly, neurotypical controls exhibit over 50,000 DMRs between these brain regions. The same comparison between ASD brain regions (i.e., BA10 vs BA24) showed only approximately 10,000 DMRs [109]. Only a small percentage of hypomethylated (3.5%) or hypermethylated (5.9%) DMRs corresponded to previously identified autism candidate genes. Of particular interest is the finding that of the significant DMRs identified in ASD, many of these were associated with immune response genes, including leukocyte migration, cytokine biosynthesis and the inflammatory response. Many of the DMRs related to immune functions are hypomethylated and correlate with increased transcription. In contrast, genes relevant to synaptic membrane function are hypermethylated and correlate with decreased expression. These data highlight an important area of investigation that is receiving attention in the literature (see transcriptomic studies discussed above). That immune response genes are enriched in the hypomethylated gene set suggests a dysregulated immune system as a potential etiological factor. Many of the same 'immune' genes are not also observed in the positive findings identified by genetic studies supporting the concept that these genes are likely induced through an environmentally-mediated epigenetic mechanism. In DNMT1/DNMT3A knockout mice, immune response genes are disproportionately represented as differentially methylated [110]. Maternal hospitalization due to viral infection is one of the environmental factors reported

to increase risk of ASD [111–113]. These studies add to the growing literature, encompassing both animal and human studies that support possible immune response-mediated epigenetic mechanisms as potential etiological factors in ASD.

The genome-wide methylation studies indicated above [101,109] differed with respect to their findings regarding methylation and ASD. Reasons for these differences could be due to differences in the methylation detection platform or in the means of processing the methylation data. However, both groups used the same detection platform, in other words, the HumanMethylation450 BeadChip. This platform covers over 485,000 CpGs and so has a much broader coverage than the HumanMethylation27 array. However, it should be noted that while the 450k BeadChip covers over 99% of known RefSeq genes, there is only limited coverage of non-CpG sites (i.e., CpH sites). In addition, the 450 k BeadChip covers only about 2% of the total CpG sites in the human genome (~28,000,000 [114]), and there are areas that are poorly represented (e.g. introns and intergenic regions) that are enriched in DMRs. One potential reason for the observed differences may be the different *in silico* analytical tools used in evaluating the methylation array data. It should be noted that results from the earlier study [101] were also examined in the later one [109]. Many of the sites previously identified, including CpGs sites upstream of *C11orf21/TSPAN32,* were also positive in the second study. Aside from technical issues related to data processing, there may also be issues related to cellular composition in postmortem tissue samples [115], as well as differences between patient/control cohorts. It should be noted that in both studies, sample sizes were relatively small. In addition, the HumanMethylation450k BeadChip does not differentiate between 5mC and 5hmC unless the conventional methodology is modified. With the recent advances that have occurred in the identification and sequencing of modified cytosines, complete coverage of the human genome at base resolution should be considered a necessity [83]. This is particularly important as we uncover new functions associated with large numbers of non-coding genes likely involved in different types of gene regulation. While a great deal of new information is available regarding genomic locations of 5mC and 5hmC in adult brain [82], much less is currently known regarding how changes in the levels of 5hmC at specific sites are facilitated and how these modifications are targeted to specific sites.

DNA hydroxymethylation in ASD

As noted above, 5hmC is abundant in the developing nervous system with levels increasing markedly between the fetus and adult brain [82]. However, there is currently little information available regarding DNA hydroxymethylation and ASD in structures other than the cerebellum. The dynamics of DNA hydroxymethylation were recently examined using immunoprecipitation of 5hmC from DNA isolated from fetal and adult human brain [116]. This group reported that while 5hmC is highly enriched at specific DNA domains including exons and untranslated regions, it is depleted at introns and intergenic regions. In addition, the amounts of 5hmC were noted to increase during cerebellar development with the largest amounts present in the adult. Moreover, differentially hydroxymethylated regions (DhMRs) were shown to overlap within highly expressed genes and CpG island shores. Interestingly, 5hmC was enriched at mRNAs that are regulated by the fragile X mental retardation protein (FMRP), as well as many genes linked to neurodevelopment and ASD [116].

We recently examined epigenetic mechanisms underlying the regulation of several candidate genes in cerebella of ASD subjects. We studied the expression of several enzymes in the DNA methylation/hydroxymethylation pathway, regional amounts of 5mC and 5hmC, and the expression of various ASD candidate genes in cerebellum of ASD subjects [117]. Our data showed that DNMT1 mRNA is unchanged and TET1 mRNA is increased in cerebella of ASD postmortem subjects. Moreover, the increase in TET1 is accompanied by enriched amounts of 5hmC in the promoters of GAD1 and RELN. While no changes were detected in MeCP2 protein levels, increased binding of MeCP2 to the same RELN and GAD1 promoter DNA stretch was noted. While there were no measurable differences in DNMT mRNA levels or in the binding of DNMT1 to the RELN and GAD1 promoters, the increased expression of TET1 was accompanied by increased binding of TET1 protein to these promoters [117]. Moreover, the study showed that increased amounts of 5hmC in promoter proximal domains negatively correlate with GAD1 and RELN mRNA levels in ASD cerebella. As opposed to the study cited above [116], our finding supports a negative role for increased 5hmC levels and expression of the corresponding gene.

A recent paper described results obtained in a similar candidate gene study of *EN2* in a cohort of ASD cerebella [118]. These investigators examined DNA modifications in the context of the regulation of *EN2*. Like the previous paper, they show increased levels of not only TET1, but also TET3, DNMT3A and DNMT3B. Increased amounts of 5hmC were observed at the EN2 promoter which correlate with increased EN2 mRNA expression [118] rather than decreased expression, as noted with RELN and GAD1 [117]. In addition, the increased levels of 5hmC at EN2 were associated with decreased levels of MeCP2 binding to the corresponding promoter sequences. MeCP2 binding has been

reported to be dependent on total 5mC/5hmC content and additional factors such as the local histone modifications [57]. The discrepancy in terms of MeCP2 binding relative to 5mC and 5hmC in these two studies is not clear although could be the consequence of various post-translational modifications.

Similar to what was reported in cerebella of ASD patients [117], the levels of RELN and GAD1 mRNA are also reduced in the frontal cortices of ASD and control postmortem brain [119]. There is a concomitant increase in TET1–3 expression, a decrease in DNMT1 and MeCP2 mRNA levels are unchanged in the frontal cortex in the same patient cohort. We also report that MeCP2 and DNMT1 binding to the RELN and GAD1 promoters is increased. At the same time, the levels of 5mC in the corresponding promoter regions decrease while the levels of 5hmC are not statistically different. The 5hmC/5mC ratio at both promoter regions is higher and negatively correlates with transcription. The finding of increased MeCP2 binding and reduced amounts of DNA methylation in the same stretch of DNA is difficult to reconcile. However, DNMT1 has been shown to interact with the transcriptional repressor domain of MeCP2 [120]. It may be that DNMT1, through protein–protein interactions with MeCP2, drives the increased binding of a DNMT1:MeCP2 repressor complex to unmethylated cytosines through the DNMT1 –CXXC- domain [121]. Biochemical studies demonstrate that MeCP2 assembles novel repressive chromatin structures independent of DNA modification [122]. Another possibility is that recognition of 5mC by the MBD of MeCP2 is facilitated by chromatin compaction [122]. Additional studies are needed to better understand how 5hmC/5mC levels change genome-wide, and how this measure impacts MBD/MeCP2 and DNMT protein binding and gene transcription.

Environmental effects

Environmental factors induce perturbations to the epigenome during critical periods of neurodevelopment, which affect cells during processes of proliferation, migration and terminal differentiation. As indicated above, at the molecular level these perturbations include changes in DNA methylation, hydroxymethylation, histone modifications and the expression of various noncoding RNAs. Reviews describing associations between environmental factors and ASD have been published in recent years [74,123–128]. However, considerably less is known regarding the mechanisms by which these factors impact the epigenome. Vast amounts of monies have been expended on identifying genes associated with ASD. We now need to invest in research that provides a better understanding of mechanisms by which environmental factors influence gene expression profiles at both the molecular and circuit levels so that we can better appreciate their effect on brain development. Below we discuss the role of maternal stress, infections and prenatal nutrition because considerably more is known regarding the effect of these factors on the epigenome.

Maternal stress

Fundamental processes facilitating changes in gene expression in response to environmental or extracellular cues include DNA and histone modifications (Figure 2). There is ample evidence to suggest that environmental factors related to the response of the brain to pre- or postnatal stress/trauma increase risk for ASD. Numerous studies have reported an association between prenatal stress and the development of autistic traits [129], and increased ASD risk [130]. The effects of prenatal stress via maternal factors crossing the placenta influence a wide range of neuronal and non-neuronal systems during fetal development depending on gestational timing. Recent evidence indicates that neuronal circuit formation in the brain is susceptible to the effects of prenatal stress. The establishment of GABAergic inhibitory circuits occurs at times when prenatal stress exerts persistent effects on the brain and behavior [130]. Prenatal restraint stress has been shown to delay tangential migration of interneuron progenitors in rodents [131]. Prenatal stress exerts a profound epigenetic influence on GABAergic interneurons by altering the levels of proteins such as DNMT1 and Tet1 and decreasing the expression of various targets such as BDNF [132,133]. Ultimately, this results in reducing the numbers of fully functional GABAergic neurons postnatally and a concomitant increased susceptibility toward hyperexcitability. The delayed migration of GABAergic interneuron progenitors results in reduced gene expression postnatally which is likely the consequence of increased amounts of DNA methylation [133]. In mice, mutations targeting the *Plaur* gene disrupt the ontogeny of the forebrain by arresting migration of inhibitory parvalbumin interneurons from the medial ganglionic eminence [134]. Parvalbumin-expressing interneurons have also been implicated in transcriptomic studies of postmortem ASD brain [25,135], and in the autism-like behavioral deficits exhibited by *Pvalb* knock-out mice [136]. The downstream effectors of the stress response, glucocorticoids, have also been shown to retard the radial migration of postmitotic neurons during cortical development [137]. The net effect of stress during early development is to disrupt the balance of excitatory/inhibitory neuronal firing due to the loss of function associated with disrupted neuronal migration and maturation.

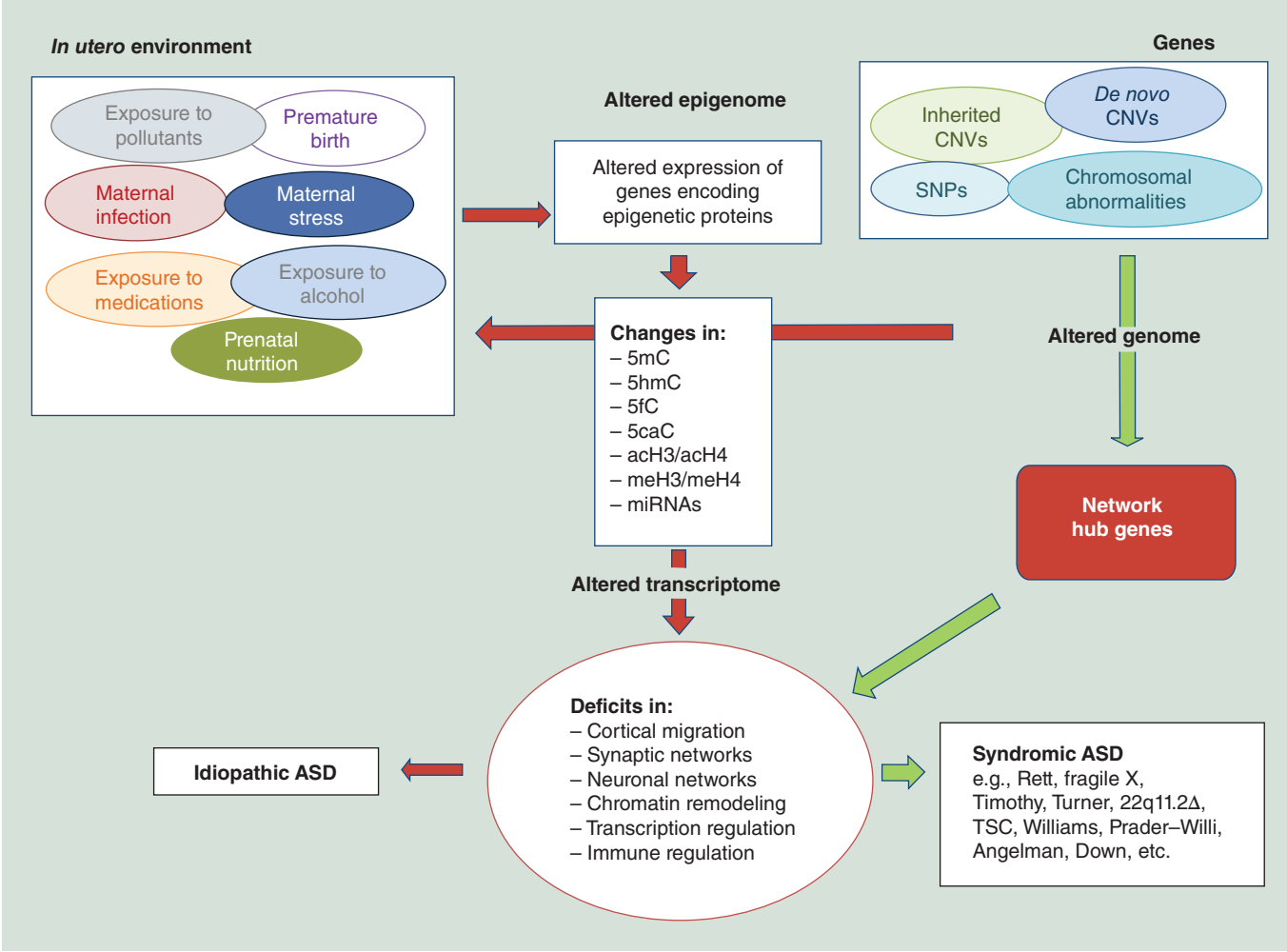

Figure 2. Epigenetic impact of the prenatal environment. Environmentally induced epigenetic mechanisms acting on the genome during prenatal and early postnatal development facilitate genome-wide changes in the epigenome that alter transcription at distinct developmental time points. The mRNA alterations that occur cause deficits in multiple processes including cortical migration, chromatin remodeling, transcriptional regulation, immune-response regulation and the formation of synaptic connections. Genome-wide association and whole exome sequencing studies have identified a large number of genes linked with ASD that encode proteins that function in synapse formation, transcriptional regulation and chromatin-remodeling pathways [12]. Current findings, highlighted in this review, indicate that there is considerable overlap between ASD-susceptibility genes and the mRNAs impacted by various environmental/epigenetic influences. Both environmental factors and genetic mutations alter the transcriptome during neurodevelopment. We propose that idiopathic ASD is the consequence of environmentally induced epigenetic influences operating on sufficient numbers of ASD susceptibility genes to produce the ASD phenotype. Individuals with mutations in ASD susceptibility genes are also subject to these same in utero environmental influences (arrow from altered genome). In contrast, syndromic ASD is characterized by genetic mutations that occur in so-called network hub genes [25]. In network theory, hub genes refer to highly interconnected nodes that are often times responsible for regulating key biological pathways or directing the cellular response to a given stimulus [185]. For example, *FMR1* encodes the RNA-binding protein, FMRP which is deficient in Fragile X, an ASD-related syndrome. FMRP regulates the translation of a set of over 800 plasticity-related genes in response to stimulation by the neurotransmitter glutamate [186]. Mutations in *FMR1* cause Fragile X syndrome, which is a syndromic ASD. The etiology and symptomatology of various syndromic ASD disorders has recently been reviewed [8,9].
ASD: Autism spectrum disorder; CNV: Copy number variant.

Maternal infections

Immune system dysfunction plays an important role during neurodevelopment and has been implicated in ASD symptoms. This includes findings of neuroinflammation, presence of autoantibodies, increased T-cell responses, increased natural killer cell responses and monocyte responses in ASD subjects [138]. Moreover, there are also reports of cytokine dysregulation in ASD which is likely the consequence of altered immune system activity [139]. Transcriptome studies of ASD brain provide data demonstrating that mRNAs associated with activated microglial and immune response genes are altered relative to non-ASD brain [20,25]. As noted above, an independent methylation analysis of

ASD brain samples shows that genes associated with immune system function tend to be hypomethylated compared with DNA isolated from non-ASD brain samples [109]. This is consistent with the increased mRNAs observed in the transcriptome studies [20,25]. Alterations in the expression of genes that function in immune-glial response pathways are likely the consequence of prenatal maternal infections or neuroinflammation (see [140] for recent review). This is supported by recent reports showing that increased risk for ASD correlates with maternal inpatient diagnosis with infection during pregnancy [111–113,141,142]. These findings are also consistent with observations that children with ASD report numerous immune system irregularities including inflammation [143,144]. Immunological factors, including inflammation, autoimmunity and maternal immune activation have long been suspected in the context of aberrant neurodevelopment and ASD risk (see [140,145] for current reviews). A recently published proof of concept study was undertaken to examine genome-wide RNA expression profiles in leucocytes from cohorts of children ages 1 to 4 years looking for gene modules as potential ASD biomarkers [146]. The authors report strong significant dysregulation of immune and inflammatory gene networks at an age where clinical risk signs of ASD are first beginning to emerge [146]. The finding that immune system dysfunction may be an important causative factor in ASD also provides a link to schizophrenia, another neurodevelopmental disorder that shows some overlap with respect to *de novo* mutations [147], and has been etiologically linked with maternal immune activation [148]. The risk for both schizophrenia and autism has been linked epidemiologically to maternal infection [111,149].

Prenatal nutrition

The maternal lifestyle during gestation can expose the fetus to a wide range of environmental insults and influences [123,125]. These include nutritional choices of the mother that directly impact neurodevelopment of the fetus and which been shown to influence epigenetic mechanisms [124,150]. Maternal nutrition is essential for fetal brain development and nutritional deficiencies due to increased metabolic demands imposed by the placenta and fetus during pregnancy are linked to neurodevelopmental disorders. The most direct association between nutrition and epigenetics occurs through pathways that involve methyl transfers, such as DNA and histone methylation, which utilize the methyl donor *S*-adenosyl-methionine (SAM). Early alterations in DNA methylation can cause cells to aberrantly differentiate from their normal lineage causing abnormal pools of differentiated neurons [151]. DNMTs utilize SAM to donate methyl groups at specific sites in DNA [33]. Similarly, histone methyltransferases (HMTs), which have been genetically linked to various psychiatric disorders [152], catalyze the transfer of up to three methyl groups to the e-amino group of specific lysines on histones H2 (H2BK5), H3 (H3K4, H3K9, H3K27 and H3K79) and H4 (H4K20). The levels of SAM available for one carbon transfers are affected by methyl groups derived from dietary choline, methionine and methyl-tetrahydrofolate. Folate is a major source of the methyl group for SAM and is readily available for pregnant women as part of a periconceptual vitamin supplement. A large case–control study provides epidemiological data showing that mothers of ASD children consumed less folic acid than mothers of typically developing children [153]. Based on results from this study, a greater risk of ASD was observed for children whose mothers had defects in genes associated with one carbon metabolism [153]. Subsequently, this group also reported that there is an inverse correlation between folic acid intake and ASD risk [154]. The finding that periconceptional folic acid supplementation is associated with a lower risk of ASD was replicated in a large Norwegian cohort of over 85,000 children [155]. The folic acid contained in these formulations is sufficient for both the prevention of neural tube defects and to ensure an adequate supply of methyl donors preceding and during gestation. Folic acid and prenatal vitamin supplementation protect against a range of human cancers, neurodevelopmental and neurodegenerative disorders [126]. A model was recently proposed that explains how exposure to a wide range of environmental toxins that impact neurodevelopment also result in global DNA hypomethylation [156]. This model was extended to connect pathways between dietary nutrition and environmental exposures in the context of DNA hypomethylation [157]. More recently, this hypothesis was expanded to show how dietary nutrients, environmental toxins, genome instability and neuroinflammation interact to produce changes to the DNA methylome [126].

In addition to folic acid, there is evidence that children of mothers that have a higher intake of polyunsaturated fatty acids before and during pregnancy have a reduced risk of ASD [158]. Vitamin D is a fat-soluble steroid hormone that regulates large numbers of genes that impact the brain during development. The levels of vitamin D are low in populations living in overcast areas that have low sun exposure. The number of ASD diagnoses in regions with low sun exposure in the USA is increased and has also been linked to vitamin D insufficiency [159]. Recent studies show that vitamin D activates the expression of tryptophan hydroxylase, the enzyme associated with serotonin synthesis [159]. This is particularly interesting because

it links findings showing decreased levels of serotonin in ASD patients [160] and reduced levels of vitamin D. However, a previous study found little evidence that maternal gestational vitamin D levels are related to the ASD phenotype among offspring [161]. In addition, while there is evidence that vitamin D may increase DNA demethylation of selected gene promoters, the mechanisms behind this effect are not clear [162]. There is stronger evidence that vitamin D acts to modulate chromatin access through interactions with histone acetyltransferases [162]. Moreover, aside from activating vitamin D responsive genes through a coactivator mediated pathway, the means by which vitamin D facilitates changes in chromatin relaxation are only beginning to be understood.

Exposure to air pollution, pesticides, prenatal valproate, cell phone radiation & ASD

Environmental toxins that are associated with negative health consequences have been shown to impact neurodevelopment and have been implicated in ASD [123]. For example, exposure to traffic-related air pollution, nitrogen dioxide and particulate matter during pregnancy and the first year of life is associated with ASD [163]. Toxicants that are associated with increased ASD risk include pesticides, phthalates, polychlorinated biphenyls, solvents, toxic waste sites, heavy metals and air pollutants [164]. An epidemiological study has linked PVC flooring material in the home to parental-reported ASD [165]. Exposure to plasticizers, such as bisphenol-A, results in higher levels of metabolites excreted in urine [166].

Respiratory distress and other markers of hypoxia have been associated with increased risks of ASD in twins [167]. Early exposure to androgenic hormones and maternal immune response represent epigenetic factors that affect sex susceptibility to ASD [168]. Residential proximity to agricultural pesticides during pregnancy is associated with increased risk of ASD and developmental delay [169]. The histone deacetylase inhibitor and antiepileptic drug, valproate, has long been known to cause congenital malformations and developmental delay in children exposed during gestation. In addition, maternal use of valproate to treat epilepsy during pregnancy is associated with significantly increased risk of ASD [170]. Finally, mice exposed to low frequency electromagnetic fields show a lack of normal sociability and preference for social novelty while maintaining otherwise normal behaviors [171]. While these studies show associations and links between factors in the environment and ASD, much less is known regarding how exposure to these developmental influences trigger changes to the epigenome.

Conclusion

Genetic studies have revealed a plethora of genes that are associated with increased ASD risk. It is well known that patients diagnosed with ASD exhibit considerable variability in both the number and severity of symptoms. Because of the genetic and symptomatic heterogeneity, ASD is also thought to be etiologically heterogeneous. However, as recently argued [19], neuropathological findings in both idiopathic and syndromic ASD support instead a common pathophysiological mechanism. The author of this study argues that multiple exogenous and endogenous factors disrupt cell division in the brain which leads to the pathological changes observed in ASD. These changes are thought to be related to alterations in cortical columnar structure, in dendritic spines and within the cortical subplate [172] manifest as various dysplasias and heterotopias [173]. In addition to these cortical malformations, environmental factors that contribute to neurodevelopment by altering folate metabolism, modifying the stress and inflammatory responses, and disrupting neuroendocrine hormonal signaling were also discussed [174]. In other words, both genetic mutations and environmental stressors impact neurodevelopment by altering the expression of modules of genes in multiple cell types.

Transcriptome studies of postmortem brain indicate that the expression of numerous genes and hence multiple pathways are disrupted in ASD pathophysiology (Figure 2). This includes pathways associated with synaptic and neuronal function (neuronal growth, migration, dendritic and neurite outgrowth, synaptic plasticity), chromatin remodeling (DNA 5mC and 5hmC writers, readers and erasers; histone writers, readers and erasers; chromatin remodeling proteins; microRNA expression) and immune function (microglial activation states). An overlapping set of genes associated with these pathways have also been identified in genetic studies of ASD. Immune function genes are the exception as the expression of these genes is likely a consequence of infection or neuroinflammation and not due to genetic mutation [175]. Moreover, mRNA changes linked to immune function genes tend to be increased rather than decreased in ASD [20]. It seems clear that both DNA mutations and environmental factors impact development in similar ways by reducing the expression of key genes associated with typical development. That is, the overlap between genes identified by genetic and transcriptomic studies supports observations that genes associated with synaptic function and chromatin remodeling are disrupted in ASD and related neurodevelopmental disorders. Loss-of-function mutations are syndromic when multiple downstream pathways are affected, as occurs in Fragile X and Rett syndromes (Figure 2). The disrup-

tion of hub (multiconnected) genes impacts multiple downstream pathways. Instead, environmental factors, which also reduce the expression of multiple genes that impact downstream pathways, often result in idiopathic ASD. This could be due to the less severe developmental effects caused by reducing gene expression, as opposed to a complete loss of function. As we move forward in defining the causative factors of ASD, it will be important to invest in research designed to fill the void in our current understanding of how individual environmental factors impact epigenetic marks across the genome. Organizations (both governmental and private) that fund ASD studies need to invest both in epigenomic and genetics research.

In the context of this review, we have discussed numerous advances that have been made in ASD research in recent years. As noted, however, ASD is comorbid with multiple additional conditions including various seizure disorders and ID. Although there is considerable overlap between ASD and ID, intellectual disability is often considered separately from ASD. This is not surprising given that there are people with ASD or ID and people that experience both ASD and ID. It is not surprising that recent genetics studies of ID have identified CNVs that are often times also present in subjects with ASD [176]. One of the problems that these issues raise is that current estimates of autism prevalence do not always account for the effects of comorbidity on the ASD diagnosis [177]. In fact, at one point it was speculated that much of the recent advances in ASD research have been the result of research on subjects who are intellectually normal [7]. While it is not clear if this remains to be a problem, it is incumbent upon researchers reporting results to provide accurate, clear and anonymous clinical descriptions of the subjects in their studies.

Future perspective

Transcriptome analyses of ASD postmortem brain tissue demonstrate that large numbers of mRNAs are altered as compared with age- and gender-matched typically developing subjects. A recent meta-analysis of transcriptome studies in ASD confirmed that there are commonalities across independent groups of individuals with ASD [178]. The mRNA changes are the consequence of gene mutations (SNPs, *de novo* and inherited CNVs, chromosome abnormalities) that impact the associated gene and environmental factors that influence the epigenome during neurodevelopment (see Figure 2). While the immediate consequences of null mutations may be predicted, the downstream consequences of these mutations are more difficult to predict. For example, a mutation in *GRIN2B* has the immediate consequence of disrupting GRIN2B mRNA and protein levels. Downstream consequences of this mutation impact the expression of genes associated with numerous signaling cascades (p53, Jak-STAT, Wnt and Notch pathways), as well as, the actin cytoskeleton and neuroactive ligand-receptor interactions [179]. In order to better understand the complex interconnections that underlie ASD-related phenotypes, we have to better appreciate how multiple gene networks overlap and the connections within each network. These studies are currently underway and some have appeared in the literature [179–183]. That is, we are currently at the point in which genes within networks show nonquantifiable relationships and it would be more informative to be able to weight the contributions of individual genes to nodes within networks to allow for quantitative predictions to be made.

Currently available data suggest that there is overlap between mRNAs misregulated in ASD due to genetic abnormalities [12,13,15,184], and the mRNAs differentially expressed derived from transcriptome studies of ASD subjects [20,25]. Epigenetic modifications likely converge on specific sets of genes depending on the nature of the environmental factor. Moreover, while numerous studies have been carried out on environmental factors in specifying ASD-related phenotypes [124–126,158]), considerably less is known regarding the direct consequences of these factors on the epigenome. Some environmental factors increase DNA methylation (as in synaptic plasticity genes), while others likely facilitate DNA demethylation (immune function genes) and others likely affect histone methylation and acetylation patterns. Ultimately, transcriptomic profiles reflect the downstream consequences of the effects of genetic mutations/abnormalities and environmental factors and how these forces interact during neurodevelopment. Understanding how the epigenome responds to environmental insults will likely provide better insight into common final pathways operative in the pathophysiology of ASD and provide for the design of better pharmacotherapeutic treatments.

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Executive summary

Autism spectrum disorder genetics

- Data from genetic studies have been used to identify a large number of genes that contribute increased risk for autism spectrum disorder (ASD). Network analysis of these genes indicate that they converge on a small set of pathways that are integral to synaptic function, chromatin remodeling and transcriptional regulation.

Transcriptome profiles in ASD brain

- RNA-seq studies have provided information regarding transcriptome changes in various brain regions of ASD subjects as compared with typically developing individuals. Network correlation analysis of these mRNAs provides evidence that in addition to ASD risk genes, modules of genes associated with immune function, synaptic transmission and neuronal function are altered in ASD.

DNA methylation in ASD

- Members of the DNMT and TET family of proteins modify DNA by either adding a methyl group or by oxidizing the methyl to form 5hmC, 5fC and 5caC. Changes in the levels of 5mC and 5hmC have been noted at specific genes in brains of subjects diagnosed with ASD. Comparisons of genome-wide DNA methylation data with transcriptome data in ASD show that genes associated with immune regulation are hypomethylated and overexpressed, while genes related to synaptic function are hypermethylated and underexpressed.

DNA methylationreaders

- Members of the methyl binding domain family of proteins, which includes the Rett syndrome protein, MeCP2, bind to 5mC residues. While MeCP2 is ubiquitously expressed in neurons and astrocytes it localizes to repressed chromatin domains facilitating transcriptional silencing.

DNA hydroxymethylation in ASD

- DNA hydroxymethylation is the first step in the DNA demethylation cascade. Changes in the levels of 5hmC in ASD postmortem brain have not been widely studied. Thus far, the research has focused on ASD candidate genes and results show that the increased 5hmC is associated with both increased (En2) and decreased (RELN, GAD1) mRNA expression. This suggests the possibility that the 5hmC epigenetic marks may be inappropriately read by the transcriptional machinery.

Environmental effects

- Exposure to various environmental factors, including stress, infections, nutrition, substance abuse, medications, pollutants and toxins have been linked to increased ASD risk. These factors are likely to impact the epigenome through changes in DNA methylation and related mechanisms that regulate local chromatin conformation. Collectively, the current literature suggests that the effects of environmental factors converge on epigenetic pathways that result in altered 5mC and 5hmC marks. There is a growing need to better understand how each of the environmental influences impact chromatin structure in neurons, astrocytes and microglia of the brain.

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Review

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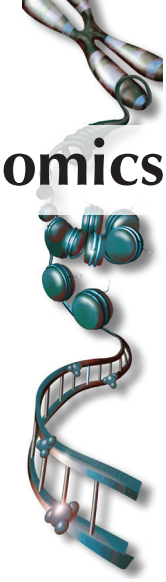
Epigenomics



Regulatory RNAs and control of epigenetic mechanisms: expectations for cognition and cognitive dysfunction

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The diverse functions of noncoding RNAs (ncRNAs) can influence virtually every aspect of the transcriptional process including epigenetic regulation of genes. In the CNS, regulatory RNA networks and epigenetic mechanisms have broad relevance to gene transcription changes involved in long-term memory formation and cognition. Thus, it is becoming increasingly clear that multiple classes of ncRNAs impact neuronal development, neuroplasticity, and cognition. Currently, a large gap exists in our knowledge of how ncRNAs facilitate epigenetic processes, and how this phenomenon affects cognitive function. In this review, we discuss recent findings highlighting a provocative role for ncRNAs including lncRNAs and piRNAs in the control of epigenetic mechanisms involved in cognitive function. Furthermore, we discuss the putative roles for these ncRNAs in cognitive disorders such as schizophrenia and Alzheimer's disease.

Keywords: chromatin • epigenetics • long noncoding RNA • neuroplasticity • neuroscience • short noncoding RNA

Epigenetic mechanisms have emerged as critical components of the memory formation process and are involved in cognition and cognitive disorders. As related to the nervous system, the term epigenetics refers to long-term, potentially heritable changes in gene expression patterns that do not result from mutations in the DNA sequence. This broad definition encompasses a number of chemical modifications to DNA residues as well as alterations to closely associated molecules such as histone proteins or chromatin-associated RNAs [1]. These epigenetic modifications (also referred to as epigenetic marks) are involved in numerous cellular and molecular functions, ultimately influencing nuclear organization and transcriptional activity at chromatin regions [1]. The semipermanent nature of these epigenetic modifications thus allows genetically identical cells to differentiate into distinct lineages, express unique gene patterns, and perform unique functions in a lineage-dependent fashion [2,3].

As the epigenome plays an important role in driving cellular development, it is not surprising that epigenetic mechanisms critically control the development of the nervous system [4–7]. However, in the past decade it has become increasingly clear that despite the postmitotic state of most neurons, epigenetic mechanisms continue to play a critical role in controlling transcription into adulthood (reviewed in [8]). This is especially evident in the context of transcription-dependent cognitive processes such as long-term memory formation, where altered epigenetic processes can either impair or improve performance in memory tasks.

While the importance of epigenetic regulation in cognition has been well established, the mechanisms by which epigenetic marks are targeted to particular genes or loci remain relatively unexplored. Instead, most studies to date have examined either global changes in the levels of epigenetic marks within specific brain regions or the presence of epigenetic marks at particular genes and promoters with known cognitive function. As a result of these studies, it is becoming increasingly clear that the neuro-epigenetics

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landscape can differ significantly within and across brain regions, and that dysregulation of chromatin-modifying enzymes (CMEs) can have profound effects on brain function, and thereby, cognition and cognitive disorders [9–11]. Indeed, evidence suggests that dysfunction of epigenetic processes is involved in the etiology of many cognitive disorders, including schizophrenia, bipolar disorder and major depressive disorder [9]. Such studies have advanced our knowledge of the role of epigenetic mechanisms in specific brain regions, and have given rise to the rapidly expanding field of cognitive epigenetics. However, as very few CMEs have demonstrated sequence-specific DNA binding, the question of how CMEs are directed to their target gene regions remains largely unsolved. Some observations have indicated that DNA binding proteins, such as the transcription factors including nuclear factor-κB, Nanog, and Oct4, interact with CMEs to direct site-specific epigenetic gene regulation [12–14]. Additionally, recent studies suggest that multiple families of regulatory RNAs also mediate epigenetic targeting.

To date, a majority of the research studies have described regulatory RNAs with little to no protein-coding potential, thus defined as noncoding RNAs (ncRNAs). Although poorly described relative to protein-coding genes, ncRNAs comprise a major portion of the mammalian transcriptome [15]. While estimates differ as to the abundance of ncRNAs, the general consensus of the field is that ncRNAs are quite plentiful, particularly in brain tissues [15–19]. It remains plausible that regulatory RNAs have both translation-independent functions and protein-coding potential, and indeed, translation-independent activity of mRNAs has been identified in well-studied pathways such as p53 signaling [20–22]. However, the field of epigenetics has largely focused on characterizing such functions in ncRNAs, in part as a precaution against experimental confounds.

Common practice in the epigenetics field describes ncRNAs as either long or short, with the division being set at a length of 200 nucleotides. While seemingly arbitrary, this division allows for the useful separation of the many characterized classes of small functional RNAs, including miRNAs, piRNAs (PIWI-interacting RNAs), siRNAs (small interfering RNA), snoRNAs (small nucleolar RNAs) and tRNAs (transfer RNAs) from the less well characterized long noncoding RNAs (lncRNAs) [23]. Among other functions, both short and lncRNAs have attributable roles in neuronal epigenetic mechanisms [24,25] – a finding with exciting implications for cognitive sciences. In this review, we will highlight key findings that are beginning to elucidate a role for ncRNAs in the control of neuronal and cognitive function via epigenetic mechanisms. Our goal for this review is to draw attention to this phenomenon and encourage new investigations into ncRNA-mediated control of the epigenome in cognition and cognitive disorders. Further, while miRNAs are well studied with regard to cognitive disorders, they are poorly studied in the context of epigenetic function. In contrast, while piRNAs and lncRNAs are better characterized in the context of epigenetic function, they are poorly studied with regard to cognitive disorders – even though many such disorders involve dysregulation of the epigenome. Nonetheless, emerging studies are beginning to explore the interesting idea that ncRNAs are involved in the epigenetic process underlying cognition and may be altered in cognitive disorders.

Short ncRNA

Mechanisms of canonical RNA interference

When Lee and colleagues [26] first demonstrated that the small (22-nucleotide) ncRNA dubbed lin–4 represses the translation of several developmental genes in *Caenorhabditis elegans*, the scientific community failed to recognize this discovery as anything more than a curious feature of the invertebrate model's genetics. As a consequence, few of these ncRNAs were identified or characterized until the discovery of RNA interference (RNAi), a post-transcriptional regulatory process that will be reviewed and discussed in later sections. However, we begin our discussion with small regulatory RNAs initially shown to be highly conserved in plants and animals in the early 2000s [27]. Today, the known roles of miRNAs and their related structures have expanded to encompass the view that as many as 60% of protein-coding RNAs are regulated by miRNA activity [28,29]. Since the days of Ambrose and Lee, tens of thousands of miRNAs have been annotated [30–34], and miRNAs have been shown to regulate such diverse biological processes as developmental pattern formation [27], pluripotency [35], cell signaling [36], cardiovascular disease [37], cancer [38], diabetes [39], neural plasticity and memory [40], among others [41]. This review will largely describe miRNAs as they relate to the epigenome; however, in order to better understand how miRNAs have come to be associated with epigenetic activity, it is first necessary to understand the canonical post-transcriptional pathway by which these miRNAs regulate gene expression.

In the canonical pathway (reviewed in [42]), the nascent miRNA begins as a transcript of intronic or intergenic DNA, a miRNA precursor molecule known as a primary miRNA (pri-miRNA). While still in the nucleus, this pri-miRNA is bound and cleaved by a microprocessor complex composed of Drosha and

Dgcr8. Processing of the pri-miRNA by this complex leads to the formation of a shorter hairpin-like structure defined as a precursor miRNA (pre-miRNA) [42,43]. The pre-miRNA is then exported into the cytoplasm via Exportin 5 where it undergoes further cleavage by the RNAase enzyme *Dicer*, thereby forming a complementary duplex of two miRNA strands. Unwinding of this duplex releases one of the RNA strands, while the remaining strand is bound to an AGO protein in the RNA-induced silencing complex (RISC). The mature miRNA, coupled with RISC (now referred to as miRISC), then functions to detect complementary sequences inside messenger RNAs, usually found in the 3′-untranslated region of the target mRNA [43,44]. The binding of this miRISC complex to a target mRNA results in silencing of the mRNA, which may occur either by degrading the target transcript via the endonuclease activity of AGO2, or by simply preventing translation of the target transcript in cases of less perfect complementarity.

Although studies have generally focused on the regulation of mRNA by the canonical RNAi pathway, there is considerable evidence that interaction between canonical RNAi and other ncRNA signaling pathways occurs and may have broad ramifications in neuroplasticity and cognition (reviewed in [45]). Many miRNAs are expressed in the brain, and a number of studies have examined their role in cognitive disorders (Table 1) [46]. Indeed, roles for miRNA have been attributed to molecular signaling in neurodevelopment [47], neural stem cell differentiation [48,49] and cortical neurodegeneration [50,51], where knockout of the enzyme *Dicer* results in the dysregulation of these processes. Intriguingly, studies using an inducible *Dicer1* knockout mouse model demonstrate improved performance in several memory tasks, suggesting that miRNA signaling may also act to restrict memory formation in some cases [52].

Canonical miRNA signaling in schizophrenia

Among miRNA-related cognitive disorders, schizophrenia is perhaps the most extensively studied. Patients suffering from DiGeorge Syndrome often experience microdeletions affecting the miRNA microprocessing gene Dgcr8, and are 30-times more likely to suffer from schizophrenia or schizoaffective disorders [66]. This observation suggests a critical importance for miRNA signaling in schizophrenia. Postmortem studies in the brains of schizophrenic patients have identified dysregulation of two miRNAs, miR-132 and miR-219, both of which have been linked to schizophrenia-associated cognitive or behavioral impairments [60,67–69]. Both miRNAs are dysregulated in response to NMDA-receptor blockade [42], an interesting finding in light of the hypothesis that hypofunction/downregulation of the NMDA receptor plays a critical role in schizophrenia pathophysiology. A third candidate, miR-195, is increased in the brains of patients with schizophrenia, where it regulates several schizophrenia-related genes, including *Bdnf* [70], *Reln*, *Vsnl1*, *Htr2a* and *Grin3* [71]. Indeed, *in silico* studies of miRNAs associated with transcription factors suggest that miR-195 plays a central role within a regulatory network of schizophrenia-related genes [63].

Because schizophrenia is a heterogeneous and complex disorder that involves several brain regions and cell types, the role miRNAs in the control of gene transcription must be considered in this context as well. For example, patients with schizophrenia are known to exhibit a wide range of up-regulated miRNAs in the superior temporal gyrus and prefrontal cortex, including miR-107, miR-15a, miR-15b, miR-16, miR-195, miR181b, let-7e, miR-20a, miR-26b, miR-19a [71]. Interestingly, none of the miRNAs described above are specific to neurons: miR-219 regulates oligodendrocyte maturation [72], while miR-195 and miR-132 are expressed in astrocytes [73–75]. Comprehensive studies should further elucidate the role of miRNAs in the nervous system, as well as test the possibility that global changes in miRNA processing may result in the disease's pathogenesis [71].

While the miRNAs described above target schizophrenia related genes directly, there is also evidence for miRNA-mediated epigenetic dysfunction in schizophrenia, and some schizophrenia-related miRNAs are known to be more directly involved in epigenetic regulation via the targeting of CMEs (Figure 1). Such regulatory miRNAs include miR-132, miR-212 and miR-195 [64,71,76–77]. miR-132, which was described above, is downregulated in the prefrontal cortex (PFC) of patients with schizophrenia, and regulates expression of DNA methyltransferase 3 α (DNMT3-α), thereby regulating DNA methylation [60]. This is especially interesting, considering that DNMT3-α is known to be upregulated some brain regions in schizophrenia – including the PFC (see [78] for review). Another promising candidate is miR-137, an miRNA identified as having a SNP strongly associated with schizophrenia in a meta-analysis of genome-wide association studies [79]. miR-137 targets multiple chromatin-modifying genes [80], including the histone-lysine *N*-methyltransferase *EZH2* [81], as well as the lysine-specific histone demethylase 1A (*KDM1A*), which governs neuron differentiation and migration [62]. While such epigenetic regulation may occur, future studies are required to determine the exact nature and extent of miRNA-mediated epigenetic regulation in

Table 1 Examples of noncoding RNAs in epigenetically-linked cognitive disorders.

Name	Type	Putative epigenetic MOA	Disorder	Ref.
17A	lncRNA	None indicated	AD	[53]
1810014B01Rik	lncRNA	None indicated	AD, PD	[53]
BACE1-AS	lncRNA	None indicated	AD	[54]
GDNFOS	lncRNA	None indicated	AD	[53]
Gomafu	lncRNA	Binds to the polycomb repressor complex, PRC1	Scz, Anx	[55]
Malat–1	lncRNA	Associates with the EZH2 subunit of the polycomb repressor complex	Alc	[56]
NAT-Rad18	lncRNA	None indicated	AD	[53]
Sox2OT	lncRNA	None indicated	AD, PD	[57]
miR-106b	miRNA	None indicated	Scz	[58]
miR-125a	miRNA	–	MDD	[59]
miR-132	miRNA	Targets the DNA methyltransferase DNMT3-α	Scz, MDD	[60,61]
miR-137	miRNA	Targets the EZH2 subunit of the polycomb repressor complex; targets the histone demethylase KDM1A	Scz	[62]
miR-16	miRNA	None indicated	Scz	[58]
miR-182	miRNA	None indicated	MDD	[59,61]
miR-195	miRNA	None indicated	Scz	[63]
miR-212	miRNA	None indicated	Scz	[64]
miR-219-3p	miRNA	None indicated	Scz	[58]
miR-298	miRNA	None indicated	MDD	[59]
miR-29c	miRNA	None indicated	AD	[65]

AD: Alzheimers disease; Alc: Alcoholism; Anx: Anxiety; MDD: Major depressive disorder; MOA: Mechanism of Action; PD: Parkinsons disease; Scz: Schizophrenia.

the development and pathophysiology of cognitive disorders such as schizophrenia.

Canonical function of siRNA-mediated RNA inteference

There are significant functional similarities between miRNA- and siRNA-mediated RNAi. In this section we will highlight some of the more unique aspects of siRNA generation and regulation. Similar to miRNAs, siRNAs are short (~21 nucleotide), noncoding transcripts that are canonically generated from exogenous dsRNAs. When siRNAs were first discovered in plants by David Baulcombe and colleagues [82], they appeared to function as part of a natural, antiviral immune response. Upon exposure, exogenous, double-stranded RNAs (dsRNAs) – often from viruses – are digested by Dicer in the cytoplasm, generating short RNA duplexes. These RNA duplexes are bound by AGO as part of the RISC complex and guide the complex to a complementary target, in this case, a copy of the viral RNA. Once bound, the endonuclease 'slicer' activity of AGO2 is further activated by the complementation of the siRNA-target interaction, thus mediating target destruction [83]. In this fashion, canonical siRNA-mediated RNAi initiation turns viral RNA against itself for destruction. Interestingly, newly discovered noncanonical mechanisms of endogenous siRNA (endo-siRNA) generation and function are gaining increased relevance in cognitive neuroscience. Below, we discuss emerging roles for siRNA-directed epigenetic regulation of gene expression changes.

Emerging mechanisms of siRNA directed epigenetic regulation

siRNAs participate in epigenetic regulation of genes through DNA methylation as well as by histone modification (Figure 2) [84,85]. The precise mechanism of siRNA generation depends on the organism involved. In *Schizosaccaromyces pombe*, endo-siRNAs are generated by an RNA-directed RNA polymerase complex (RDRC), and epigenetic regulation is carried out by the RNA-induced transcriptional silencing (RITS) complex, with the latter being dependent on siRNAs gener-

ated by the former (for review see [86]). In *Arabidopsis thaliana*, this process involves two plant-specific RNA polymerase II related RNA polymerase enzymes: Pol IV and Pol V [87]. First, transcripts from Pol IV are used as templates by the RNA-dependent RNA polymerase RDR2 to form dsRNA which is reduced into 24-nucleotide duplexes by the *Dicer* protein DCL3. From the cytoplasm, one strand of a duplex is then loaded onto AGO4, which translocate into the nucleus [88] and binds to complementary, nascent transcripts created by Pol V [89,90]. Once stabilized to a target transcript by Pol V and the Pol V transcript binding protein KTF1, AGO4 associates with the DRM2 DNA methyltransferase, a writer of the 5 mC epigenetic mark at CHH sites [91]. RDM1, a subunit of the final complex responsible for linking AGO4 to Pol V and DRM2, has itself an affinity for methylated DNA, a finding that suggests a predilection of the Pol V-AGO4 complex for pre-existing sites of methylated DNA. While still speculative, these studies suggest a parallel between siRNA-directed histone modification and siRNA-directed DNA methylation insofar as both may be mediated as part of a self-perpetuating feedback loop [92].

Although endo-siRNA generation and epigenetic function is well-characterized in *S. pombe* and *A. thaliana*, other studies speculate on a potential role in endogenous production of siRNAs and their epigenetic function in human cells [96,97]. Mammalian endo-siRNAs are known to be generated from hybridized mRNAs and antisense transcripts [97], which may then regulate the epigenome. An alternative pathway for the generation of such endo-siRNAs has also been identified in which a complex composed of human TERT (hTERT), Brahma-related gene 1 (*BRG1*) and nucleostemin (NS) – together referred to as the TBN complex-produces dsRNAs. These dsRNAs can then be processed into siRNAs that facilitate the formation of heterochromatic regions [98].

Promisingly, several studies demonstrate endo-siRNA-mediated histone methylation and DNA methylation in cultured mammalian cells [84–85,99]. The mechanistic actions of mammalian endo-siRNA are poorly characterized; moreover, a neurological role for these endo-siRNAs also remains to be established, as little neuroepigenetic research has focused on these mechanisms. Importantly, targeted sequencing studies

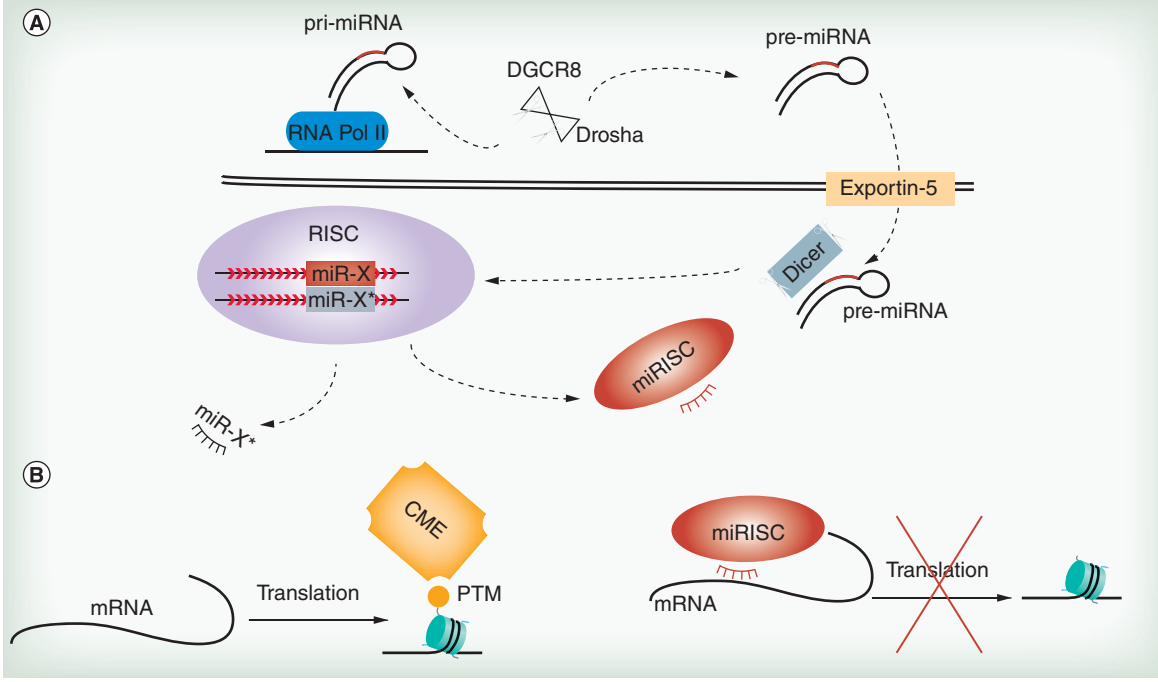

Figure 1. Canonical mechanism of miRNA generation and epigenetic regulation. (A) Schematic of canonical miRNA biogenesis. Pri-miRNA are transcribed by RNA Pol II, and stem-loop regions are processed by Drosha and DGCR8. The resulting pre-miRNA is exported through the nuclear membrane into the cytoplasm, where Dicer further cleaves the pre-miRNA into a short double-stranded RNA region. A guide strand is selected and bound by AGO within the RISC complex, while the passenger strand is cleaved and degraded. **(B)** miRNA and siRNA in conjunction with the cytoplasmic RISC complex target proteins involved in epigenetic regulation at the mRNA level. This post-transcriptional silencing, ultimately results in global alterations in the epigenome and cellular function. AGO: Argonaute; CME: Chromatin-modifying enzyme; miRISC: miRNA in complex with RISC; PTM: Post-translational modifications; Pri-miRNA: Primary miRNA; Pre-miRNA: Precursor miRNA; RISC: RNA-induced silencing complex.

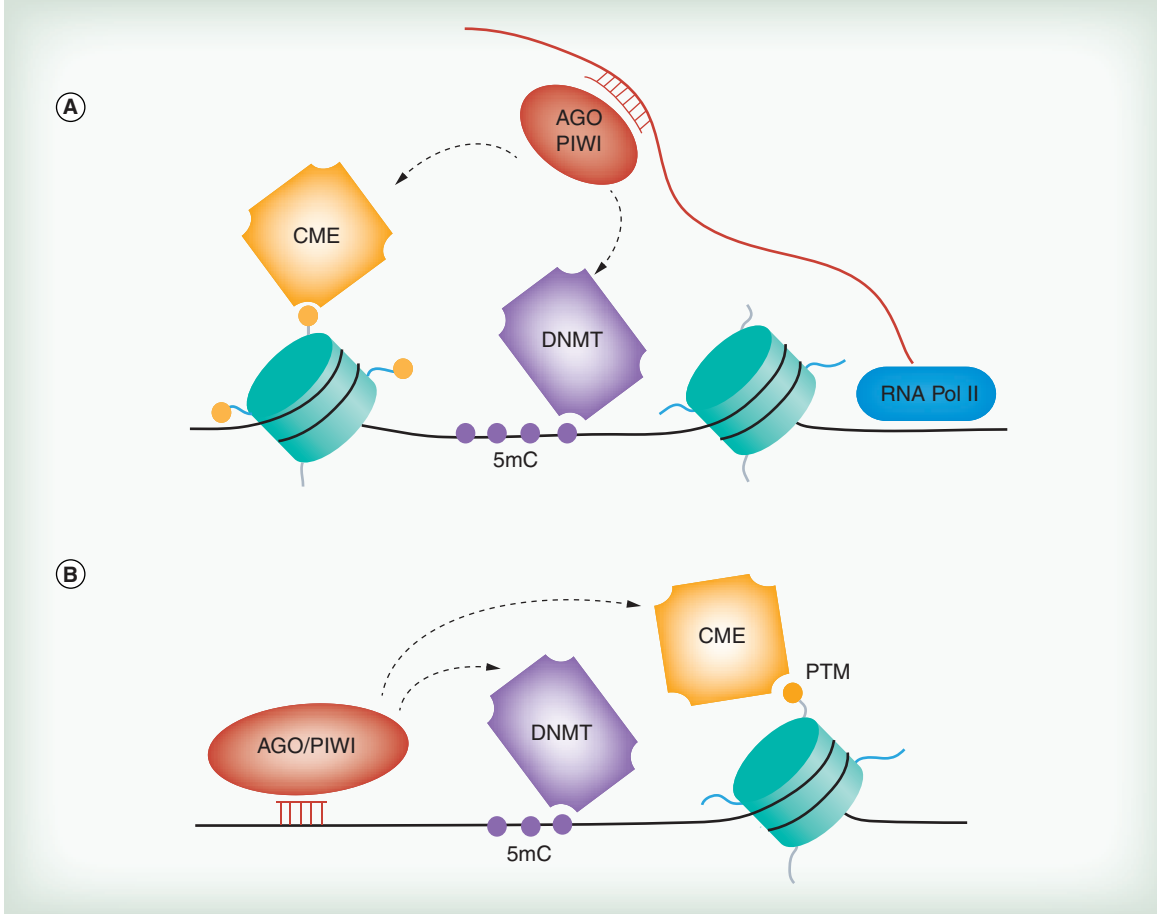

Figure 2. Epigenetic regulation by nuclear short noncoding RNA. Proposed mechanisms of sncRNA-mediated epigenetic regulation. **(A)** Studies have demonstrated short non-coding RNA (sncRNA)-mediated targeting of mRNA cotranscriptionally. This results in the recruitment of AGO or PIWI proteins to the gene locus of nascent transcripts and may result in the recruitment of CMEs and epigenetic regulation via DNA methylation or the post-translational modification of associated proteins, such as histones [93,94]. **(B)** sncRNAs in complex with AGO/PIWI also associate directly with DNA. This results in the recruitment of CMEs and epigenetic regulation [95]. AGO/PIWI indicates a member of either the argonaute or PIWI family of proteins.
CME: Chromatin-modifying enzyme; DNMT: DNA methyltransferase; PTM: Post-translational modifications; RNA Pol II: RNA polymerase II.

demonstrate a large number of these RNAs in human somatic cells [100], and recent studies have identified putative endo-siRNA populations in hippocampal tissues [101,102]. Thus, indicating a potential epigenetic role for endo-siRNAs in neuronal alterations.

piRNAs: regulators of the epigenome and neuroplasticity

In exploring the role for ncRNAs in cognition and cognitive sciences, piRNAs have become a topic of some intriguing investigations. piRNAs are distinguished from siRNAs by their size (they are slightly longer at 26–31 nt rather than 20–24 nt), and association with PIWI proteins, a clade of the AGO family [103]. Unlike miRNAs and siRNAs, piRNAs are generated from single-stranded RNA species in a *Dicer*-independent manner [104]. piRNAs are preeminently expressed and best characterized in the context of germ cell development [105–107]. Indeed, the name 'PIWI' has its humorous origin owing to the discovery of the 'P-element induced wimpy testis' in the gonadal cells of *Drosophila*. PIWI proteins translocate into the nucleus in an RNA-dependent manner, guided by piRNAs [107,108] where they serve to silence transposons in the nuclei of germ cells [109], ostensibly for the purpose of genome protection in the vulnerable germline DNA. However, this functionality is not exclusive, as protein-coding genes may also code for piRNAs [110]. Moreover, recent studies provide evidence for numerous piRNAs expressed in adult organ tissues, including in the brain, suggesting new possibilities for epigenome regulation in neurons [111,112].

With regard to epigenetic initiation and targeting, piRNAs have been shown to target heterochromatic

regions with the help of bound heterochromatin protein 1a (HP1a) as part of a PIWI-piRNA complex [113]. This complex typically associates with repressive histone lysine methylation marks, but may also facilitate transcription [114], and some evidence suggests that piRNA could form an initiator complex on chromatin that recruits other chromatin-modifying agents [115]. Additionally, Carmell and colleagues provide an interesting set of studies that suggests chromatin regulation by piRNAs. Specifically, Carmell and colleagues demonstrate that knockout of a murine PIWI results in increased transposon expression due to a loss of inhibitory DNA methylation at transposon sites [116]. Further elucidation of this mechanism by Aravin and colleagues revealed that piRNA-mediated silencing of transposons by PIWI orthologs plays a significant role in maintaining the genome integrity of the mouse testis [117,118]. Interestingly, some transposable elements have been identified as sources of dsRNAs, which feed into the endo-siRNA pathway, suggesting a degree of redundancy between endo-siRNA and piRNA pathways [104]. While still largely unexplored in mammalian systems, at least one population of piRNAs has been identified in the murine hippocampus via next-generation sequencing [119].

With regard to the neuroepigenetic function of piRNAs, recent studies suggest an epigenetically-active population of serotonin-induced piRNAs in the CNS of *Aplysia*. These studies demonstrate (via knockout of PIWI) the necessity of PIWI for serotonin induced long-term facilitation [111] – a synaptic correlate for memory formation. piRNAs have also been demonstrated to silence CREB2 – a suppressor of memory formation- in an activity-dependent manner in *Aplysia* [111], further supporting the idea that piRNA signaling is necessary for memory formation. Collectively, these results are suggestive of a broader role for piRNAs in epigenetic regulation than was previously expected and future studies will likely uncover additional piRNAs mediating neuroepigenetic regulation.

lncRNA

Discovery & characterization of lncRNAs

Typically, sncRNAs can be separated into distinct classes by clearly defined homologies of structure and function, while lncRNAs represent a heterogeneous and often modular set of transcripts [120–122]. As a result, the most common working nomenclature of lncRNA structure describes transcripts on the basis of their genomic location relative to nearby protein-coding genes [23,120]. Among these subcategories of lncRNAs are antisense, bidirectional, intergenic, intronic and overlapping transcripts (Figure 3).

Although the first functional role attributed to a lncRNA was described prior to the discovery of sncRNAs [123], it is only recently that the abundance of lncRNAs in the mammalian transcriptome has been fully recognized. Recent studies have identified thousands of lncRNA genes in the human transcriptome [19,124]. While these studies have expanded our knowledge of the transcriptome, most observations are still limited in scope to cultured cells and resting state expression profiles within tissues. Given the highly specific expression profiles of many known lncRNAs and their comparatively lower expression levels (ten-fold lower than protein coding genes, on average) [125,126], it is likely that many functional lncRNA transcripts are expressed below the power of detection for such studies. Indeed, novel deep-sequencing methodologies

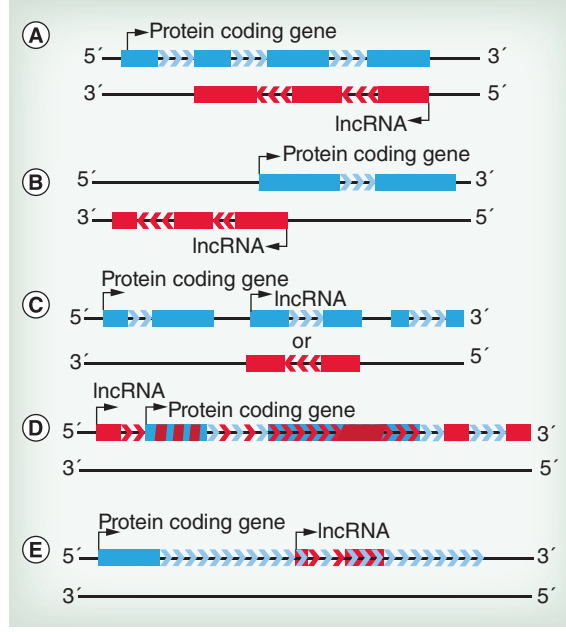

Figure 3. Origins of long noncoding RNAs.
(A–E) Many lncRNA genomic loci are colocalized with protein coding genes, and they are often described in relation to these genes. A number of common naming conventions have come into general use to describe the various protein coding gene associated lncRNAs. **(A)** Antisense transcripts overlap protein coding genes, but are transcribed from the antisense strand. **(B)** Bidirectional transcripts share transcription start sites with protein coding genes, but are transcribed in the opposite direction. **(C)** Intergenic transcripts do not overlap with protein coding genes. **(D)** Overlapping transcripts overlap significantly with or encompass protein coding genes on the sense strand. **(E)** Intronic transcripts are located within a sense-strand intron of a protein coding gene. Solid bars indicate exons of mRNAs (blue), lncRNAs (red). Diagonal stripes indicate overlapping exons. Chevron arrows indicate introns of mRNAs (blue), lncRNAs (red) or overlapping transcripts (alternating red and blue). Curved arrows indicate transcription start sites.

demonstrate that the full transcriptome is much larger than current sequencing studies have revealed [127–129]. A thorough investigation of lncRNA abundance will likely require the targeted transcriptional profiling of specific tissues, cell types or perturbations. Moreover, as many as 80% of mammalian protein coding loci express some form of antisense transcript along with their respective mRNAs [130–132]. Antisense transcripts can often impact the regulation of associated protein coding genes [133], though this is not a necessity, nor does it preclude additional *in trans* effects [23].

In addition to shared genomic loci, lncRNAs and mRNAs share characteristics that distinguish them from other small RNA species. Similar to mRNAs, lncRNAs demonstrate properties such as chromatin structure typical of RNA polymerase II (Pol II) transcription, alternative splicing sites and regulation by transcription factors [134]. Furthermore, many lncRNAs are polyadenylated and capped with 5′-methylguanisine [124]. There have even been reports of lncRNAs associating with ribosomes – although ribosome profiling experiments suggest that such associations are usually inactive [135,136]. Surprisingly, some translation of lncRNAs has been observed, resulting in the expression of small proteins products, though recent studies suggest that global translation of all ncRNAs may occur in a manner resembling pervasive gene transcription [137], though the importance of this mechanism remains to be defined in any cell system.

lncRNAs impact cellular processes via a number of molecular mechanisms. These include regulation of transcription [138–140], epigenetic regulation [141], scaffolding of protein complexes [142,143], guiding of regulatory complexes [143], acting as decoys to regulatory complexes [144] or simply being transcribed [145]. These mechanisms of action often rely on the ability of RNAs to bind both proteins and nucleic acids in a targeted manner. An RNA molecule's primary structure – that is, the linear sequence of nucleotides – allows RNA transcripts to bind homologous DNA regions via canonical or noncanonical base pairing. Recently, tools have been developed for the computational prediction of lncRNA DNA-binding motifs and binding sites [146]. Similar hybridization also allows single stranded RNA to fold into complex secondary and tertiary structures, and to bind with other RNA molecules. It is these structural arrangements, in addition to sequence specificity, that often underlie interactions with RNA-binding proteins (RBPs) [147,148]. Many currently known mechanisms of lncRNA activity rely largely on interaction with RBPs and alterations in localization, activity or association with other proteins. RBPs are a functionally and structurally diverse class of molecules, and recent studies have estimated that 40% of RBPs (out of a cohort of 1542 RBPs) are involved in ncRNA-related processes [149]. Additionally, lncRNAs have been observed to bind and regulate other small RNA molecules such as miRNAs [150,151], and extensive noncoding interactomes have been proposed [152].

In the nucleus, lncRNAs modulate gene expression via regulation of transcription and the epigenetic landscape (Figure 4) [134,153]. Indeed, lncRNAs can bind to a number of CMEs, usually 'writers' of epigenetic marks [153]. The extent of such a phenomenon was established in 2009, when it was shown that some 20% of lncRNAs (out of a cohort of 3300) associate with the PRC2, a histone methyltransferase that catalyzes the addition of repressive H3K27 methylation [154]. Additionally, binding of lncRNAs to CMEs can prevent or restrict CME activity, as was recently demonstrated to occur at the CEBPA locus, where an overlapping lncRNA (sometimes described as an extracoding RNA or ecRNA) preferentially binds the DNA methyltransferase DNMT1 [144], ultimately leading to decreased local DNA methylation. Interestingly, this phenomenon is not restricted to the CEBPA locus but occurs at multiple methylation sites across the epigenome [144].

lncRNAs in cognitive disorders

The importance of epigenetics is become increasingly recognized in neuronal alterations and cognitive function. A recent GWAS study of common cognitive disorders found that epigenetic – specifically, histone methylation – pathways were strongly associated with impaired cognition [9], and a number of screening studies suggest that lncRNA dysregulation is associated with neurodevelopmental and cognitive disorders [155], including Rett syndrome [156], autism [157] and Fragile X syndrome [55]. While the widespread mechanisms of ncRNA-mediated regulation have been established for some time, only in very recent years have these mechanisms been investigated in a neurological or cognitive context. lncRNAs have been found to be co-expressed with genes that are critical for neuronal activity, including *C-fos*, *Arc* and *BDNF*, suggesting an extensive network of protein coding and noncoding genes involved in neuronal plasticity [55,158]. Additionally, lncRNAs are known to play a role in normal brain development [159]. While the majority of lncRNAs transcripts have been characterized either in cell culture or during development, efforts to examine the functional roles of neuronal lncRNAs in cognition are still continuing. Here, we will recount some of the better-characterized examples of lncRNA functioning in the context of the adult brain, and their impact on cognition or cognitive disorders.

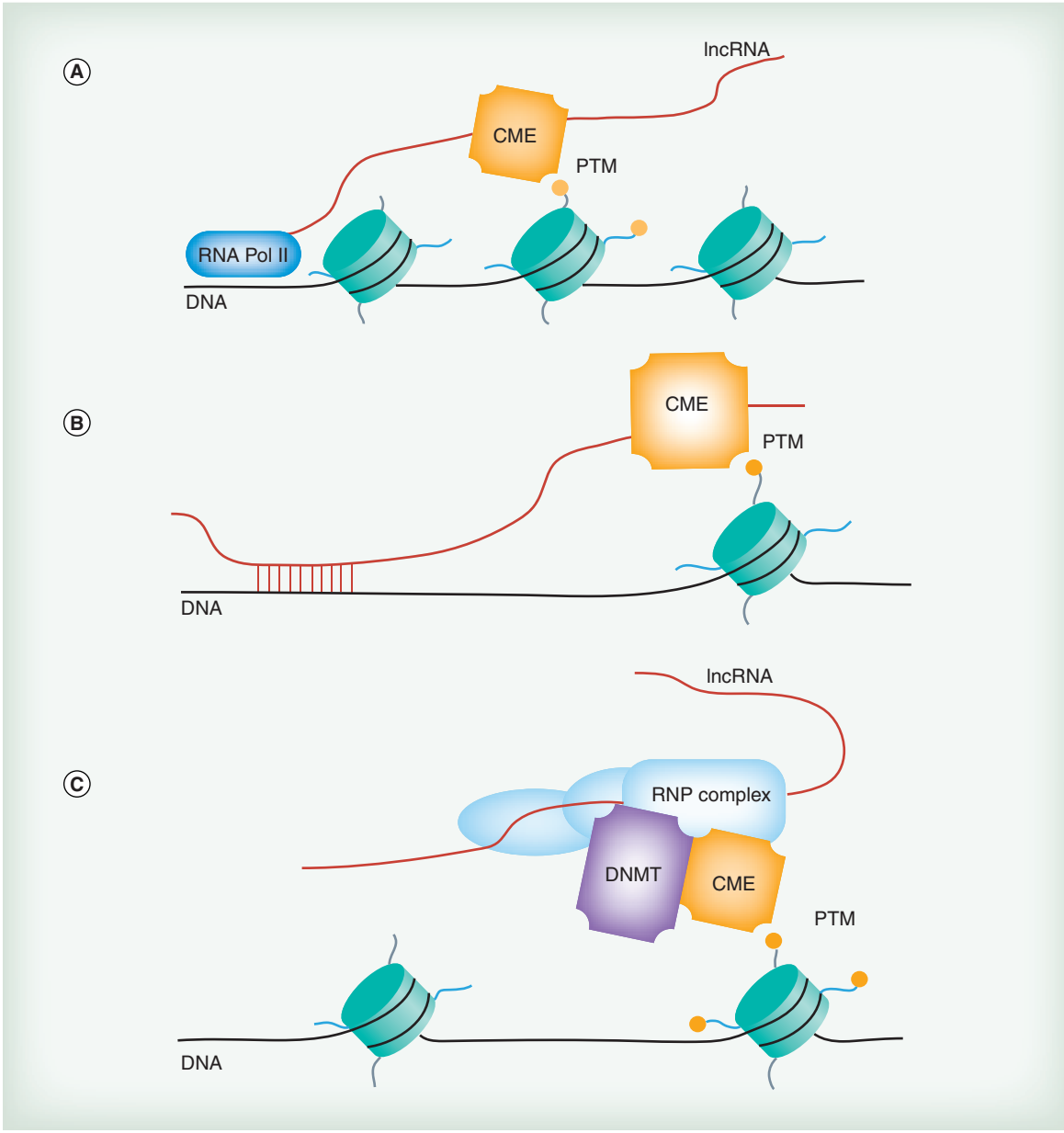

Figure 4. lncRNA-mediated epigenetic regulation. lncRNAs possess a number of mechanisms by which they initiate or facilitate epigenetic regulation, and multiple archetypal functions are often utilized within a single lncRNA transcript. **(A)** lncRNAs often recruit chromatin-modifying enzymes in *cis*, thereby mediating epigenetic regulation of nearby genes (dimitrova, zhang, redrup). **(B)** lncRNAs may also act as guides, targeting associated CMEs to target loci in *trans*, potentially through direct interaction with target regions. **(C)** lncRNAs may act as scaffolding factors, and mediate the assembly of ribonucleoprotein complexes with multiple regulatory functions. This may occur either in *cis*, as occurs with the direct CMEs to target loci in *trans*.
CME: Chromatin-modifying enzyme; DNMT: DNA methyltransferase; PTM: Post-translational modifications; RNA Pol II: RNA polymerase II; RNP complex: Ribonucleoprotein complex.

Malat1

One lncRNA that has been observed to regulate neuronal activity is Malat1 (also known as Neat2). This highly conserved nuclear lncRNA is expressed in numerous tissues, with a high degree of expression in neurons [160]. Knockdown studies of Malat1 have resulted in decreased synaptic density in cultured hippocampal neurons [160], and postmortem studies have demonstrated that Malat1 is upregulated in multiple brain regions in both human alcoholism as well as rodent models of alcoholism [56]. Malat1 can regulate gene expression *in cis*, thus controlling the expression of proximally located genes which are involved in nuclear function [138]. Conversely, Malat1 can associate with

hundreds of sites *in trans*, where it preferentially binds the gene body of active genes in a transcription-dependent fashion [161]. Epigenetically, Malat1 has been shown to associate *in vivo* with EZH2, a subunit of the PRC2 [162]. Interestingly, and despite many functional associations, Malat1 knockout in mice does not affect viability or normal development [138,163].

Gomafu

The lncRNA Gomafu has also been shown to play multiple roles in the adult brain. Gomafu has been observed to govern SZ-related alternative splicing by acting as a splicing factor scaffold for QK1 and SRSF1, and it is known to be dysregulated in postmortem studies of schizophrenia patients [164]. Recently, an additional study has suggested that Gomafu functions *in cis* to mediate epigenetic regulation of gene expression via the PRC1 complex, and that knockdown of Gomafu in adult mice results in abnormal behavioral phenotypes and increased anxiety [55].

BACE1-AS

Another example of lncRNAs involved in neuronal disorders, is the antisense lncRNA, BACE1-AS that has been implicated in Alzheimer's disease (AD). AD is a progressive neuro-degenerative disorder which has been previously associated with epigenetic dysregulation, particularly in histone acetylation [165,166]. A characteristic marker of AD pathology is the accumulation of β amyloid plaques consisting of oligomerized amyloid β peptides. These plaques form as a result of the processing of amyloid precursor protiens (APP), the rate limiting step of which is the cleavage of APP by the Beta-secretase enzyme (BACE1) [167]. Dysregulation of BACE1 contributes to AD pathology via the overproduction of Aβ [167]. Recent studies have identified an antisense lncRNA at the BACE1 locus (BACE1-AS) which physically associates with and stabilizes BACE1 mRNA, increasing BACE1 expression both *in vitro* and *in vivo*, and ultimately resulting in increased generation of Aβ [54]. BACE1 mRNA is targeted by the miR-485–5p, which normally results in BACE1 repression; however, BACE1-AS prevents this repression by competitively binding the miRNA target site [168]. Both the BACE1-AS lncRNA and BACE1 mRNA are overexpressed in the parietal lobe and in the cerebellum of postmortem AD patients, suggesting a relevant mechanistic link between the BACE1-AS lncRNA and the pathophysiology of AD [168]. Interestingly, knockdown of BACE1 or BACE1-AS results in reduction of Alzheimer's pathology in an APP mouse model of AD [169].

While the dysregulation of lncRNAs has been implicated in cognitive disorders, the task of exploring the role of lncRNA-mediated epigenetic regulation in normal cognitive function remains incomplete.

Transgenerational impacts of ncRNA-mediated epigenetic regulation

Since the discovery of epigenetics, there has been much curiosity and speculation as to the transgenerational heritability of epigenetic marks. In mammals, much of the epigenome is erased during the processes of fertilization and generation of primary germ cells (reviewed in [170]); nonetheless, evidence of a transgenerationally altered epigenome has steadily accumulated, including heritable cognitive changes and behavioral phenotypes [171–175]. A simple explanation for this phenomenon would be incomplete erasure of DNA and histone modifications. While there is some evidence in support of this hypothesis (reviewed in [176]), other studies have demonstrated the existence of an indirect mechanism of chromatin regulation via generational transfer of ncRNAs.

Recently developed mammalian epimutation models – in which phenotypes are derived from a heritable change in gene expression, as opposed to an altered genome – have demonstrated the sufficiency of parental RNA to alter the epigenome of treated progeny [177]. Additionally, in a rodent stress model, treatment of fertilized mouse oocytes with ncRNAs from the sperm of stressed males is sufficient to recapitulate heritable stress-related behavioral and metabolic phenotypes [173]. These results indicate that alterations in the transcriptome are sufficient for the transfer of epigenetic information across generations, and play a critical role in cognitive function.

The most direct evidence for a neuronal role in transgenerational epigenetic phenomenon comes from *C. elegans*, where neuronally expressed RNA species are transported to the cells of the germline. These RNAs then initiate the transgenerational epigenetic silencing of particular genomic loci, thereby impacting gene expression in the germ line and, potentially, in any progeny [178]. It is tempting to speculate that an analogous mechanism could exist in mammals, by which somatic tissues such those of the brain may regulate the epigenome of cells distant in both space and time. Clearly, such a finding would have far-reaching consequences for cognitive science.

Conclusion & future perspective

While numerous studies have found associations between ncRNAs, cognition and cognitive disorders, few have fully investigated and characterized the diverse mechanisms that can be attributed to ncRNAs. In this review, we provide insights for future direction in the investigation of different classes of ncRNAs and discuss regulatory RNAs that

have both established roles in cellular and molecular processes and a defined relationship to the epigenome. Thus, we present the provocative research idea that ncRNAs might serve to control epigenetic mechanisms involved in cognition by illustrating the few cases of such phenomena that have been described in the literature.

To date, only a few regulatory RNAs have been discovered to have both epigenetic and cognitive relevance. However, these few examples underscore the extent to which the numerous and heterogeneous classes of regulatory RNAs are still unexplored. Anatomically, these species are expressed primarily within the cognitive centers of the brain, and indeed, their relevance to cognition is well established. However, emerging studies are beginning to explore beyond the canonical pathways of regulatory RNA function established in previous decades. The studies we have reviewed here demonstrate the long-term impact of regulatory RNAs on the epigenome and thereby cognition. Although the canonical functions of ncRNAs involve diverse mechanisms, new insights suggest that several classes of ncRNAs impact the epigenome, a common ground where both protein and RNA species converge to regulate cellular function. Groundbreaking studies are beginning to demonstrate that epigenetic regulation by ncRNAs is – to a yet poorly explored extent – actively influencing neuronal and cognitive function. Therefore, it is likely that future studies will focus on increasing knowledge of ncRNA-mediated epigenetic regulation on well-characterized cognitive functions, such as memory formation. Moreover, we fully expect that further investigations into the role of regulatory RNAs will reveal novel epigenetic roles for this versatile class of molecules in cognitive disorders.

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Executive summary

- This review discusses mechanisms of multiple classes of nonoding RNAs (ncRNAs) and how they relate to the epigenome.
- Schizophrenia, a cognitive disorder with epigenetic dysfunction, is used to describe a possible role for canonical miRNA species in the regulation of epigenetic mechanisms.
- A description of small interfering RNA mechanism of action is provided, and newly defined mechanisms of epigenetic regulation not yet investigated in cognition or cognitive dysfunction.
- An introduction to PIWI-interacting RNAs is provided along with studies describing the potential role of PIWI-interacting RNAs as regulators of the epigenome in neuroplasticity.
- We present a description of long noncoding RNAs, a newly defined class of ncRNAs, and describe the roles of several long noncoding RNAs in cognition and cognitive disorders. Moreover, we highlight new studies indicating that long ncRNAs impact cognition via their role as epigenetic regulators.
- Importantly, we highlight recent studies on the transgenerational impact of ncRNAs and the epigenome on cognitive function.
- The key findings presented throughout this review warrant future studies of ncRNAs, as related to the epigenome and cognitive function.

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Editorial

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Epigenomics

Does DNA methylation influence the effects of psychiatric drugs?

"While sequence polymorphisms of DNA are enduring genetic factors, the extent of CpG methylation may have multiple influences, both environmental (in the broadest interpretation of that term) and genetic."

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The introduction, in the middle of the last century, of specific drug treatments for schizophrenia, bipolar illness and depression brought relief to many of those suffering from these disorders. Subsequently developed drugs have offered some improvements in tolerability, through changes in side effect profiles, but not more than marginal changes in efficacy. Thus severe mental illness, despite the undoubted value of current pharmacotherapy, still represents a huge economic and social burden, reflecting the limited efficacy of drug treatments. Treatment of depression achieves response rates of little more than 50%, while even lower proportions of people with schizophrenia achieve adequate symptom relief with the antipsychotic drugs.

Thus there are profound differences between individuals with severe mental illness in how they respond to drug treatments. In addition to the wide variability in symptom response mentioned above, the experience of adverse drug effects can also vary greatly between individuals. These differences in efficacy and side effects are thought to reflect, at least in part, genetic differences between patients; thus pharmacogenetics in psychiatry has much to offer in the eventual identification of risk factors for these limitations of drug treatment.

Over the past two decades, research into the pharmacogenetics of psychiatric drugs has identified several genetic variations that reliably associate with some of the effects of drug treatment, as well as many more that provide intriguing but inadequately replicated indications of gene associations. There appears to have been more success in identifying genetic factors relating to side effects of drug treatment than in finding associations with symptom response. This may be because the phenotypes related to adverse effects, such as weight gain, are often easily defined and objectively measurable, while disease severity in psychiatry is multifactorial with complex and subjective measures.

Nevertheless, some consistent findings relating to treatment response of symptoms of depression and schizophrenia have been found, particularly when one considers neurotransmitter systems rather than individual genes or SNPs. For example, variations in genes related to serotonin (5-HT) synthesis, disposition and its receptors have variously been implicated in antidepressant response. Many of the various pharmacogenetic findings involve functional SNPs in 5′ regulatory regions. Often these polymorphisms are within islands of CpG sequences, sites for DNA methylation that contribute to the epigenetic control of transcription. Thus, it seems very plausible that the extent of CpG methylation of specific promoter sequences that are recognition sites for transcription factors might correlate, as SNPs can associate, with the consequences of drug treatments.

While sequence polymorphisms of DNA are enduring genetic factors, the extent of

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CpG methylation may have multiple influences, both environmental (in the broadest interpretation of that term) and genetic. It is well established that, in addition to genetic variability, environmental factors such as childhood trauma and maltreatment can contribute to an impaired response to antidepressants. Furthermore, such factors can interact with SNPs in their association with response to treatment [1]. This provides a potential indication of, and justification for studies in, epigenetic mechanisms that may link environmental factors to treatment outcome.

Certainly there is increasing evidence that DNA methylation may be involved in the influence of early life stress on depression and antidepressant drug response. As one example, DNA methylation of the serotonin transporter *SLC6A4*, perhaps the best studied of genes in psychiatric pharmacogenetics, is greater in subjects reporting child abuse, and can influence gene expression by interacting with a common functional promoter SNP, the HTTLPR [2]. Accumulating evidence suggest that this methylation is itself affecting treatment response independent of other influences [3,4]. Few other specific genes associated with antidepressant response have been investigated for their methylation status. Interestingly, DNA methyltransferase inhibitors have been reported to have an antidepressant effect in an animal model [5]. It would be valuable to know if this effect extended to human depression, and whether it might address those poorly responding patients with a history of childhood trauma.

> "How much this modulation of methylation might contribute to the clinical consequences of drug treatments remains unquantified, but it certainly provides a potential target for therapeutic intervention."

Of course, the interactions between drugs and a dynamic biological factor such as DNA methylation can be in two directions. Not only can DNA methylation influence the effects of drug treatment, but also psychiatric drugs might modify DNA methylation either globally, or in specific genes, with consequent effects on gene expression that may contribute to drug action. Certainly DNA methylation in blood cells appears to be affected by antipsychotic drug treatment [6]. The major site of action of the antipsychotic drugs is the dopamine D2 receptor; methylation of almost one half of 40 genes related to dopamine neurotransmission is changed following administration of olanzapine to rats, primarily by increased methylation [7]. It seems likely, although not studied, that this might be a common action of all the D2 receptor antagonist antipsychotic drugs.

While the dopamine system is the target for the effect of antipsychotic drugs on positive symptoms of schizophrenia, such as hallucinations and delusions, there is evidence from both pharmacogenetics and clinical pharmacology that the less responsive negative symptoms (e.g., withdrawal, lack of self care) may involve 5-HT neurotransmission [8]. One replicated pharmacogenetics study showed that a functional SNP rs6295 in the 5-HT1A receptor was associated with negative symptom response [9]. A DNA methylation study of the promoter sequence around this SNP, thought to have its effects by affecting transcription factor binding, has identified how methylation of a single CpG site, itself within a transcription factor recognition site, has a strong correlation with negative symptom response to treatment, paralleling pharmacogenetic findings [10].

Quetiapine, an antipsychotic that also has some efficacy against depressive and manic symptoms, can along with several other mood stabilizers decrease methylation in the promoter region of *SLC6A4*, perhaps counteracting the *SLC6A4* hypermethylation seen in mood disorders [11]. Equivalent results have been obtained from investigation of *BDNF* promoter methylation in bipolar disease patients undergoing treatment, with similar conclusions being drawn [12].

Understanding the action of valproate is providing clues as to the importance of DNA methylation in psychiatric pharmacotherapy. Valproate is an important drug in the treatment of bipolar disorder as well as epilepsy. Its mechanism of action is not fully understood, although it is an inhibitor of histone deacetylases and induces DNA demethylation, effects that appear to correlate with its ability to relieve symptoms of bipolar illness [13,14]. This results in widespread changes in gene expression, some of which have been proposed as important in symptom relief, including increases in some indicators of GABAergic neuronal activity [13].

Another example of a psychiatric problem that has attracted some interest from epigenetic studies is attention deficit hyperactivity disorder (ADHD). Here too there are established environmental risk factors that may underlie disturbances in DNA methylation, as well as recent evidence for hypermethylation in specific and biologically plausible genes [15,16]. One study has identified how increased promoter methylation in a strong candidate gene – *SLC6A3*, coding for the dopamine transporter which is a site of action of methylphenidate, the main drug treatment for ADHD – was correlated with poorer response to treatment [DING ET AL., UNPUBLISHED].

A concern that runs through all human epigenetic studies of diseases associated with brain dysfunction is the relevance of methylation of DNA from peripheral sources, primarily cells in blood or saliva. Nevertheless there are several studies indicating concordance

of DNA methylation between these peripheral cells and brain tissue in neurological and psychiatric disease [17,18]. Notably a site-specific hypomethylation of the gene for the 5-HT2A receptor, a site of action of newer antipsychotic and antimanic drugs, was found in both brain- and saliva-derived DNA in schizophrenia and bipolar disorder [19].

Conclusion & future perspective

Thus accumulating evidence of correlations of clinical outcomes of psychiatric drug treatments with DNA methylation from peripheral samples may well reflect changes in brain DNA. This in turn is likely to result in effects on central gene expression. In this way, DNA methylation changes can be compared with SNPs, although the former is likely to be more subtle while the latter is dichotomous in its effects on gene function. DNA methylation may therefore provide a further measure to enter into personalized medicine algorithms.

In this editorial, we have only cited a few examples of recent research to illustrate the role of DNA methylation in psychiatric pharmacotherapy. Although there are many more valuable studies, work so far has still only scratched the surface of the problem and there are many questions to be answered. Laboratory studies have shown us that DNA methylation can be highly dynamic, and that the extent of methylation in specific sites with potentially functional effects can be differentially modified by a variety of factors including psychological and physiological stress, toxins and psychiatric drugs. The mechanisms underlying these various environmental influences on DNA methylation remain obscure. How much this modulation of methylation might contribute to the clinical consequences of drug treatments remains unquantified, but it certainly provides a potential target for therapeutic intervention. Such an approach is not straightforward; drugs such as valproate that decrease DNA methylation may be unselective in the genes they target and can also be teratogenic, presumably for the same reason.

Although also true for association studies of promoter region SNPs, the intermediate steps that link DNA methylation to drug response and side effects have not been considered in much of this research. Few studies identifying 'pharmacoepigenetic' links have looked further into the mechanisms, which are likely to include effects on transcription factor binding, to assess the role of changes in gene transcription in determining clinical outcomes. A greater understanding of the precise mechanisms whereby changes in DNA methylation lead to different pharmacotherapeutic outcomes, as well as how they interact with functional SNPs, will be invaluable in identifying novel targets that better address the substantial unmet needs in the treatment of major psychiatric disorders.

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