Published in final edited form as:
Trends Neurosci. 2013 August ; 36(8): 460–470. doi:10.1016/j.tins.2013.05.001.

Epigenetic layers and players underlying neurodevelopment

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Abstract

Since epigenetic mechanisms convey information above and beyond the sequence of DNA, they are predicted to be critical in the complex regulation of brain development and explain the long-lived effects of environmental cues on pre- and early post-natal brain development. Neurons have a complex epigenetic landscape, which changes dynamically with transcriptional activity in early life. Here we summarize progress on understanding the discrete layers of the dynamic methylome, chromatin proteome, noncoding RNAs, chromatin loops, and long-range interactions in neuronal development and maturation. Many neurodevelopmental disorders have genetic alterations in these epigenetic modifications or regulators, and these human genetics lessons have demonstrated the importance of these epigenetic players and the epigenetic layers that transcriptional events lay down in the early brain.

Introduction

Epigenetics has historically referred to heritable modifications resulting in phenotypes that were not directly dependent on the DNA sequence inherited. However, in recent years the rapid expansion of epigenetic research into DNA methylation, histone modifications, and chromatin structure has broadened the definition of epigenetic heritability to include long-lived but reversible modifications to nucleotides or chromosomes. With this larger umbrella of epigenetic mechanisms has come greater complexity in defining the set of underlining epigenetic rules. Box 1 provides a current definition of epigenetics, lists the major categories of epigenetic layers and players, gives examples of what is and is not epigenetics, and dispels some epigenetic “urban myths”.

Box 1

What is epigenetics?

Epigenetics: Long-lived and reversible modifications to nucleotides or chromosomes that do not change the sequence but can alter gene expression and phenotype.

Epigenetic layers: DNA methylation, stably maintained histone modifications (e.g., H3K27me3, H3K9me3), chromatin loops, chromosomal organization and location within the nucleus, noncoding RNAs (bound to DNA)

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Epigenetic regulators or "players": chromatin-binding factors, chromatin remodeling complex proteins, noncoding RNAs, DNA methyltransferases, histone modifying enzymes

What is epigenetic? allelic differences in transcription not determined by sequence, cell lineage inheritance, long-lived modifications from past environmental exposures

What is NOT epigenetic? regulation of transcription without accompanying long-lived modifications, transcription factors (although they may direct epigenetic states)

Metastable: a physics term describing a state of precarious stability, which is also a useful term for labeling the long-lived but reversible characteristics of epigenetics [5]

Common epigenetic myths:

1. DNA sequence does not matter for epigenetic modifications.

   While this statement may be true for allelic states such as imprinting and X chromosome inactivation, sequence features such as promoter CpG content or other nucleotide bias (such as G-skew on one DNA strand) can be very important in determining deposition of the epigenetic modification DNA methylation [6–8*] and methylation patterns are heritable with genotype [*9].

2. Epigenetic marks determine transcription (but not vice-versa).

   While this was the historical thinking in the field, many recent examples demonstrate that transcription is required for the establishment of some epigenetic modifications. Epigenetic modifications may actually be historical marks of past transcriptional events that influence later transcriptional responsiveness.

3. Transcriptional differences between genetically identical individuals must be epigenetic and environmentally driven.

   While an attractive idea, not all transcriptional differences are epigenetic and many epigenetic differences are stochastic, rather than environmentally determined.

4. DNA methylation patterns, once established in early development, are stably maintained.

   While overall, methylation patterns appear to be much less variable between individuals or cell types than transcriptional patterns, there are some concrete examples of changes to methylation levels in neurons, following activity-dependent responses [1, 2, 10].

5. DNA methylation is always associated with transcriptional silencing.

   *While this is true for CpG-rich promoters that are subject to imprinting, X chromosome inactivation, or cell fate determination (*Table 1), outside of these situations the rules change, and higher DNA methylation levels is generally associated with higher expression [11, 12].

Neuroscientists have understandably been drawn to the field of epigenetics because it can help explain complex transcriptional regulation that is not fully explained by inducible and tissue-specific transcription factors alone. Epigenetics is appealing as well because it offers a potential explanation for variable phenotypic outcomes from similar genetic backgrounds. Specifically, how do early life experiences during critical periods for learning and memory or establishment of the hypothalamic-pituitary-adrenal (HPA) axis have long lived effects on

transcription lasting into adulthood? How are transcriptional patterns that define neuronal networks maintained and further refined with activity? How does drug abuse in adolescence set up a behavioral pattern of addiction? Since epigenetic modifications appear to be especially important for genes encoding proteins acting at the synapse [1–3], epigenomic investigations may point to potential "druggable" protein targets using existing medications in the treatment of neurodevelopmental and psychiatric disorders. In addition, since epigenetic mechanisms exist in a "metastable" state, an attractive possibility is the design of therapies to reverse epigenetic states in the brain without disrupting the underlying genetic state.

But caution should be exerted in not putting the cart before the horse in this line of research. Epigenetics is still a young field and the rules of how epigenetic modifications affect gene expression and may be involved in specific disease states have yet to be fully understood. With the advent of genome-wide technologies, the view of the epigenome has broadened considerably, but this new information has often challenged many of the past assumptions about epigenetic control of gene expression [4]. Here, we review the current understanding about the individual layers of epigenetic information and how they are important to neurons, primarily during early life. We define "layers" as structural components of DNA and chromatin, in contrast to "players" that are active complexes and enzymes required for epigenetic layers.

The neuronal methylome

The addition of a methyl group to the 5th carbon of cytosine (5meC) is primarily observed at CpG sites in the mammalian genome, although non-CpG methylation is also observed in plants as well as embryonic stem (ES) cells and neurons in mammals [13]. Brain DNA has one of the highest levels of 5meC in the human body. 5meC can also be converted to 5-hydroxymethylcytosine (5hmC) in the presence of TET1 [14, 15], and 5hmC is implicated in demethylation of the paternal genome in early mouse (*M. Musculus*) embryos and the self-renewal capacity of mouse embryonic stem cells, perhaps through demethylation of the *Nanog* promoter [16]. 5hmC levels are particularly high in brain compared to other tissues and Purkinje compared to granular neurons of the cerebellum [14]. Although 5hmC appears to be an important transition state in the dynamic methylation patterns of neurons, it is far less abundant than 5meC even in Purkinje neurons. Furthermore, most methods used to investigate DNA methylation that involve either bisulfite conversion or methyl-sensitive restriction enzymes cannot distinguish between 5meC and 5hmC. Methods for discriminating 5hmC are beginning to reveal differences in tissue and location from 5mC [17, 18], but the functional relevance of 5hmC is still unknown. Therefore, what we refer to in this review as "DNA methylation" and the "neuronal methylome" will actually be a collection of 5meC, 5hmC, and perhaps other less characterized modifications of cytosines [19].

The relationship of DNA methylation to gene expression does not abide by one universal rule, but rather is heavily dependent on underlying sequence, transcription state, and location of the genomic DNA (Figure 1). The canonical view of DNA methylation as a repressive mark is observed for CpG island promoters of genes on the active versus inactive X chromosome (XCI) and imprinting control regions which are clear examples of allele-specific methylation [20, 21]. However, recent genome-wide DNA methylation studies have shown that high CpG methylation is common in gene bodies (genomic loci spanning exons and introns) where it positively correlates with transcription [22, 23]. Furthermore, gene bodies contained in partially methylated domains (PMDs, continuous domains of <70% methylated CpGs) [12] showed decreased expression compared to those contained in highly methylated domains (HMDs). Interestingly, tissue-specific differences in the presence of

PMDs have revealed a subset of developmentally regulated genes that act at the synapse are highly methylated and expressed in human immature neurons (SH-SY5Y) cells compared to fetal fibroblasts [3] or placenta [24].

Neurons appear to have a unique methylomic landscape compared to other cell types. The contrast between very low levels of methylation in CpG islands and very high methylation in gene bodies is sharper in human brain and neurons compared to non-neuronal cells [3]. DNA methylation is highly dynamic in mammalian postnatal neurons and these cells express the corresponding DNA methyltransferases (DNMT) at high levels [25]. Importantly, mice deficient for both DNMT1 and DNMT3A in forebrain excitatory neurons showed deficits in learning and memory [26], similar to deficits in fear memory observed with DNMT1 chemical inhibition [27]. Likewise, DNMT3A regulates emotional behavior and spine plasticity [28] and ensures the expression of key neurogenic genes by targeting *de novo* non-promoter DNA methylation around active genes [29]. DNA methylation changes were observed at the *Bdnf* promoter in neuronal cultures following activity [30] and an in vivo paradigm of learning and memory resulted in significant changes to methylation patterns [31].

Dynamic changes in DNA patterns have been observed genome-wide in adult mouse dentate granule neurons after neuronal activation that remained stable for at least 24 h [1]. Interestingly, these activity-modified CpGs were specifically enriched in low-CpG density regions [1] that are more characteristic of PMDs [3] than the high CpG density regions typically examined for methylation differences. Furthermore, not all of the observed changes in DNA methylation correlated with an immediate transcriptional change, suggesting that altered methylomes may have subtle, long-lasting effects [1]. Interestingly, active demethylation in the adult brain can be potentially explained by the action of TET1, which hydroxylates 5mC to 5hmC, leading to demethylation [32]. Therefore, while once considered a stable mark in somatic tissues, DNA methylation is emerging as an important metastable epigenetic layer in postnatal neurons.

The neuronal chromatin proteome

The neuronal methylome is intricately intertwined with the numerous proteins that bind to DNA in neuronal nuclei. The most abundant and best characterized factors in neurons are the histone core proteins H2, H3, and H4, linker histone H1, methyl CpG binding protein 2 (MeCP2), and chromatin insulator CTCF. The neuronal chromatin proteome is an important epigenetic layer because it both “reads” the methylome and influences the degree of compaction and accessibility of the DNA to transcription factors and the transcriptional machinery [33–35]. Like DNA methylation, the marks of the neuronal chromatin proteome can be both long-lived and dynamically altered in response to transcription or activity, resulting in metastable “chromatin states” defined by combinations of genome-wide analyses of histone modifications and transcription in the recent human ENCODE (Encyclopedia of DNA elements) project [36] (Figure 2).

While the specific chromatin states are less well defined for neurons as for the ENCODE cell lines, the importance of dynamic changes to chromatin states is acutely evident for neuronal development and activity. Activity and energy in the form of ATP is required for changes to chromatin states during neuronal lineage commitment in the epigenetic process of “chromatin remodeling” [37]. Interestingly, mutations in several chromatin remodeling factors, including *ATRX* and *ARID1B* are associated with human neurodevelopmental disorders [38–40]. Furthermore, neurons have a unique chromatin remodeling complex, defined by the neuron-specific subunit BAF53b, expressed in postmototic neurons around embryonic day 12.5 [41]. Deficiency of BAF53b in mouse results in significant deficits in

long-term memory and transcriptional changes to the noncoding RNA miR132 implicated in synaptic plasticity [42].

Histone core proteins reflect and/or influence accessibility of the DNA through multiple post-translational modifications. Different combinations of histone core protein modifications make up what has been called the “histone code” which has been reviewed extensively [43–45] but debated as to whether most histone modifications fit the definition of being long-lived epigenetic states [46]. Perhaps the removal of histone methylation marks is particularly critical for neurons, as mutations in genes encoding histone demethylases have been found in human X-linked autism-spectrum disorders, including JMJD1C/TRP8 that demethylates H3K9 and KDM5C/JARD1C that demethylates H3K4 [47, 48].

Beyond the core nucleosome particle and associated modifications, other protein factors are important in defining chromatin states. The CCCTC-binding factor (CTCF) is a sequence-specific chromatin organizer of chromosomal domains [49]. Interestingly, CTCF has been implicated as a master regulator of alternative splicing of the highly alternatively spliced protocadherin gene clusters in neuronal connectivity [50], and protocadherin genes are also highly enriched within N-HMDs of the neuronal methylome [3, 24]. DNA methylation within gene bodies can influence alternative splicing by blocking CTCF binding and pausing of RNA Pol II near weak exons [51].

In contrast to CTCF which is ubiquitously expressed, has distinct binding sites, and a higher affinity for unmethylated DNA [49, 52], methyl CpG binding protein 2 (MeCP2) is highly expressed specifically in the central nervous system, binds widely throughout the genome, and has increased affinity for methylated DNA [53–55]. Mutations in *MECP2* cause the X-linked autism-spectrum disorder Rett syndrome [56]. MeCP2 levels increase with neuronal maturity, becoming as abundant as histone proteins in mature neurons and displacing of portion of H1 linker histones [35]. Similar to histones, MeCP2 is extensively post-translationally modified by phosphorylation, acetylation, and ubiquitinylation [57]. Although the functional relevance of the “MeCP2 code” is much less clear than it is for the histone code, phosphorylation of S421 is implicated in psychostimulant responsiveness [58] and synaptic scaling [59], and differential phosphorylation of S80 and S229 of MeCP2 discriminate cofactor associations [57].

Long, non-coding RNAs as epigenetic players and layers in neurodevelopment

Recent lines of evidence have shown that long, non-coding RNAs (lncRNAs) are critical regulators of epigenetic mechanisms [60, 61] (Figure 3). That RNA is a regulatory keystone is not surprising given the hypothesis that the first living, biological molecules were RNA [62]. Furthermore, most cellular processes are governed by self-regulating feedback loops so the presence of RNA as a regulator of transcription fits with general biological principles. The advent of transcriptomic analyses revealed that many long and short non-coding transcripts exist [63] and that many exhibit tissue-specific patterns of regulation [64–66]. Many different forms of RNA that regulate transcription have been described, including microRNAs (miRNAs), long noncoding RNAs (lncRNAs) [67], enhancer RNAs (eRNAs) [68], and circular (circRNAs) [69]. While miRNAs and circRNAs play a critical role in neurodevelopment, they are not epigenetic layers per se, but can regulate other epigenetic layers and players as well as be epigenetically regulated themselves. For instance, the miRNAs miR-483-5p and miR-132 regulate MeCP2 levels [70, 71], and miR-132 itself is regulated by the neuronal chromatin remodeling complex containing BAF53b [42].

Here, we will focus on the role of lncRNAs since they are emerging as a key-regulator of epigenetic processes by targeting chromatin remodeling complexes (player) or by being a part of the chromatin structure (layer). LncRNAs certainly also have functions beyond directing epigenetic mechanisms, since they can regulate alternate splicing [72], miRNA abundance [69, 73] protein-protein interactions, and molecular signals [61]. Since many lncRNAs exhibit brain region specific patterns of expression [74], they are predicted to regulate the diversity of neuronal subtypes and populations in the brain. Comprehensive studies of lncRNA function during neurodevelopment have focused on control of neurogenesis from ESC to differentiated neurons in cell culture systems [75, 76], leaving unresolved their potential roles in the postnatal stages of neuronal maturation, and in mediating activity-dependent changes in chromatin structure.

A neurodevelopmental role for lncRNAs has been shown in differentiation of neurons from human embryonic stem cells [75, 76] and in specifying subpopulations in the retina, where loss of function of two lncRNAs *RNCR2* and *Six3OS* led to changes in neuronal populations [77, 78]. The loss of *Six3OS* may cause changes in chromatin states because of its interaction with the PRC2 complex member Ezh2, which mediates H3K27 methylation and interacts with thousands of additional lncRNAs [64, 79]. By binding PRC2, these lncRNAs act to repress target polycomb genes and provide specificity to a repressive complex that lacks a protein-DNA targeting factor. Although lncRNAs are also predicted to target activating complexes in a similar manner by binding Trithorax group proteins [80], a systematic identification of RNAs bound to activating complexes has not been performed.

While some lncRNAs appear to regulate only one or two genes [81], many lncRNAs apparently regulate multiple genes by forming a nuclear subdomain [64, 82–85]. Studies of the lncRNAs *Xist* and *Kcnq1ot1* demonstrate how a lncRNA recruits repressive complexes to a whole chromosome (*Xist*) or an imprinted domain (*Kcnq1ot1*) [60]. Since neurons are postmitotic, formation of nuclear subdomains by lncRNAs may be one mechanism by which long-term epigenetic regulation occurs, although this has not yet been demonstrated. One nuclear lncRNA, *116HG,* is implicated in the pathogenesis of the imprinted neurodevelopmental disease Prader-Willi syndrome (PWS) [86] and expression of *116HG* may regulate a large-scale chromatin decondensation [87], although the precise mechanism is unknown.

While emerging evidence points to lncRNAs as epigenetic “players” in neurodevelopment by directing epigenetic mechanisms, noncoding transcripts can also act as an epigenetic “layer” on chromosomes. As an example, transcription of *Ube3a-ATS* leads to paternal silencing of *Ube3a* in neurons. The precise mechanism of silencing is not known, but a strong similarity to the imprinted *Airn*/*Igf2r* locus suggests that the most likely mechanism is the act of transcription in the opposite orientation through the *Ube3a* gene and promoter rather than a direct action of the *Ube3a-ATS* ncRNA itself [88, 89]. Such a model needs to be confirmed, but strand-specific RNA-sequencing experiments in mice demonstrate that the paternal *Ube3a* promoter initiates transcription but terminates prematurely, supporting a polymerase clash model [90]. In neurons, expression of *Ube3a-ATS* coincides with a large-scale chromatin decondensation of the paternal allele in the presence of transcription [87], raising the possibility that transcription drives the change in chromatin structure, although it is not known whether this transcription acts as a “layer” of the chromatin or a “player” to recruit chromatin modifying complexes.

Noncoding RNA also appears to act as an epigenetic layer at enhancers. Activation of neurons with KCl treatment results in productive transcription at thousands of enhancers to form enhancer RNAs (eRNAs) [68]. Although the function of these eRNAs is unknown, they could be acting as signaling molecules to mark active enhancers, or as a molecular tag

within the chromatin. Another possibility is that eRNAs could be part of the enhancer activity by binding Mediator in order to facilitate chromatin looping and neighboring gene activation [91].

An open question has been how CGI promoters are protected from de novo methylation during development. Recently, the formation of RNA:DNA hybrids called “R-loops” at CGI promoters was demonstrated as a protective mechanism from DNA methylation [92]. As a result, the presence of RNA as an RNA:DNA hybrid acts as an epigenetic layer that regulates the epigenetic layer of DNA methylation.

Chromatin loops and long-range interactions in neurodevelopment

Genomic chromatin loop organization is an important additional layer of epigenetic regulation containing several sub-layers at different scales (Figure 4). For example, the packaging of approximately two meters of genomic DNA into a series of “fractal globules” compartmentalizes DNA into open and closed chromatin loop regions in mammalian nuclei [93]. However, starting at the level of individual genes, chromosome conformation capture studies combinined with massively parallel sequencing have revealed complex looping interactions between enhancers and promoters. The CREB binding protein (CBP, encoded by *CREBBP*) is responsive to neuronal activity by recruiting RNA Pol II to enhancers such as *Arc* [68] (Figure 4A). As mentioned above, the chromatin factors CTCF and cohesin (SMCA1) mediate chromatin looping interactions between distal enhancers and alternate promoters of the protocadherin alpha gene locus [50]. Furthermore, in perhaps the most comprehensive study to date in non-neuronal cells, complex looping interactions were observed between transcription start sites in promoters and distal elements up to 120 kb away, with positive effects on gene expression [94].

Next, on a broader scale sub-level of chromatin organization, chromatin looping can organize genomic regions into functional domains. While enhancer-promoter looping interactions are typically in the 1–100 kb range, the genome appears to be organized into a series of larger topologically grouped domains. Evidence for this comes from the chromatin structure assay called HiC, a derivative chromosome conformation capture (3C) assay, performed in non-neuronal cell lines. From this genome-wide analysis, a series of megabase (Mb) sized chromatin looped “topological” domains were observed bound by CTCF, transfer RNAs and retro-transposons [95]. Remarkably, these topological looped domains were conserved across cell types and between species. As a positive control, results from an earlier analysis of looping patterns in the *HoxA* cluster (Figure 4B) were confirmed that organize this locus into two functional domains [96]. The identification of megabase-sized looped chromatin domains is also supported by results using an independent ChIA-PET technique which mapped CTCF looped domains in ES cells, and identified a portion of these that contained repressed genes located at the nuclear lamina [97]. As with enhancer-promoter loop interactions, chromatin domains have been characterized primarily in non-neuronal cells, but the mapping of regulatory elements in multiple tissues supports the hypothesis that the neuronal genome is also subdivided into a series of co-regulated domains [97].

Although stable topological domains may be common in multiple cell types, the additional epigenetic mechanisms that specify gene expression in neurons may not occur at this layer of chromosomal organization. In the case of specific neuronal genes, regulation may occur at the level of long-range multi-megabase looping interactions. The first example of regulation by long-range chromatin looping was shown at the human 15q11–13 locus [98] (Figure 3C). In this study, chromosome conformation capture on microarray (4C) analysis in neurons revealed that the Prader-Willi imprinting control region forms a series of long-range

interactions with loci throughout 15q11–13. One of these interactions was observed over 7 megabases between the Prader-Willi ICR and the *CHRNA7* locus (encoding nicotinic acetylcholine receptor alpha 7 subunit). Furthermore, deficient MeCP2 expression in Rett and autistic brain cortices was correlated with reduced *CHRNA7* expression [98] suggesting that MeCP2 and perhaps the CTCF/cohesin complex [99] are involved in this long range loop interaction (Figure 3C) that was previously identified at the *Gtl2/Dlk1* locus [39]. MeCP2 and CTCF also were shown to form long-range complexes with both ATRX and cohesion [39], which are implicated in human neurodevelopmental disorders. A prime example of tissue-specific epigenetic regulation by chromatin looping was shown by MeCP2 ChIP-loop analysis of the striatum where MeCP2 anchoring and silent chromatin looping prevented the ectopic expression of *Dlx5* [100]. In summary, multiple levels of chromatin looping constitute the epigenetic layer of chromatin organization that contribute to neuronal gene expression and phenotype.

Higher-order nuclear organization of neuronal nuclei

The last epigenetic layer is one that can be observed at the light microscopic level as the high degree of nuclear architechiture in neurons (recently reviewed in [101]). One form of nuclear compartmentalization is the existence of chromosome territories and the propensity of a genomic region to associate more readily with other regions from the same chromosome as compared to direct inter-chromosomal interactions [93, 102]. Interestingly, chromosome territories defined by DNA fluorescence in situ hybridization (FISH) also appear to exhibit higher-order organization with exons of some genes appearing to be present beyond the periphery of their chromosome territories [103]. Such higher-order organization could relate to the formation of transcription factories, which are thought to facilitate transcription of large domains or repeated transcription events on gene loops [104]. However, debate exists as to whether the formation and localization of transcription factories are a consequence or a determinant of transcription [105]. Furthermore, transcription factories are defined as distinct foci of RNA Pol II by immunofluorescence and active transcription, and investigation of transcription factories alone does not address the additional layer of chromosome-wide domains of association and transcription regulation.

Some chromosomal organizations appear to be non-random in neuronal nuclei, including the homologous pairing of maternal and paternal alleles of human 15q11–13 loci in differentiated neurons that is disrupted by loss of MeCP2 [106] or maternal duplication of chromosome 15q [99, 107]. In mouse ES cells and other tissues, homologous pairing is observed in a subset of both imprinted and nonimprinted gene loci and corresponds with transcriptional activity [108]. Therefore, it may not be the specific epigenetic marks at imprinted loci *per se* that are driving non-random interactions within neuronal nuclei, but the transcriptional events and locations in common between chromosomal loci containing complex epigenetic regulation at multiple layers.

Concluding remarks

In conclusion, an emerging theme in common to all the epigenetic layers is that they are more likely to be the consequences of past transcriptional events rather than simple signposts of current transcriptional activity. But by poising specific genes either positively or negatively for transcriptional responsiveness to later activity, epigenetic layers and players can have profound effects on phenotype, as evident from the multiple human diseases with specific deficiencies (Table 1). New technologies have emerged for visualizing and cataloguing the epigenetic layers at genome-wide resolution that will be important to utilize in neuronal systems and brain tissue (Table 2). Perhaps by viewing the epigenetic layers through the lens of cellular historians, we can more thoughtfully interpret

the evidence laid down in multiple layers to find more long-lasting treasures relevant to human neurodevelopmental disorders (Box 2). Since by definition, an epigenetic "metastable" state means that the state is precarious and thereby changeable using the proper conditions, epigenetic layers are strong candidates for targeted therapeutic strategies for neurodevelopmental disorders such as autism, schizophrenia, and intellectual disability.

Box 2

Outstanding questions

- Why do partial methylated domains exist and how are they important for developing neurons?
- How and why do neurons change methylation patterns throughout life?
- How does a threshold of transcriptional activity in neurons set up a pattern of epigenetic memory and transcriptional responsiveness of a gene after the initial stimulus is gone?
- What is the "MeCP2 code" of isoform differences and post-translational modifications?
- Which noncoding RNAs provide specificity and/or long-lasting memory to epigenetic mechanisms in neurons?
- How do lncRNAs function in neurons, and what role do they have in the pathogenesis of neurodevelopmental disorders?
- How do genomic copy number variations affect epigenetic layers, such as DNA methylation and large-scale chromatin organization, in human neurodevelopmental disorders?
- How are chromatin topological domains formed and maintained across cell type and species?
- How can therapies shift epigenetic states?
- How does the dynamic epigenetic state fit together with dynamic transcriptional loops?

Acknowledgments

We thank the UC Davis Epigenome Club for helpful discussions and Dr. Diane Schroeder for critical reading of manuscript. Funding for research provided by the National Institutes of Health, Department of Defense Congressionally Mandated Directed Research Program, International Rett Syndrome Foundation, and the Prader-Willi Syndrome Foundation.

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Highlights

- Epigenetic modifications are long-lived layers of past transcriptional events.
- The relationship of DNA methylation to transcription depends on genomic context.
- Noncoding RNAs act as epigenetic layers and players influencing chromatin states.
- Chromatin loops and long-range interactions are observed in neurodevelopment.

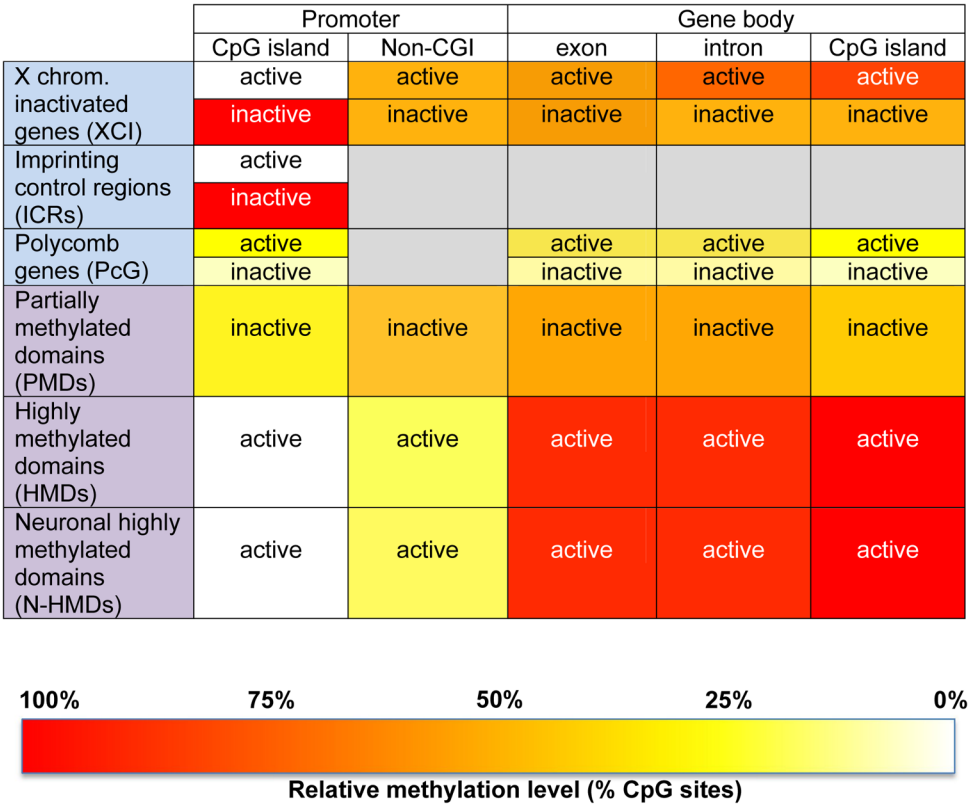

Figure 1. The context of sequence, transcription, and location in DNA methylation

For the purpose of demonstrating the importance of genetic context in interpreting DNA methylation levels, genes are broken down into identifiable parts, with promoters separated as either CpG islands or non-CpG islands (non-CGI), and parts of the "gene body" (defined as the genomic locus from transcription start to end sites) separated as exons, introns, or non-promoter CpG islands. The methylation levels for each of these genomic locations are color-coded according to the heat map shown at the bottom, with red representing the highest methylation levels and white representing the lowest. Grey shading represents unrepresented or unknown categories. Rows with headings labeled in blue represent different subcategories of genes or controlling regions with distinct methylation patterns from other genes in the genome. Rows with headings labeled in purple represent the different whole genome landscape categories of partially methylated domains (PMDs, <70% methylated CpGs) [12], highly methylated domains (HMDs, >70% methylated CpG at non-CGIs) [3], or neuronal HMDs (N-HMDs, HMD in neuronal SH-SY5Y cells, but PMD in IMR90 fibroblasts and placenta) [24].

A

Chromatin state color code	Histone methylation	Histone acetylation	Transcript	Protein binding
Active promoter	H3K4me2/me3	H3K27, H3K9		
Weak Promoter	H3K4me/me2/me3	H3K9		
Inactive/poised promoter	H3K27me3, H3K4me/me2/me3			
Strong enhancer	H3K4me/me2/me3	H3K27, H3K9	low	
Weak/poised enhancer	H3K4me/me2		low	
Transcriptional elongation	H3K36me3, H4K20me		high	
Weak transcribed			moderate	
Polycomb-repressed	H3K27me3			
Heterochromatin, low signal				Nuclear lamina
Insulator				CTCF

B

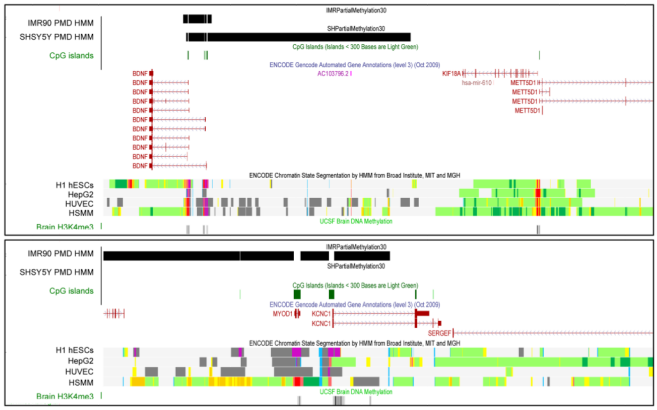

Figure 2. Chromatin states defined by histone modifications and protein binding

A. A color-coded guide to the chromatin state maps derived from hidden Markov model (HMM) segmentation of histone modifications and CTCF binding sites in non-neuronal human cell lines from the human ENCODE project [36]. Histone methylation and acetylation was analyzed by ChIP-seq using antibodies to the specific modifications and sites listed. Transcript analysis was determined by RNA-seq on the same cell lines. Genes involved in developmental and tissue-specific functions exhibit H3K27 trimethylation (H3K27me3) blocks characteristic of polycomb-repressed genes (dark grey) and have inactive but "poised" bivalent promoter states (purple) of both silent (H3K27me3) and active (H3K4me3) histone modifications in human embryonic stem cells (hESCs).

B. Two examples of chromatin state maps combined with PMD state maps in the UCSC Genome Browser for neurologically relevant genes. PMDs mapped from HMM analysis of MethylC-seq data in fibroblast (IMR90) or neuronal (SH-SY5Y) cell lines [3] are shown as black bars, with gaps at CpG islands (green) because they were removed from the PMD analysis. ENCODE tracks (middle) include annotated genes and chromatin state segmentation by HMM color coded as in **A.** While neuronal tissue was not included in the preliminary ENCODE analysis of chromatin states, a track of "Brain H3K4me3" is shown at the bottom that identifies active genes in human adult brain. Notice the difference in chromatin states between regions covered by tissue-specific PMDs (*BDNF*, *MYOD1*, *KCNC1*, inactive poised promoter, polycomb-repressed, and heterochromatin) compared to genes with HMD in all tissues (*METT5D1*, *KF18A*, active promoter and transcribed). Also, notice the difference in chromatin states at the *MYOD1* locus in human skeletal muscle myotubule (HSMM) cells compared to embryonic stem cells (H1 hESCs, inactive/poised promoter) and liver (HepG2) or umbilical vein epithelial cells (HUVEC) which have the H3K27me3 marks of polycomb repression. Both *MYOD1* and *KCNC1* are transcriptionally active in brain (H3K4me3 promoter peaks) and are within a neuronal highly methylated domain (N-HMD, defined as PMD in IMR90 but HMD in SH-SY5Y cells). In contrast,

BDNF is inactive but poised for transcriptional activity differentially at different promoters and in cell lines and the alternative *BDNF* promoters are within a PMD in both SH-SY5Y and IMR90 cells. The nuclear lamina, a heterochromatic protein matrix at the nuclear periphery made of lamin and scaffold proteins, overlaps with both PMDs and light grey “off the map” locations in the chromatin state maps. CTCF (blue) shows multiple distinct sites genome-wide that are both intergenic and close to promoters and ubiquitous as well as tissue-specific.

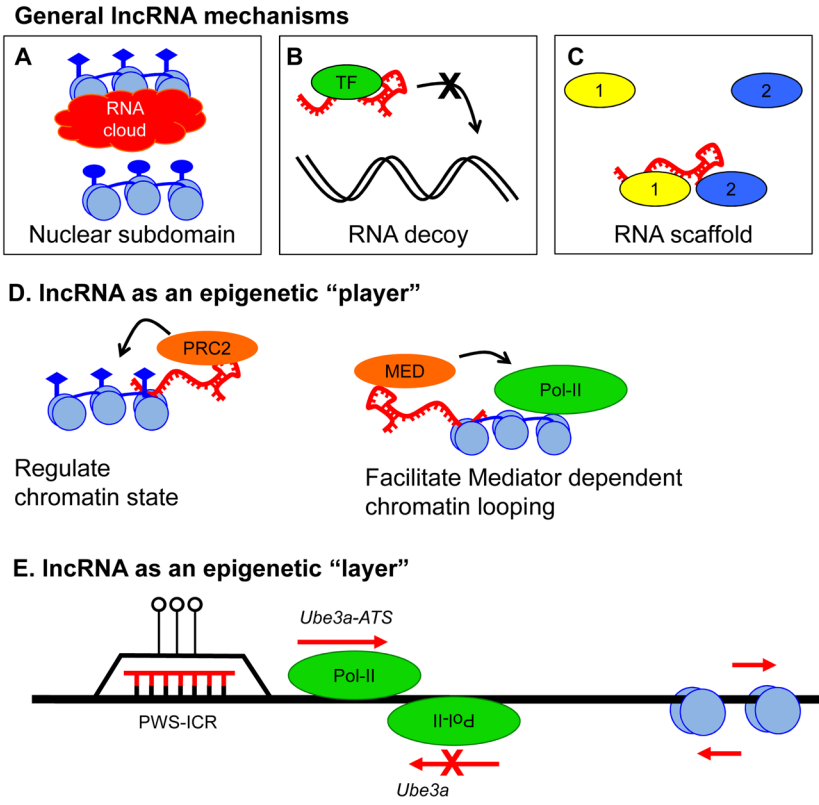

Figure 3. Noncoding RNA as an epigenetic layer and player

A. LncRNAs can function to demarcate a nuclear subdomain by forming and "RNA cloud" (red), as seen for *Xist* and *Kcnq1ot1*, in order to coat chromosomes or genomic regions (blue) and regulate transcription [60]. Loci coated by the RNA cloud are most often silenced via recruitment of repressive chromatin complexes, but RNA clouds may also upregulate transcription [80]. **B.** LncRNAs (red) also act as molecular decoys either for DNA binding transcription factors (*Gas5*) or miRNAs (*PTENP1*). **C.** LncRNAs can have multiple protein interacting partners and thereby act as a molecular scaffold for larger complexes. **D.** In recent years a novel role for lncRNAs as epigenetic "players" has been described. Many lncRNAs were found to interact with the repressive chromatin modifier PRC2 [64, 79], and thereby regulate the chromatin structure of large domains, or even of whole chromosomes. Recently, activating lncRNAs were also described that bind to the Mediator complex and facilitate enhancer-promoter loops [3]. This function may potentially overlap with enhancer RNAs (eRNAs) which are transcribed bidirectionally from enhancers [68]. **E.** RNA also functions as an epigenetic "layer" by modifying the structure of the DNA or regulating sense transcription via antisense transcription. Such a role has been described at the neurodevelopmentally critical PWS/AS locus on chromosome 15q11-q13, where RNA:DNA hybrid (R-loop) formation at the Prader-Willi imprinting control region (PWS-ICR) protects against DNA methylation [8], and processive transcription to produce *UBE3A-ATS* silences paternal *UBE3A* in neurons [89]. Similarly, eRNAs may act as molecular signals to mark active enhancers in response to stimulation [68].

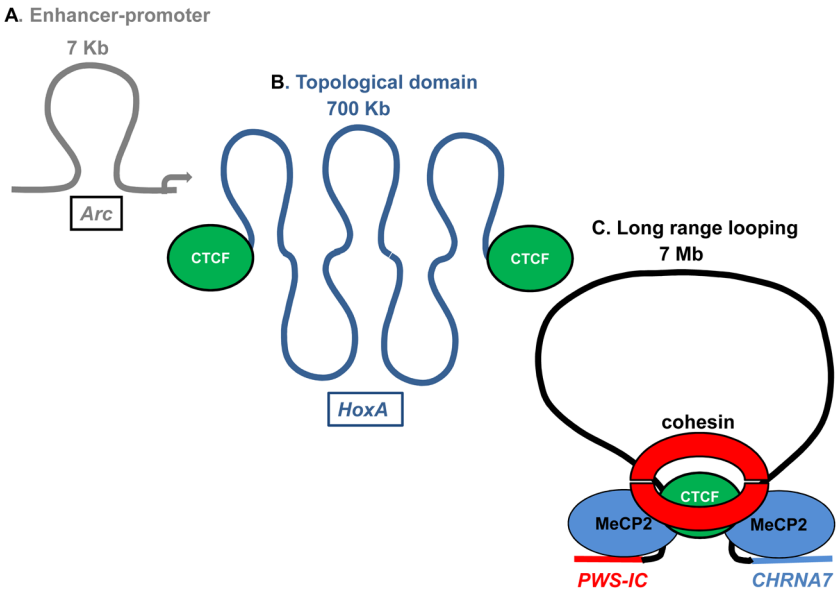

Figure 4. The epigenetic layer of chromatin looping is composed of at least three sublayers

A. At the sub-layer of specific transcription units, enhancers and other distal units make contact with gene promoters such as *Arc,* a neuronal factor required for the regulation of AMPA receptors, via kb scale chromatin loops. Pol II (red) is recruited by enhancers where transcription initiates before transfer by looping to the *Arc* promoter [68]. **B**. Chromatin domains up to 1 Mb organize chromosomes into a series of functional units in mammalian cells. The insulating factor CTCF (green) regulates formation and maintenance of this intermediate layer in the *HoxA* locus and other domains genome wide [96]. **C**. Long range, multi-megabase chromatin loop interactions regulate gene expression between distant loci above the domain layer. At this layer, the PWS-IC (red) contacts the locus encoding *CHRNA7* (blue) via a 7 megabase looping interaction via MeCP2 and potentially CTCF (green) and cohesion (red) [98, 99]. This long-range interaction modulates expression of *CHRNA7* and other neurologic genes in 15q11–13 [98].

Table 1

Epigenetic layers and players and their human disease associations

	Full name and function	Human neuronal disease association	Refs
Layers	**Modifications of DNA or chromatin**		
5hmeC	5-hydroxymethylcytosine		[13]
5mC	5-methylcytosine		[14, 15]
PMD	Partially methylated domain		[12]
HMD	Highly methylated domain		[3]
N-HMD	Neuronal highly methylated		[3, 24]
ICR	Imprinting control region	Prader-Willi, Angelman	[20, 21]
PcG	Polycomb group gene		[36] [24]
H3K27me3	Histone H3 trimethylation at lysine 27		[36]
H3K4me3	Histone H3 trimethylation at lysine 4		[36]
H3K36me3	Histone H3 trimethylation at lysine 36		[36]
H3K27ac	Histone H3 acetylation at lysine 27		[36]
H3K9ac	Histone H3 acetylation at lysine 9		[36]
CGI	CpG island, nonrandom cluster of CpG sites, association with gene promoters		[92]
R-loop	RNA/DNA hybrid formed at transcribed regions with high G skew, protects active promoter CGIs from DNA methylation		[92]
eRNA	Enhancer RNA, noncoding RNAs transcribed from active enhancers, implicated in long- range looping interactions		[68]
antisense RNA	Noncoding RNA transcribed in the opposite strand orientation from a protein coding gene, inhibition of transcriptional elongation of sense transcript		[70] [91]
Players	**Enzymes or protein complexes that direct epigenetic layers**		
DNMT1	DNA methyltransferase 1, maintenance DNA methylation		[25] [27]
DNMT3A	DNA methyltransferase 3A, *de novo* DNA methylation		[26] [28]
DNMT3B	DNA methyltransferase 3B, *de novo* DNA methylation	immunodeficiency, centromeric instability, facial dysmorphism (ICF)	[109]
PRC1, PRC2	Polycomb repressive complex 1 and 2, regulation of polycomb group genes		[64, 79]
EZH2	Enhancer of Zeste, *Drosophila* homologue 2, methyltransferase for H3K27		[64, 79]
MeCP2	Methyl CpG binding protein 2, abundant nuclear factor in mature neurons, activity- dependent transcriptional responses	Rett syndrome, *MECP2* duplication syndrome	[56] [110]
ATRX	Nuclear factor with Zinc finger, ATPase, and helicase domains, similarities to SNF2H chromatin remodeling protein	Alpha-thalassemia/Mental retardation, X linked	[111]
BAF53b	Actin-like component of neuronal chromatin remodeling complex, implicated in synaptic plasticity		[41] [42]
ARID1B	Component of neuronal BAF chromatin remodeling complex	Coffin-Siris syndrome	[40, 112]
JMJD1C/TRP8	Histone demethylase for H3K9, hormone-dependent transcriptional activation	X-linked intellectual disability, autism	45 [113]
KDM5C/JARD1C	Histone demethylase of H3K4, gene repression	X-linked intellectual disability, autism	[47, 114]

	Full name and function	**Human neuronal disease association**	**Refs**
SMCA1/cohesin	Cohesion, structural maintenance of chromosomes 1A, chromatin looping	Cornelia de Lange	[115]
Mediator	Multiprotein complex and general regulator of transcription, transcriptional coactivator, chromatin looping, activating-RNA binding		[91]
CTCF	CCCTC-binding factor, chromatin insulator, chromatin looping		[51] [50]
CREBBP (CBP)	CREB binding protein, H3K56 acetyltransferase, activity-dependent transcriptional responses	Rubinstein-Taybi syndrome	[116] [117]
TET1	TET oncogene, family member 1, methylcytosine dioxygenase, converts 5mC to 5hmC resulting in demethylation		[14, 15]
MLL1	Mixed lineage leukemia 1, H3K4 methyltransferase	Wiedemann-Steiner syndrome	[118]
lncRNA	Long, non-coding RNA, >200nt with low-protein coding potential		[72] [73]
Xist	X chromosome inactivation specific transcript		[60]

Table 2

Methods utilized for identifying epigenetic layers and players

Method abbreviation	Method description	Refs
ChIP-seq	Chromatin immunoprecipitation with high throughput sequencing to identify DNA bound to target proteins	[36]
RNA-seq	RNA-sequencing to identify genome-wide transcriptome	[36]
3C, 4C	Chromosome conformation capture (3C) on microarray (4C)	[98] [39]
HiC	3C variant interrogating genome wide with high throughput sequencing	[95] [96]
HMM	Hidden Markov model, a computer learning tool used for defining and segmenting chromatin and methylation states based on sequencing data	[36] [3]
MethylC-seq	Methyl cytosine bisulfite conversion plus high throughput sequencing	[3, 12]
FISH	Fluorescent *in situ* hybridization to characterize the subcellular localization and expression of RNAs and homologous pairing of chromomsomes	[60] [86] [87]
ChIA-PET	Chromatin interaction analysis with paired end tag sequencing	[97]
ChIP-loop	Chromatin immunoprecipitation combined with loci specific PCR	[100]