The 3D Genome in Brain Development: An Exploration of Molecular Mechanisms and Experimental Methods

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ABSTRACT: The human brain contains multiple cell types that are spatially organized into functionally distinct regions. The proper development of the brain requires complex gene regulation mechanisms in both neurons and the non-neuronal cell types that support neuronal function. Studies across the last decade have discovered that the 3D nuclear organization of the genome is instrumental in the regulation of gene expression in the diverse cell types of the brain. In this review, we describe the fundamental biochemical mechanisms that regulate the 3D genome, and comprehensively describe in vitro and ex vivo studies on mouse and human brain development that have characterized the roles of the 3D genome in gene regulation. We highlight the significance of the 3D genome in linking distal enhancers to their target promoters, which provides insights on the etiology of psychiatric and neurological disorders, as the genetic variants associated with these disorders are primarily located in noncoding regulatory regions. We also describe the molecular mechanisms that regulate chromatin folding and gene expression in neurons. Furthermore, we describe studies with an evolutionary perspective, which have investigated features that are conserved from mice to human, as well as human gained 3D chromatin features. Although most of the insights on disease and molecular mechanisms have been obtained from bulk 3C based experiments, we also highlight other approaches that have been developed recently, such as single cell 3C approaches, as well as non-3C based approaches. In our future perspectives, we highlight the gaps in our current knowledge and emphasize the need for 3D genome engineering and live cell imaging approaches to elucidate mechanisms and temporal dynamics of chromatin interactions, respectively.

KEYWORDS: Neurogenetics, synaptogenesis, epigenetics, 3D genome, neural development, gene regulation, Alzheimer's, genomics, schizophrenia, autism, bipolar disorder

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Structural Features of the 3D Genome

Eukaryotic genomes consist of large DNA molecules that are efficiently folded into small nuclei to organize genetic information in 3-dimensional space, allowing dynamic temporal regulation of gene expression. DNA is wrapped around complexes of histone proteins called nucleosomes that are further condensed into chromatin fibers. Chromatin fibers are compacted into chromosomes, which are positioned within distinct nuclear regions called chromosome territories. Studies using chromosome conformation capture (3C) based methods have elucidated other complex features of 3D chromatin structure.[1-7] Chromosomes are segregated into two distinct compartments, called A and B, which are enriched for active and repressed chromatin, respectively.[4] The genome is also partitioned into self-interacting insulated neighborhoods called topologically associated domains (TADs), whose borders are bound by the DNA binding architectural protein CTCF.[6] TADs are thought to be formed through a process of loop extrusion, which involves the loading of the ring-shaped protein cohesin onto DNA, followed by ATP-driven translocation of cohesin that is ultimately blocked in both directions by CTCF at sites with inward facing CTCF binding motifs.[8,9] Earlier studies assumed a hierarchical model of 3D genome organization in which TADs were considered to be smaller genomic blocks within broad compartments[6,10] (Figure 1A). However, cohesin depletion experiments and biophysical simulations have shown that the process of loop extrusion disrupts fine scale compartmentalization, but strengthens TADs.[11,12] Recent work shows that compartments can indeed be very fine scaled at only a few kb in size.[13,14] Therefore, the relationship between different features of the 3D genome is not static and hierarchical, but dynamic and modulated by complex biochemical processes (Figure 1B).

Various models of phase separation have been proposed to explain the mechanisms driving chromatin compartmentalization, but the details have not been fully elucidated.[12,15-19] Liquid-liquid phase separation (LLPS) can occur when proteins form multivalent interactions with each other via intrinsically disordered domains that have low amino acid complexity, resulting in a liquid droplet that is separated from the surrounding aqueous environment in a membrane-less manner.[17]

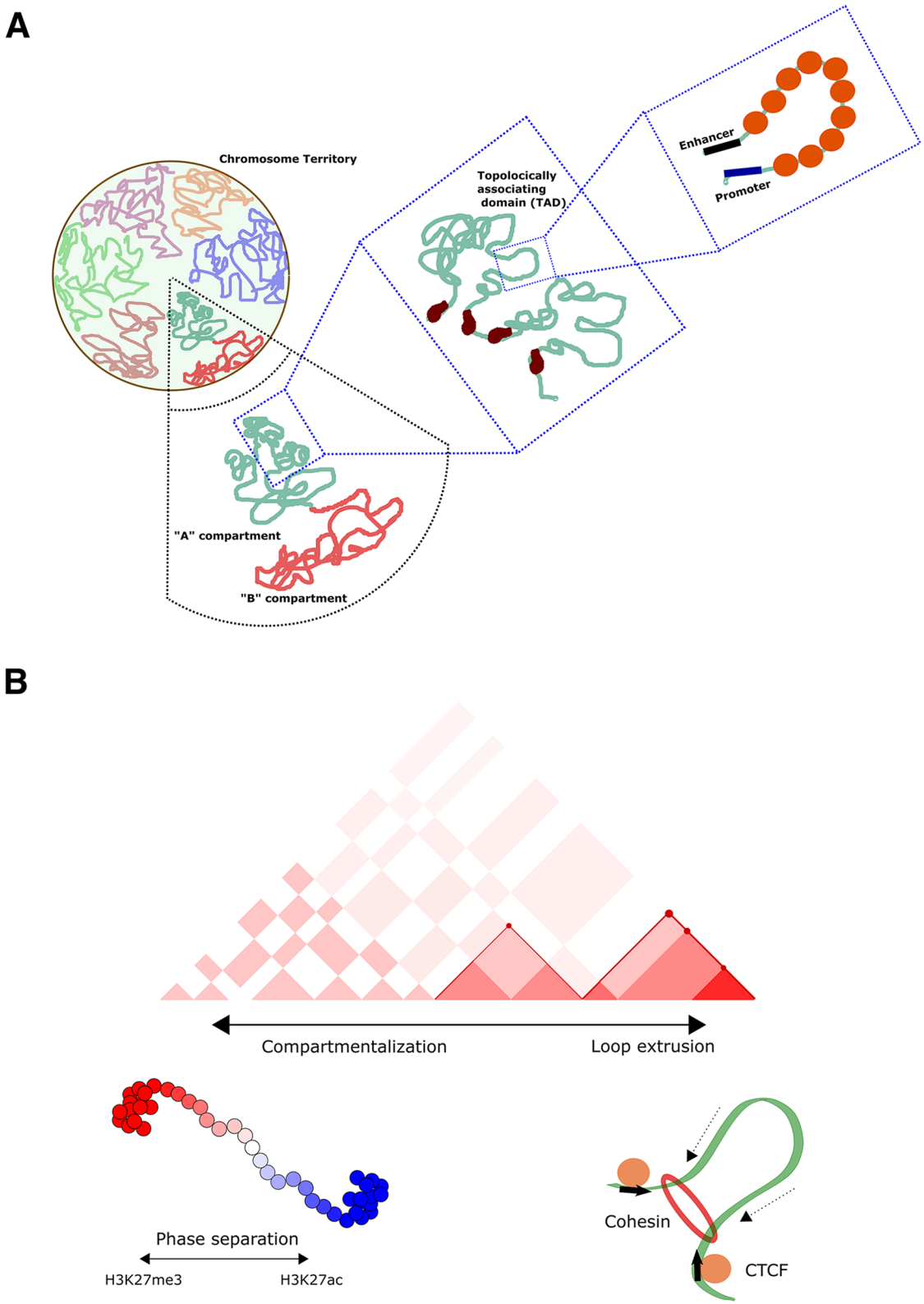

Figure 1. (A) Hierarchical organization of the 3D genome and (B) biophysical forces that drive chromatin folding.

Polymer-polymer phase separation (PPPS) occurs when proteins act as bridging factors to link different segments of chromatin.[17]

Multiple lines of evidence show LLPS as a potential driver of both constitutive and facultative heterochromatin compartmentalization.[15,16,19-21] HP1, a protein that binds to H3K9me3 marked nucleosomes and induces the compaction of the underlying repressed constitutive heterochromatin, was shown to have the ability to phase separate into liquid droplets.[15,16,19] Notably, DNA and nucleosomes can be partitioned into these droplets, suggesting a potential role for phase separation in heterochromatic gene silencing.[15] Polycomb repression may

also involve LLPS, as the Polycomb Repressive Complex 1 (PRC1), which binds to the facultative heterochromatin associated H3K27me3 modification deposited by Polycomb Repressive Complex 2 (PRC2), contains the protein CBX2 that is able to phase separate via a low complexity disordered region.[20] Interestingly, mutations of charged residues within this disordered region that disrupt phase separation in vitro and the formation of puncta in cells were also shown to inhibit chromatin compaction and disrupt proper body patterning during mouse development in previous studies.[22,23] Therefore, phase separation mediated heterochromatin compartmentalization could play an important role in the maintenance of gene repression that is required for proper development.

Although LLPS has been more extensively studied in the context of heterochromatin, euchromatin compartmentalization may involve similar phase separation mechanisms via disordered regions in proteins that are implicated in transcription activation.[24-26] In vitro experiments demonstrated that transcriptional co-activator BRD4 binds to acetylated histones, inducing phase separation of chromatin into liquid droplets that are demixed from unmodified chromatin.[24] Furthermore, both BRD4 and MED1, a subunit of the Mediator complex that links enhancers with promoters, were shown to form nuclear puncta around super enhancer clusters, exhibiting liquid condensate like properties.[25] These liquid condensate properties were required to maintain BRD4, MED1, and RNA pol II occupancy at super-enhancer loci. Furthermore, BRD4 and MED1 were shown to contain intrinsically disordered domains that allow them to phase separate. RNA pol II was also shown to have the ability to phase separate into liquid droplets in vivo via its low complexity C-terminal domain, creating RNA Pol II hubs.[26] Notably, individual RNA pol II enzymes are released from these hubs following transcription initiation associated phosphorylation of the CTD, suggesting that promoters coalesce around RNA pol II hubs prior to gene activation. Altogether, LLPS is an attractive model that can potentially explain the formation of the active A and repressed B compartments discovered from 3C-based studies. However, it has been debated whether LLPS is the major driver of compartmentalization, as other mechanisms such as polymer-polymer phase separation or processes distinct from phase separation may explain nuclear puncta formation.[18,27,28] Therefore, further robust biochemical tests are required to discount alternative hypotheses. Furthermore, although compartmentalization correlates with gene regulation, the functions of long range compartmental interactions are poorly understood.[18]

Improvements to the original HiC (high throughput chromosome conformation capture) methodology, along with deeper sequencing, helped to resolve finer features of the 3D genome, revealing the presence of chromatin loops within TADs, many of them linking enhancers with promoters.[7] Disruption of TAD boundaries can lead to aberrant enhancer-promoter loops across TADs, while disrupting the enhancer promoter loops within TADs, resulting in transcription misregulation.[10,29] Early work suggested that TADs are mostly conserved across human cell types, and conserved across evolution from mouse to human as well.[6] However, analysis of HiC datasets across multiple studies showed considerable variability in TAD structure between tissues and cell types suggesting the biological importance of TADs in cell type specific gene regulation.[30] The extent of evolutionary conservation of TADs has also been debated, as much of the evidence is anecdotal, focusing on a few candidate loci, or extrapolated from the conservation of other genomic features that are correlated with TADs.[31] Although TADs were initially described as non-overlapping megabase-scale features of the 3D genome, deeper sequencing reveals TADs at smaller scales, often nested within larger TADs.[7,32] Furthermore, different studies have used different algorithms to identify TADs, implying that there may be some computational bias that can obscure any meaningful biological interpretation.[33] The complexity of TAD organization as well as differences in sequencing depths and algorithms across studies makes inter-species comparisons of TAD structure challenging.

Nonetheless, evolutionary studies have revealed interesting insights on both conserved and divergent TAD structures. A study on human and *Drosophila* HiC data showed that clusters of conserved noncoding elements (CNEs) called genomic regulatory blocks (GRBs) are associated with TAD boundaries, allowing them to infer that at least a subset of TADs may be considered as evolutionarily ancient features.[34] Another study on mouse, macaque, rabbit, and dog showed that the conservation of TADs across species is correlated with conserved CTCF binding sites with inward facing motifs.[35] Divergent CTCF motifs were shown to be associated with species specific intra-domain loops within the broader conserved TADs. A recent study comparing TAD boundaries across human, primate, and rodent species found that a much smaller proportion of them were strongly conserved than previously assumed.[36] Ultraconserved TAD boundaries may have been established in an ancient common ancestor, and they were shown to be more strongly insulated with higher CTCF enrichment than newly evolved species specific TAD boundaries.[36] Therefore, the structural and functional characteristics of TADs may have been modified across evolution.

In this review, we discuss the roles of the 3D genome in gene expression regulation in the brain, which is a complex organ with multiple cell types. In particular, we discuss mechanistic insights on 3D chromatin structure and transcription regulation during neuronal development, and its relevance to psychiatric disease. We take a comprehensive view of the 3D genome, discussing the biological importance of compartments, TADs, and loops. We also explore single cell multi-omics approaches that simultaneously investigate the 3D

genome along with transcription or epigenetic modifications such as DNA methylation as well as non 3C based genomics and imaging methodologies, which have all provided new insights. We conclude by discussing future directions for 3D genome research, which should move beyond descriptive studies and focus on 3D genome engineering approaches to obtain functional insights as well as live cell imaging approaches to monitor chromatin interaction dynamics and transcription in real time.

Early In Vitro Studies of Chromatin Conformation Changes During Early Neural Development

During neural development, the 3D genome is remodeled at the levels of A/B compartments, TADs, and enhancer-promoter loops.[37] A study on mESCs (mouse embryonic stem cells) differentiated into NPCs (neural progenitor cells), and then to neurons, demonstrated that most TAD boundaries remain stable, while interactions between TADs and within TADs (at the sub-TAD level) change during differentiation.[38] Importantly, interactions between TADs produce higher order meta-TAD structures that are correlated with gene expression and epigenomic features such as RNA Pol II-Ser5-phosphorylation, which marks promoters primed for transcriptional activation, as well as H3K27me3 enrichment. Thus, both gene activation and polycomb repression mechanisms are associated with higher-order genome topology. Differentiation of hESCs into 4 different lineages, including NPCs, show genome-wide compartment switching of genes from A-B or B-A, and increased intra-domain interactions between upregulated genes that are enriched in active histone marks.[39] Interestingly, although there is a high degree of plasticity in A and B compartments during early development, the effects on gene expression are subtle, suggesting that while compartments contribute to gene regulation, they don't necessarily play a deterministic role in cell type specific expression patterns.[39] Similar to what was shown during mESC differentiation, most TAD boundaries appear to be stable during hESC differentiation. Intriguingly, a later study investigating changes in 3D genome architecture during mouse neural differentiation both in vitro and ex vivo using ultra-deep HiC revealed many cell-type specific 3D chromatin features, including de novo TAD boundaries at transcriptionally active neural specific genes.[40,41] Furthermore, neural differentiation induces the formation of contacts between neural transcription factor bound sites, as well as enhancer-promoter loops. Thus, obtaining higher resolution HiC contact maps allowed to elucidate regulatory features at finer scales of the 3D genome during neural differentiation. A study on the roles of architectural proteins during early mouse neural lineage commitment provided further mechanistic insights on 3D genome remodeling during the earliest stages of neural differentiation.[42] Notably, differentiation from ESCs to NPCs leads to the loss of CTCF occupancy at many sites, concurrent with the loss of CTCF associated loops, but a subset of CTCF occupied sites persist in NPCs with minimal gain of de novo CTCF binding sites. Many of the looping interactions gained during the ESC to NPC transition link NPC specific enhancers to target genes and were shown to be mediated by the transcription factor YY1 (ying yang protein 1). YY1 mediated loops often occurred within broader CTCF anchored domains, showing that constitutive TADs already present in the pluripotent state can serve as a framework to facilitate nested regulatory interactions during neural differentiation.[41,42]

3C Studies in Human Tissue Illuminating the Etiology of Psychiatric and Neurological Disorders

Studies in human tissue initially used the 3C approach to investigate chromatin loops at specific genes involved in neuronal function and implicated in psychiatric disease. For example, an early study discovered a loop that is evolutionarily conserved from mouse to human between a distal noncoding region and the promoter of *GAD1*, a gene involved in the synthesis of the inhibitory neurotransmitter GABA.[41,43] The loop was induced in an activity dependent manner during neural differentiation, and was correlated with GAD1 activation. Interestingly, another study described a hub of activity-dependent loops in mouse and human prefrontal cortex targeting *GRIN2B*, which encodes the NMDA glutamate receptor.[41,44] It was later discovered that common risk variants associated with schizophrenia are primarily located within distal noncoding loci that are predicted to act as enhancers.[41,45] In particular, one noncoding risk variant was shown to downregulate expression of the calcium channel gene *CACNA1C* and is located within an enhancer that was shown by 3C to physically interact with the *CACNA1C* promoter.[41,45]

Several recent studies have explored the roles of the 3D chromatin interactome in the human brain on a genome-wide level.[41,46-49] A study in human fetal brain that integrated Hi-C data with noncoding variants from schizophrenia GWAS revealed that most schizophrenia associated single nucleotide polymorphisms (SNPs) interact with distal genes that are involved in pathways related to neuronal development.[46] Furthermore, CRISPR editing in neural progenitors of an enhancer containing a schizophrenia associated SNP that physically interacts with *FOXG1*, a gene encoding a transcription factor implicated in early brain development, led to downregulation of *FOXG1* expression.[46]

A study on human induced pluripotent stem cell (hIPSC) derived NPCs differentiated into excitatory neurons and astrocyte-like glia demonstrated the cell type specificity of the schizophrenia risk interactome.[41,50] Briefly, short-range chromatin loops are pruned out during neural differentiation while longer loops are induced, many of which link common variants associated with schizophrenia to target genes involved in neural connectivity, synaptic signaling,

and chromatin remodeling.[41,50] Schizophrenia risk associated distal enhancers were functionally validated with CRISPRi and CRISPRa.[50] Furthermore, the schizophrenia associated neuronal genes that are linked through the chromatin interactome also show co-regulation at the levels of mRNA and protein expression.[50]

Another study investigated the 3D genomes of neurons and glia isolated from adult human brains, gaining insights on cell type specific gene regulatory networks associated with brain disorders.[41,47] Notably, integrating the chromatin interactome and the enhancer profiles of the GABAergic and glutamatergic neuronal subtypes with risk factors for schizophrenia and bipolar disorder (BD) revealed common pathways associated with parvalbumin-expressing interneurons, but also distinct schizophrenia and BD specific pathways in deeper layer projection neurons and in upper layer neurons, respectively.[41,47] Another study discovered that cis-regulatory domains (CRDs) of H3K27ac enriched enhancers that are dysregulated in a large cohort of schizophrenia and BD brains are embedded within neuronal TADs in the prefrontal cortex.[48] These CRDs were shown to overlap with sub-TAD like features, implying the role of a nested 3D chromatin structure in dynamic gene regulation.

Cell type specific chromatin interactome analysis has also helped to elucidate the molecular mechanisms implicated in genetic variants associated with neurological disorders such as Alzheimer's.[49] Unlike psychiatric disorders, in which heritable variants are mostly found within neuronal enhancers and promoters, heritability of SNPs associated with Alzheimer's is strongest within regulatory elements in microglia, mainly enhancers.[49] Imputed causal variants of Alzheimer's were mostly found in microglia enhancers that interact with target gene promoters via long range chromatin interactions. Furthermore, CRISPR deletion of the microglia associated enhancer that interacts with the *BIN1* gene, containing one of the highest risk scoring AD variants, in human pluripotent stem cells (hPSCs) resulted in nearly complete inhibition of *BIN1* expression in differentiated microglia, but not in differentiated neurons and astrocytes.[49]

Altogether, these studies highlight the importance of investigating the 3D genome in distinct cell types in the human brain to link GWAS loci to their target genes to understand the disruption of gene regulatory networks driving both neurological and psychiatric disorders (Figure 2).

The Regulatory Roles of Different Features of the 3D Genome

Neural differentiation from hIPSCs primarily involves dynamic changes in loops rather than compartment switching.[51] Differentiation associated enhancer-promoter hubs can encompass large genomic neighborhoods, and mediate co-regulation of multiple genes.[51] Furthermore, chromatin loops outperform eQTLs (expression quantitative trait loci) as a means of predicting neurological GWAS target genes, revealing that the variability in disease relevant target mRNA expression is mechanistically linked to distal regulatory elements.[51]

A recent study characterizing cell type specific 3D epigenomes during human corticogenesis, identified promoter-centric interactions in radial glia, intermediate progenitor cells, excitatory neurons, and interneurons isolated from human mid-gestational fetal cortex.[52] Notably, it was shown that disease associated variants and transposable elements are located at distal interacting loci in a cell type specific manner. Furthermore, promoters with a high level of chromatin interactivity, named super-interactive promoters, are enriched for lineage specific genes.[52] Distal interacting regions were also functionally validated through CRISPRview, an imaging-based approach that combines CRISPRi with cell type specific immunostaining and nascent transcript quantification to elucidate cell type specific regulatory mechanisms in heterogeneous populations of primary cells.[52]

A study comprising distinct cell types in the adult brain and stages of development from fetal to adult revealed insights on epigenomic regulation at multiple scales of the 3D genome.[53] It was found that neurons have weaker A/B compartmentalization relative to non-neuronal cell types, but stronger TADs that are established during fetal development. A subset of these are associated with differential TAD boundaries enriched for active promoters that are involved in neurodevelopmental processes. Neurons also tend to be enriched for repressive B type compartments and polycomb associated loops that are mostly pre-established in the fetal cortical plate, but, across development, show an increase in enhancer-promoter loops associated with upregulation of genes implicated in synaptic activity. Furthermore, chromatin loops specific to the fetal cortical plate are enriched for autism GWAS loci, implicating the disruption of chromatin interactions during fetal development in the etiology of autism.

Studies from other groups have corroborated some of the results described above.[54,55] Notably, the hIPSC-to-neuron transition is associated with an expansion of repressive B type compartments for dopaminergic, GABAergic, and glutamatergic neuronal subtypes.[54] Developmentally regulated compartment switching was similar across these neuronal subtypes, with limited A/B compartment differences between them. Furthermore, weak compartmentalization and long range polycomb loops have also been identified in neurons sorted from the Wernicke's area of the human brain.[55] Additionally, the same study revealed higher protein levels of the cohesin component RAD21 in neurons relative to glia, which corroborates with the relative, albeit subtle, increase in RAD21 mRNA expression in neurons in the study cited previously.[53] Taken together, a likely interpretation of these findings is the involvement of a loop extrusion mechanism that disrupts compartmentalization in neurons. Furthermore, the

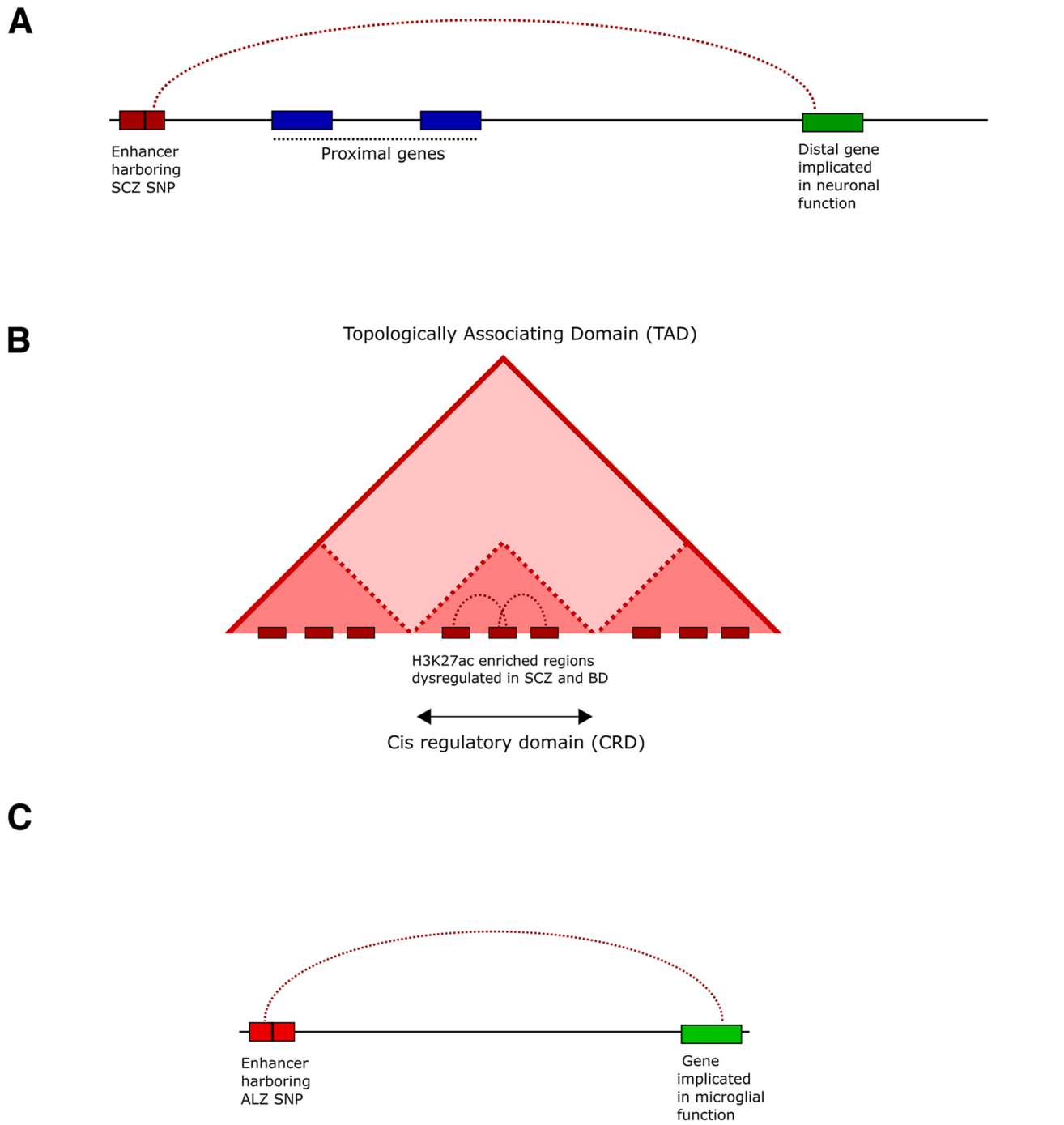

Figure 2. (A) Chromatin interactions link enhancers enriched for schizophrenia associated genetic variants with nonproximal genes involved in neuronal functions, (B) cis-regulatory domains dysregulated in schizophrenia and bipolar disorder are embedded within TADs in the prefrontal cortex, and (C) chromatin interactions link enhancers enriched for Alzheimer's disease associated genetic variants with genes involved in microglial functions.

prevalence of repressive B type compartments and polycomb associated loops in neurons reveals a previously underappreciated role of the 3D genome in maintaining gene repression during neural development. Notably, long range polycomb repressive loops were shown to be enriched for non-neural developmental genes.[53] Altogether, recent studies suggest the importance of TADs in potentially activating neurodevelopmental genes, as well as long-range polycomb repressive interactions in suppressing non-neural developmental genes.

Mechanistic Insights on the Roles of Chromatin Architectural Proteins

The role of cohesin in the regulation of expression of genes involved in neuronal maturation has been investigated in mouse neurons.[56] Importantly, RAD21 depletion disrupts CTCF anchored loops and downregulates genes involved in neuronal connectivity and activity, which results in reduced morphological complexity, as shown by reduced numbers of dendritic spines. Interestingly, RAD21 depletion was shown to

primarily affect the chromatin organization and expression of genes engaged in long range loops. The study by Powell et al[54] indicates that genes linked to distal schizophrenia risk loci tend to be enriched for cell adhesion processes, which is also one of the biological functions most enriched across genes downregulated upon RAD21 depletion in mouse neurons.[56] Rahman et al[53] also showed that genes with transcriptionally active promoters near differential TAD boundaries in neurons tend to be enriched for neurodevelopmental processes such as cell-to-cell adhesion and synapse organization. Investigation of HiC contact maps showed that these genes are associated with large loop domains. The earlier finding by Rajarajan et al,[50] which revealed the pruning out of short-range loops and gain of long-range loops during the NPC-neuron transition, also suggests a loop extrusion mechanism in the regulation of genes crucial for neural development, corroborating these findings. CTCF was shown to bind adjacent to lineage associated promoters in mouse neural progenitor cells, helping to promote interactions with distal enhancers.[57] Interestingly, Rahman et al show that while CTCF footprints occur more frequently across open chromatin regions in glia, they tend to be enriched more strongly at TAD boundaries in neurons.[53] Thus, multiple recent studies on both mouse and human neurons suggest that CTCF and cohesin play specific roles in neuronal gene regulation beyond their functions as general regulators of 3D genome architecture.

Loop extrusion may offer a mechanism to scan chromatin in 1D for target specific enhancers, allowing efficient control of gene expression, whereas enhancer-promoter communication by compartmental interaction may involve a less efficient 3D diffusion process, which could partly explain the tendency toward stronger TADs, but weaker compartments in neuronal lineage determination. Furthermore, cause and effect relationships are still poorly understood, as 3D genome structure may regulate transcription, but transcription may also regulate 3D genome structure. Notably, Rahman et al[53] showed that CRISPRi on the TSS of *CNTNAP2*, an important neurodevelopmental gene, results in decreased insulation at the TSS proximal boundary, suggesting that epigenetic and transcriptional memory could be a driver of local chromatin folding. Interestingly, although a previous study showed that mouse neural differentiation is associated with the formation of de novo TADs at transcriptionally active neuronal genes, CRISPRa did not result in increased insulation at their targeted loci.[40] Therefore, relationships between transcription and 3D chromatin structure are complex and likely to be locus specific. Several studies have now shown interactions between CTCF, loop extrusion factors, and the transcriptional machinery.[58-61] A recent study investigated the role of SATB2, a DNA binding protein that is selectively expressed in pyramidal neurons of the cortex and hippocampus, in regulating 3D genome architecture in the mouse cortex.[62] It was found that loss of SATB2 results in changes at all hierarchical levels of the 3D genome, from compartments to enhancer-promoter loops, affecting gene expression. Importantly, genes that are dependent on SATB2-mediated 3D epigenome modeling are functionally associated with cognition and contribute to the risk for neuropsychiatric and neurodevelopment disorders.[62] SATB2 was shown to induce loops both independently and in cooperation with CTCF. Altogether, further work is needed to uncover mechanistic insights on the interplay between gene expression and the 3D genome during neural development, in particular trying to decipher how transcription factors may interact with CTCF, cohesin, and the transcriptional machinery (Figure 3).

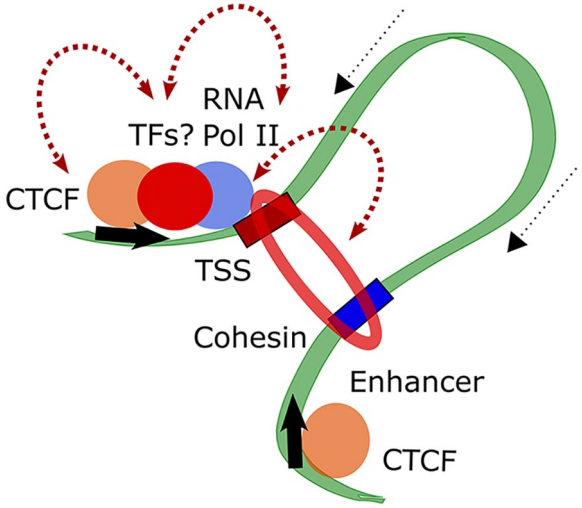

Figure 3. Mechanistic model linking neural lineage determining transcription factors with chromatin architectural proteins and transcriptional machinery.

Evolutionary Insights on Novel and Conserved 3D Genome Features in Human Brain Gene Regulation

A recent study highlighted evolutionary innovations in human 3D chromatin structure that are important for corticogenesis by comparing ultra-deep 3D genome data from fetal macaque brain with previously published data from human fetal brain[46] and mouse brain.[40,63] Human gained TADs (hgTADs) in fetal brain are enriched for evolutionarily recent transposable elements at their boundaries.[63] Genes at human specific loops are predominantly expressed in the subplate lamina of the developing brain.[63] Furthermore, human specific chromatin loops mainly comprise enhancer-enhancer interactions that form complex hubs to co-regulate multiple genes. Another recent study on the evolution of TADs across human, primate, and rodent species found that human specific TAD boundaries are enriched for pathways pertaining to the regulation of synapse assembly.[36] Synapse formation is one of the processes of brain development that distinguishes humans from other primates,[64] disruptions of which is associated with psychiatric disorders such as autism and schizophrenia. Interestingly, deletion of a human specific TAD boundary 200 kb upstream of the *AUTS2*

(Autism Susceptibility Candidate 2) locus resulted in upregulation of the gene in hiPSC differentiated dopaminergic neurons.[36] Therefore, human specific TAD boundaries may play a crucial role in the evolution of the complex human brain, and disruptions of these boundaries can potentially lead to transcription mis-regulation resulting in neurodevelopmental disorders.[36] Altogether, evolutionary innovations in human 3D chromatin structure may be associated with specific gene regulation mechanisms in the human brain.

Distinct nuclear compartments are associated with specific genome architecture features in the developing mammalian brain.[65] Notably, neural precursors from different regions of the mouse and human forebrain have differences in LAD (lamina-associated domain) architecture corresponding to their regional identity. Interestingly, LADs in the human and mouse cortex contain transcriptionally active sub-domains depleted of H3K9me2.[65] Furthermore, LADs conserved across human, mouse, and macaque brains are enriched for transcriptionally active neural genes involved in synaptic function. Therefore, evolutionarily conserved 3D genome features play an important role in human neuronal gene regulation in addition to the evolutionarily novel 3D genome features described above.

Insights From Single Cell 3C Based Methods

Historically, 3D genome studies were conducted on populations of cells, in which the data represented statistically averaged ensemble chromatin interaction patterns, thus obscuring the dynamic changes in chromatin structure in individual cells, as well as cell-to-cell heterogeneity. Several recent studies have attempted to decipher 3D chromatin structure along with epigenetic signatures in individual cells using single cell 3C/HiC multiome approaches.[66-70] Notably, a study in 2019 developed a single cell multi-omics approach called single nucleus methyl-3C (sn-m3C), which allowed the joint profiling of DNA methylation and chromatin interaction profiles in single nuclei by combining the conventional in situ 3C/HiC protocol with single cell bisulfite sequencing.[66] This methodology was applied to postmortem human frontal cortex samples, identifying 14 cell types by clustering the cytosine methylation (mC) profiles, and then identifying cell type specific 3D chromatin structures from these clusters. A strong, cell type specific relationship was observed between mC and 3D genome structure, suggesting extensive co-regulation of these epigenomic features.[66] It should be noted that neuronal subtypes could be distinguished at much higher resolution using the mC signature alone compared to only using the chromatin interactome profiles. However, clustering using chromatin interactions alone or jointly with mC can robustly resolve non-neuronal cell types.[66] These findings imply that single cell 3C/HiC data may still be too sparse to resolve subtle differences between neuronal subtypes, which are physiologically more similar to each other than non-neuronal cell types, which are more distinct from each other.

The sn-m3C method was later applied to mouse brain to identify distinct cell types in the hippocampus, highlighting the dynamic nature of the 3D genome across cell types.[67] Notably, enhancers positioned at the anchors of cell type-specific loops show corresponding hypomethylation in the same cell type that the loops are detected in. Therefore, there are strong relationships between DNA methylation patterns and 3D genome structure in distinct cell types in the brain. The sn-m3C method was recently applied to multiple regions of mouse and human brains to create detailed methylome and 3D genome atlases.[68,69] In addition to discovering spatial correlations between DNA methylation, chromatin conformation, and transcription, these studies obtained multi-scale insights on 3D genome structure at the single cell level. Notably, the mouse study found that TAD boundaries tended to form around long neuronal genes, and intragenic chromatin interactions are associated with alternative isoform usage, which correlate with results obtained from bulk HiC data in the human brain.[53,68] The human brain 3D genome and methylome study found that the prevalence of short-range chromatin contacts in neurons is associated with stronger TAD associated interactions, whereas the prevalence of longer range chromatin interactions in non-neurons is associated with compartmental interactions, mainly between B compartment regions, correlating with bulk HiC studies.[53,55,69]

Application of another single cell method called Diploid-C in developing mouse brain helped to distinguish between neuronal and non-neuronal subtypes whose transcriptomes and chromatin interactomes are remodeled during post-natal development.[41,70] Notably, during neuronal development from the neonatal to the adult stage genes are shifted from the nuclear periphery toward the interior.[70] Given that the mouse brain is highly plastic during the post-natal period and influenced by sensory input, particularly through the eyes, the authors tested to see if the post-natal chromatin remodeling is predetermined genetically or influenced by sensory experience. Specifically, they performed Diploid-C on single cells from the visual cortex of dark-reared and control mice at 5 different ages during the first post-natal month. Interestingly, changes in transcription and chromatin structure in the dark reared mice occurred normally as in the control mice, implying that the restructuring of the 3D genome is not influenced by sensory experience, but genetically predetermined.[41,70]

Previous studies investigated the relationship between gene expression and the 3D genome indirectly by integrating separate HiC and RNAseq datasets. This approach is limited in providing mechanistic insights as it does not investigate chromatin structure and transcription simultaneously within the same individual cells. Several multiome methods have been recently developed that sequence RNA and the chromatin interactome simultaneously in individual cells, which allow the linking of genome structure to function.[71-75] A method called HiRES (Hi-C and RNA-seq employed simultaneously) was

applied to single cells from developing mouse embryo showing that single cell 3D genome structures are determined by both cell cycle and developmental stages, and that lineage specification over time gradually creates divergent chromatin interactomes in a cell type specific manner.[71] Furthermore, integrating transcriptional and 3D chromatin structure dynamics from the same individual cells across development showed that widespread chromatin rewiring occurs before transcription activation. A similar approach called scCAREseq further showed that transcription and 3D chromatin structure are coordinated throughout the cell cyle, exhibiting simultaneous periodic changes.[72]

GAGE-seq employs a strategy of combinatorial barcoding of both cDNA and proximity ligated DNA, thereby increasing the throughput and sensitivity of detection of both the transcriptional output as well as chromatin interactomes of individual cells.[73] In particular, the scRNA-seq component of GAGE-seq allowed to infer many rare neuronal subtypes in the mouse cortex that were not identified by HiRES. Furthermore, clustering of single cell chromatin interactomes from the mouse cortex was able to distinguish all of the transcriptome defined cell types, showing an improvement over previous studies, in which scHi-C lacked the resolution to identify as many distinct cell types as scRNAseq from complex tissue. Integrating the GAGE-seq scRNAseq with previously published MERFISH spatial transcriptomics data[76] from mouse cortex also allowed to gain insights on in situ spatial variation in the co-regulation of 3D genome structure and gene expression.

LIMCA (Linking mRNA to chromatin architecture) splits cytoplasmic and nuclear fractions to perform scRNAseq and scHi-C separately in the cytoplasm and nucleus from the same individual cells.[74] This approach detects full length mRNAs and circumvents the potential loss of sensitivity of each assay when scHiC and scRNAseq are performed together within the same nucleus, as in previous methods such as HiRes. This method was applied to developing olfactory sensory neurons in conjunction with a high sensitivity scATACseq approach to decipher the dynamic changes in enhancer-promoter interactions and chromatin accessibility that result in the silencing of all but one olfactory receptor gene, establishing the mechanistic basis for the "one neuron-one receptor" model.

A recent preprint describes a tri-omic assay that allows to investigate chromatin accessibility, interaction, and RNA simultaneously (ChAIR).[75] The study confirmed previous observations from single cell multiomics studies of the coordination of genome structure and function throughout the cell cyle[71,72] and discovered spatial variation in 3D genome structure in the mouse brain by integration with spatial transcriptomics data, similar to the GAGE-seq study.[73] Furthermore, short range chromatin contacts were associated with TAD dominant structures enriched for open chromatin and transcriptional activity and long range "megacontacts" were associated with compartment dominant structures enriched for repressed chromatin, correlating with observations from previous bulk and single cell studies.[53,55,69] Notably, neurons and non-neurons showed opposite trends in genome re-organization during cellular aging, as oligodendrocyte precursor cells shifted from short range to ultra-long range contacts during maturation, whereas neuroblasts in some brain regions tended to shift from ultra-long range to short range contacts, corroborating evidence from previous studies.[53,55,69]

Conventional 3C approaches have poor genomic coverage, as they rely on proximity ligation of DNA fragments that are digested by restriction enzymes, whose recognition motifs are unevenly distributed across the genome and may not always be accessible. An alternative proximity ligation method called Micro-C utilizes MNase to digest nucleosome free linker regions, followed by ligation, producing nucleosome level resolution maps of 3D chromatin structure.[77-81] A recent preprint describes a single cell Micro-C method that was able to characterize cohesin mediated transcription elongation loops on long genes, illustrating the ability of single cell high resolution 3D genome maps to decipher the intimate connection between 3D chromatin structure and transcription.[82] Interestingly, the human brain is biased toward the expression of long genes, which is particularly evident in neurons.[83] Dysregulation of the expression of long genes in the human brain has implications for neurological disorders, such as autism and Rett Syndrome.[84,85] Gene loops were shown in both mouse and human brain,[40,53,68] with both bulk and single cell 3C approaches showing relationships between intragenic chromatin interactions and differential isoform expression.[53,68] Along with previous evidence showing that splicing in the human brain is co-transcriptional,[86] and evidence of the involvement of cohesin in exon usage in different cell lines,[87] collectively, these studies indicate intricate mechanisms that link 3D chromatin structure with transcription and transcript processing that would merit the usage of high resolution single cell 3D genomic approaches like single cell Micro-C for further characterization. Altogether, improving the resolution of single cell 3C based methods and pairing with other modalities such as RNAseq, or methyl-Seq, as discussed, will significantly broaden our understanding of the complex dynamics of gene regulation in the human brain.

Alternative Multi-Contact Genomics Methods

Although 3C-based approaches have been most widely used in elucidating the structure of the 3D genome, they are largely limited to describing pairwise interactions generated from proximity ligation. A novel technique called Pore-C,[88] which is based on 3C chemistry, does not fragment proximity ligated DNA through sonication, thereby retaining concatemers of DNA with multiple ligation junctions, allowing for the detection of multi-way contacts. Furthermore, these concatemers are not amplified through PCR, but read directly through nanopore sequencing technology, allowing the readout of epigenetically modified nucleotides in a 3D chromatin context. A recent

study utilized an optimized version of Pore-C called HiPore-C (High-Throughput Pore-C) that was able to capture DNA methylation simultaneously with chromatin topology and determine that multi-way interactions can span multiple TADs and multiple compartments of the same type (A-A or B-B), producing higher-order 3D genome structures.[89] Furthermore, it was shown that TADs that are highly conserved across cell types can contain discrete cell type specific clusters of interactions. As shown through analysis of the B-globin locus, such clusters can correspond to enhancer-promoter hubs, which corroborates with previous findings using targeted multi-contact 3C based approaches such as multi-contact 4C and Tri-C.[90,91] Altogether, multi-contact 3C based approaches can reveal complex gene regulatory hubs that are not easily identifiable with conventional 3C methods.

3C based approaches are limited to identifying interactions that occur within a small contact radius, as the loci need to be close enough for ligation (less than 200 nm). Recently, other approaches have been developed, such as SPRITE,[92] and GAM,[93] that do not rely on proximity ligation, allowing the capture of multi-way interactions within a broader contact radius. SPRITE (split-pool recognition of interactions by tag extension) splits crosslinked fragments of DNA onto a 96 well plate, adding a unique tag onto all DNA molecules in a single well, and then pooling all the complexes together.[92] This entire process is repeated multiple times, allowing DNA fragments within specific hubs to be uniquely barcoded. This approach allowed to identify 2 major inter-chromosomal hubs centered around nuclear speckles and the nucleolus, corresponding to transcriptionally active and inactive regions, respectively. These 2 nuclear bodies were shown to shape the overall 3D packaging of the genome in the nucleus. Recently, a scSPRITE approach was developed that was able to identify inter-chromosomal hubs around nuclear speckles, nucleoli, and centromeres, features that are not easily detectable in scHiC approaches, due to their inherent limitations in detecting long range interactions.[94] Furthermore, TADs and long-range enhancer-promoter interactions showed significant cell-to-cell heterogeneity, showing that regulatory interactions are very dynamic in nature. A modification of the SPRITE method called RDSPRITE (RNA and DNA SPRITE) allowed the identification of RNA-RNA and RNA-DNA complexes.[95] Notably, they found that nuclear compartments involved in RNA processing, heterochromatin formation and gene regulation are generated through the accumulation of nascent non-coding RNAs that seed the assembly of other diffusible ncRNAs and regulatory proteins and help to organize long range DNA-DNA contacts.

GAM (genome architecture mapping) is based on ultrathin sectioning of nuclei and sequencing barcoded DNA fragments within the same nuclear plane.[93] It should be noted that while techniques such as SPRITE and GAM can capture multi-way chromatin interactions within a broader nuclear radius, they may lose the specificity of higher-order interactions that are involved in gene regulation, which are thought to occur at the 10 to 200 nm scale.[96] Nonetheless, a recent study performed a multiscale 3D genome analysis on different cell types from mouse brain tissue using immunoGAM, a variant of the original GAM protocol that immunolabels specific cell types from tissue sections.[97] Analysis of chromatin contacts in oligodendrocytes, pyramidal glutamatergic neurons, and dopaminergic neurons revealed extensive reorganization at the compartment and TAD level, with unique TAD boundaries correlating with the expression of cell type specific genes. Long transcriptionally active genes were also shown to decondense and shift away from repressive landmarks such as the nuclear lamina.[97] Furthermore, networks of contacts in neuronal subtypes are enriched for genes implicated in subtype specific function, whereby hubs specific to pyramidal glutamatergic neurons contain genes involved in synaptic plasticity, while those of dopaminergic neurons contain genes associated with addiction.

Although many studies have described chromatin interactions and gene expression, including recent studies investigating transcription and chromatin folding simultaneously in single cells,[71-75] there has been limited research investigating RNA-chromatin interactions simultaneously with the 3D genome. A novel approach called MUSIC (multinucleic acid interaction mapping in single cells) that profiles RNA concurrently with multiplex RNA-DNA and DNA-DNA interactions revealed chromatin interaction and transcriptional signatures correlated with aging and Alzheimer's disease pathology in human frontal cortex tissue from older donors.[98] Importantly, it was found that nuclei with decreased local chromatin interactions tended to show expression patterns associated with cellular aging, which correlates with findings in mouse brain,[75] in which aging was associated with a loss of short range contacts but gain in ultra long range contacts, particularly in oligodendrocytes. Furthermore, investigation of XIST, the lncRNA implicated in X chromosome inactivation in females, shows heterogeneity in XIST-chromatin interactions across female cortical cells, along with diverse spatial organizations of the X chromosome, in which XIST + X chromosomes show a tendency toward longer range interactions than XIST-.[98]

Spatial Imaging Methods

Although 3C and non-3C based genomics approaches can measure the relative frequencies of interaction between different segments of chromatin, they do not provide the spatial context of these interactions within the nucleus. DNA FISH based approaches image chromatin directly in intact nuclei by hybridizing fluorescently labeled probes against specific genomic regions. Diminishing costs of DNA synthesis have allowed multiplexing whereby multiple genomic regions can be imaged simultaneously with large oligonucleotide

libraries.[99] Oligopaint technology involves designing short oligos with extended non-genomic sequences which can be used as priming sites for PCR amplification of probes or as hybridization sites for secondary labeled oligos.[100-103] The use of secondary labeled readout probes increases multiplexing capabilities as unlabeled primary probes with different readout sequences can target multiple regions, which can then be imaged sequentially across multiple cycles of hybridization and removal of different readout probes. Therefore, the number of loci that can be imaged is not limited by the number of available fluorescent dyes. Techniques such as seqFISH and MERFISH that are based on the Oligopaint principle were initially developed to image the transcriptome at scale by hybridizing secondary barcoded readout probes to multiple target RNAs across multiple rounds of sequential hybridization.[76,104-111] These approaches have been adapted to image the chromatin interactome in a similarly high throughput manner.[112,113]

Tiling probes at high density and utilizing super resolution imaging approaches such as STORM (Stochastic Optical Reconstruction Microscopy) can resolve fine scale chromatin folding features.[103,114] STORM uses photo-switchable fluorophores that are stochastically activated across the biological sample over multiple imaging cycles to resolve densely labeled features over time that are spatially unresolvable due to the optical diffraction limit.[115] Individual fluorescently labeled molecules are then localized to reconstruct a high-resolution image. Using STORM in conjunction with a sequential hybridization approach using secondary readout probes to image chromatin segments along tiled megabase-sized regions in different cell types allowed the reconstruction of high-resolution chromatin structure in single cells.[114] Notably, chromatin was found to be segregated into globular TAD-like structures with sharp boundaries, showing for the first time through microscopy that TADs are not merely population averaged features in bulk HiC contact maps but actually exist in single cells. However, domain boundaries were shown to be quite variable, but predominantly occurring at sites bound by CTCF and cohesin. Interestingly, while cohesin depletion results in loss of TADs in population averaged contact maps, as shown previously,[11,12] TAD-like structures persist in single cells, albeit with the loss of the preferred CTCF occupied boundaries. Therefore, while cohesin may not necessarily be implicated in the formation of TADs, it still plays an important role in establishing proper boundaries via its interaction with CTCF. Altogether, microscopy-based approaches can validate chromatin interaction features detected in population averaged HiC contact maps, but offer further insights into their dynamic nature and mechanism of formation.

Other Oligopaint based approaches such as ORCA (Optical Reconstruction of Chromatin Architecture) and Hi-M used sequential hybridization of secondary readout probes against densely tiled sequence specific probes for high resolution chromatin tracing in intact *Drosophila* embryos.[116,117] Both approaches were paired with RNA labeling to simultaneously detect 3D chromatin structure and transcription. Hi-M offered a high throughput, high-resolution, and high coverage approach to monitor global changes in TAD organization during early embryogenesis and transcriptional activation.[117] ORCA was used to decipher the chromatin structure at the *Hox* gene cluster in different cell types positioned along the *Drosophila* embryo, providing cell-type specific and spatial insights.[116] Notably, 2 types of cell-type specific domains were identified, those that separate active from polycomb repressed chromatin, and polycomb-independent domains whose boundaries are enriched for CTCF and cohesin, thus resembling the TADs more commonly found in mammalian species. Deletion of the boundaries of the polycomb-independent domains led to ectopic enhancer-promoter contacts, aberrant gene expression, and developmental abnormalities. Therefore, the architectural proteins CTCF and cohesin may have played an important role in cell type specific gene regulation prior to vertebrate evolution.

Multi-omics studies in single cells can decipher cell-to-cell heterogeneity in transcription and epigenetic features, but it is also imperative to study these features within intact nuclei in a native tissue context to obtain insights on spatial gene regulation, especially in a complex organ such as the brain. Integrative seqFISH and MERFISH methods have both been recently used to investigate transcription and chromatin structure simultaneously in different regions of the mouse brain.[113,118] Notably, the first study simultaneously imaged DNA loci, RNA, histone modifications, and nuclear bodies in single cells in tissue sections of the adult mouse cerebral cortex.[113] This was achieved using integrated spatial genomics, which uses RNAseq FISH and DNAseqFISH to hybridize probes against multiple RNA and DNA targets, respectively, and utilizes probes against oligo-congugated antibodies targeting histone modifications and nuclear body associated proteins. This approach enabled to determine the differential spatial positioning of DNA loci with respect to histone markers and nuclear bodies within intact nuclei across different cell types and its correlation with differential expression. Furthermore, co-localization of loci within specific physical distances correlates with read counts from bulk HiC data. Interestingly, however, domain boundaries show variability in single cells, implying that TADs in bulk HiC datasets represent statistically averaged chromatin structures that are very dynamic in nature when examined in individual nuclei, corroborating results from other imaging and single cell omics studies.[94,114]

The other study, currently in preprint, used an alternative multiplexed integrated RNA and DNA FISH approach based on the MERFISH technology that allowed to infer the spatial organization of chromatin and transcription simultaneously within individual nuclei across different cell types in the primary mouse motor cortex.[118] Notably, neurons were shown to

have larger nuclei and larger chromosome territories than non-neurons, correlating with overall higher transcriptional activity. Despite the larger nucleus size, neurons tend to show an enrichment of short-range contacts relative to non-neurons, correlating with previous bulk and single cell 3C studies.[53,55,69] However, in contrast to previous bulk and single cell 3C studies, neurons were shown to have stronger A/B compartmentalization, which could reflect the biases of different methods, or potentially the higher sensitivity of DNA FISH to detect fine scaled compartments. Interestingly, the methylated DNA binding protein MeCP2, implicated in a neurological disorder known as Rett syndrome, was shown to regulate transcription differentially in neurons in a nuclear radial position dependent manner, whereby MeCP2 represses transcription of genes near the nuclear periphery but activates transcription of genes near the nuclear interior. The activity of MeCP2 was shown to be linked to its ability to modulate A/B compartmentalization, which was hypothesized to involve a phase separation mechanism, as previous studies have shown the ability of MeCP2 to form phase separated condensates via an intrinsically disordered region.[119,120]

Altogether, spatial imaging approaches that combine the detection of RNA and DNA can reveal not only the heterogeneity in transcriptional activity and chromatin folding mechanisms, but also the importance of the intra-nuclear positioning of genes and regulatory elements. Future studies that combine detection of RNA and DNA with detection of protein in different sections of mouse and human brains will enable us to obtain further insights on spatial gene regulation.

Future Perspectives

Within the last decade, there have been substantial advancements in elucidating the role of the 3D genome in brain development. However, given the technical challenges in generating high resolution data at large scale, the amount of available data is still limited. Furthermore, the lack of standardized methodologies makes comparisons of data across different studies very challenging. Also, much of our current knowledge about the 3D genome is based on bulk cell population studies, and therefore we know very little about the cell-to-cell heterogeneity of chromatin structures. As described in this review, single cell 3C/HiC approaches have been developed to decipher chromatin organization at the single cell level in both mouse and human brain samples. However, relative to other omics methods, the data generated from single cell 3C/HiC is very sparse, and lacks the resolution to provide a detailed cellular taxonomy.

As previous studies have largely been descriptive, future research should also focus on functional characterization of chromatin interactions as well as understanding the dynamics of chromatin interactions in real time through live cell imaging methods. Several approaches have been developed for 3D genome engineering.[121-123] The CRISPR-GO (CRISPR-genome organization) method enables the repositioning of genomic loci to different nuclear compartments to study the effect of spatial location on gene expression. Briefly, it involves targeting a specific genomic locus with a nuclease-deficient Cas9 (dCas9) that is fused to a heterodimer that can be chemically induced to bind to its cognate heterodimer, which is fused with the nuclear compartment protein of interest. Notably, this approach revealed that targeting loci to the nuclear periphery or cajal bodies results in gene repression.[121] As neurons have been shown to reposition genes toward the nuclear interior,[70,97] it could be interesting to use CRISPR-GO to reposition neuronal genes toward the nuclear lamina or other repressive landmarks and examine the transcriptional and phenotypic consequences during neural development.

CLOuD9 and LADL are 2 intriguing techniques that can engineer chromatin loops, using chemically and optically induced protein dimerization, respectively.[122,123] CLOuD9 targets 2 different genomic loci with dCas9 constructs from different species, each fused with one-half of a dimerization product that can be chemically induced, thus forming a loop.[122] Similar to CRISPR-GO, the engineered interaction is reversible upon washing out the ligand, and forced chromatin looping results in epigenetic changes and transcription upregulation at the targeted locus. LADL (light activated dynamic looping) involves targeting dCas9 fused to the CIBN protein from *A. thaliana* (dCas9-CIBN) to 2 different genomic loci and forcing a bridging interaction via another protein CRY2 in response to blue light.[123] The method was successfully used in mouse embryonic stem cells to reposition a super-enhancer away from its endogenous *Klf4* target gene and to the *Zfp462* promoter, and transcription upregulation at the newly targeted locus was validated in single cells using single molecule RNA FISH.[123] Interestingly, the CLOuD9 approach has recently been used to validate neuronal subtype specific loops obtained from HiC.[54] Two long range chromatin loops detected in induced GABAergic neurons, one linked to the well-known eQTL-based schizophrenia risk gene *SNAP91*, and the other linking a schizophrenia risk locus to the distal *BHLHE22* gene were successfully re-created in induced glutamatergic neurons, resulting in transcriptional upregulation and electrophysiological changes. Altogether, 3D genome engineering approaches show incredible promise in functional characterization of chromatin interactions, and may be applied to other cell types in the brain as well.

Genomics and fixed cell imaging approaches can only provide static snapshots, and thus a mechanistic understanding of chromatin interactions will require live cell imaging approaches. There are different models of enhancer-promoter looping, such as the stable contact model and the hit-and-run model (dynamic contact).[124] The evidence for stable contact comes from studies showing that forced enhancer-promoter looping is sufficient to activate B-globin expression.[124-126] However, another study that simultaneously visualized DNA and RNA by FISH in *Drosophila* showed only a small correlation between

transcription and enhancer-promoter proximity for the 3 genes studied.[116,124] The latter study seems to support a dynamic hit-and-run model, whereby the enhancer may transiently contact the promoter, and once transcription occurs, contact is broken. Furthermore, to distinguish between whether transcription requires physical enhancer-promoter contact or merely proximity will require high spatial resolution imaging. Therefore, a robust mechanistic understanding of chromatin looping and transcription regulation will require super-resolution live cell imaging (SRLCI) approaches.[124]

SRLCI approaches have been used to monitor transcription and enhancer-promoter communication simultaneously in the same cells over extended periods of time as demonstrated in *Drosophila* embryos and mouse embryonic stem cells.[124,127,128] Interestingly, these studies came to opposing conclusions, as one study clearly showed a role for enhancer-promoter looping in mediating gene activation,[128] while the other reported no role for enhancer-promoter proximity in gene activation.[124,127] Altogether these results show the complex relationship between chromatin conformation and transcription, and imply that there may be no universal model to explain gene regulation through 3D genome structure, as mechanisms may be locus specific. Given the challenges of designing SRLCI experiments, which involves the consideration of multiple factors such as choice of fluorescent labels, targeting strategy, microscope resolution, and duration of experiments, it is imperative to choose appropriate candidate loci and formulate precise hypotheses. Although many challenges lay ahead in understanding how the 3D genome shapes the development of diverse cell types in the brain, we now have a wealth of genomics data that enables us to generate interesting hypotheses for validation in the years to come.

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