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Perspective

3D spatial genome organization in the nervous system: From development and plasticity to disease

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SUMMARY

Chromatin is organized into multiscale three-dimensional structures, including chromosome territories, A/B compartments, topologically associating domains, and chromatin loops. This hierarchically organized genomic architecture regulates gene transcription, which, in turn, is essential for various biological processes during brain development and adult plasticity. Here, we review different aspects of spatial genome organization and their functions in regulating gene expression in the nervous system, as well as their dysregulation in brain disorders. We also highlight new technologies to probe and manipulate chromatin architecture and discuss how investigating spatial genome organization can lead to a better understanding of the nervous system and associated disorders.

INTRODUCTION

The completion of the human genome sequencing project in 2001 provided a blueprint of the genome at the linear sequence level (Lander et al., 2001; Venter et al., 2001). The human genome is encoded by approximately 3.2 billion nucleotides of DNA, and most human cells, which are diploid, contain about 6 billion base pairs, divided into 46 chromosomes. These 6 billion base pairs, equivalent to about 2 m of linear DNA, are folded into three-dimensional (3D) structures and packaged into nuclei, which are about 5–10 μm in diameter. Histone proteins compact DNA to form nucleosomes, which allows packaging of the DNA into the microscopic nuclear space. Recent technical advances such as chromosome conformation capture (3C) analysis, which detects DNA-DNA interactions between close genomic loci within the 3D space of the nucleus, have led to new insights into the spatial organization of chromatin. Interphase chromosomes are spatially organized at hierarchical levels, from chromatin loops that allow associations between promoters and other regulatory elements, such as enhancers, over short- and long-range linear genomic distances, to chromatin domains, topologically associating domains (TADs), and A/B compartments; moreover, entire chromosomes themselves occupy defined regions in the nucleus, termed chromosome territories (Figure 1A). These hierarchical structures are essential for gene expression control, and disturbances in these structures have been implicated in various human disorders (Yu and Ren, 2017). Although traditionally investigated in cancer cell lines, recent studies have started to profile 3D genomes in the nervous system and address their functions and mechanisms of action. Here, we review recent studies on 3D genome organization and its dynamics underlying neural development and neuronal plasticity. We further highlight the disorganization of spatial chromosome architectures in brain disease states and discuss how 3D genome knowledge can advance our understanding of contributions of genome variants to different brain disorders. Finally, we discuss future perspectives and questions to be addressed.

Hierarchical chromatin structure

Multiple layers of chromatin structural organization

There are multiple layers of chromosome hierarchical structure, including epigenetic modifications to the linear genome such as DNA methylation and histone modifications, as well as chromatin loops, TADs, A/B compartments, and 3D genomic locations in the nucleus (Figure 1A).

When cells are not in mitosis, chromosomes tend not to intermingle but instead occupy distinct chromosome territories (Cremer and Cremer, 2010). Hi-C, a 3C-derived technology that allows the complete detection of "all versus all" long-distance chromatin interactions across the entire genome via sequencing, confirmed the presence of chromosome territories and further revealed intra-chromosomal compartmentalization into regions of open and closed chromatin, termed "A" and "B" compartments, respectively (Lieberman-Aiden et al., 2009). "A" compartments include genomic loci that are generally gene rich, transcriptionally active, and DNase I hypersensitive; conversely, loci found in "B" compartments are relatively gene poor, transcriptionally silent,

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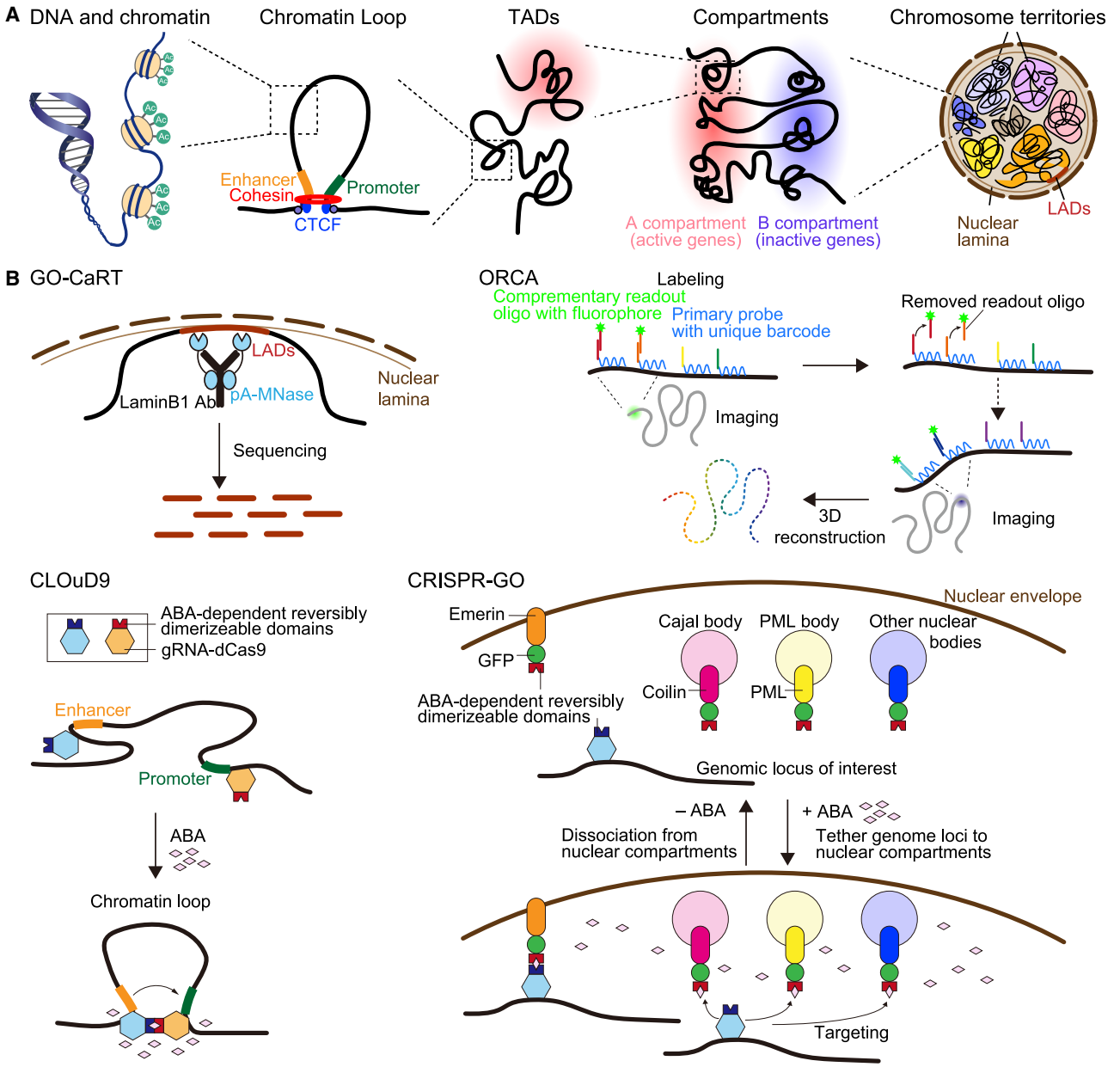

Figure 1. Hierarchical chromatin structure

(A) Illustration of multiple layers of chromosome structural organization in the nucleus.

(B) Examples of technologies to profile different forms of chromosome structural organization and induce chromatin looping. Genome organization using CUT&RUN technology (GO-CaRT) uses LaminB1 tethered proteinA-MNase and enables the cleavage of nuclear lamina proximal DNA, followed by sequencing and LAD calling. Chromatin loop reorganization using CRISPR-dCas9 (CLOuD9) induces abscisic acid (ABA)-dependent reversible chromatin looping. Optical reconstruction of chromatin architecture (ORCA) enables the reconstruction of the trajectory of a genomic region of interest by tiling the region in short sections with an average resolution of 17 kb. CRISPR-genome organizer (CRISPR-GO) induces ligand-mediated dimerization of CRISPR-dCas9 and proteins specific for versatile nuclear compartments.

and harbor heterochromatic sequences. The spatial segregation of A/B compartments was confirmed by imaging-based methods (Su et al., 2020; Wang et al., 2016).

Another major type of chromatin organization involves megabase-size folding entities termed TADs (Nora et al., 2012; Rao et al., 2014). TADs were characterized using Hi-C maps at 40-kb resolution and are markedly smaller than A/B compartments—the median sequence size is 800 kb for TADs and 3 Mb for compartments. It has been proposed that restricting interactions between genes and their regulatory sequences is one major function of TADs. In mammals, TAD boundaries are usually demarcated by zinc-finger binding protein CCCTC-binding factor (CTCF) and the cohesin complex (Rao et al., 2014). The cohesin complex forms a ring-like structure composed of four

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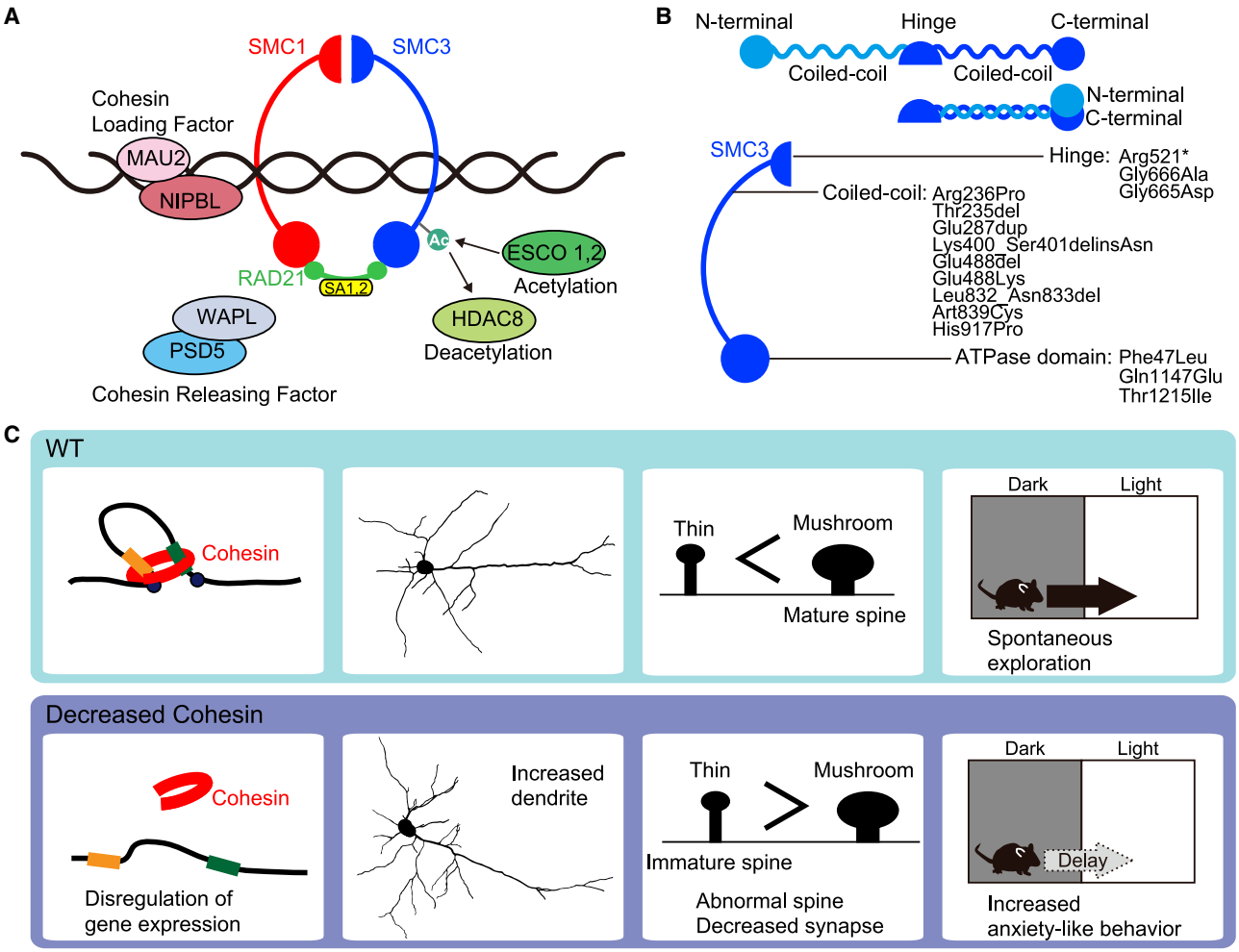

Figure 2. Molecular machineries regulating chromosome structural organization
(A) A schematic diagram of the cohesin complex.
(B) A model of SMC structure and the locations of SMC3 mutations found in human patients.
(C) Impact of mutations of cohesin complex components on the nervous system, including gene expression, dendritic growth, dendritic spine morphologies, and behavior in mouse models.

core subunits: SMC1, SMC3, RAD21, and SA1/2 (Figures 1A and 2A). Cohesin cooperates with CTCF to form a chromatin loop and functions with the general transcriptional co-activator, the Mediator complex (Wendt et al., 2008). Some studies proposed that CTCF and cohesin promote "loop extrusion," which contributes to TAD formation (Ganji et al., 2018; Sanborn et al., 2015). In this model, cohesin is loaded onto DNA by cohesin loaders, including nipped-B-like protein (NIPBL) and MAU2 sister chromatid cohesion factor (MAU2), then slides along the chromatin and extrudes it outward until it reaches the chromatin boundaries that are often formed by CTCF. Real-time visualization has recently confirmed that cohesin and its loaders induce genomic interphase DNA into loops by extrusion (Bauer et al., 2021). Direct interaction of the N-terminal segment of CTCF with cohesin contributes to loop stabilizing activity. DNA is spontaneously translocated by cohesin's hinge, and NIPBL separates from the hinge and clamps DNA onto the head of SMC3 in an ATP-dependent manner.

In most cell types, heterochromatin normally resides at the nuclear periphery, whereas euchromatin situates toward the nuclear interior. In one exception, this organization is inverted in rod photoreceptor neurons of nocturnal retinas, in which the dense heterochromatin localized in the nuclear center may serve as collecting lenses to enhance light transduction efficiency (Falk et al., 2019; Sofueva et al., 2013; Solovei et al., 2009). Lamina-associated domains (LADs), which are genomic regions that are in close contact with the nuclear lamina, are also thought to help organize chromosomes inside the nucleus and have been largely associated with gene repression (Guerreiro and Kind, 2019; van Steensel and Belmont, 2017).

Advanced technologies to probe 3D genome and investigate its function

Since the initial 3C technology, various methodologies have been developed to investigate chromatin architecture. First, there have been significant improvements in the mapping resolution and in the reduction of cell numbers required, even to

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the single-cell level, along with advances in assays for chromatin-protein interactions and general chromatin architecture, such as single-cell Hi-C, cleavage under targets and tagmentation (CUT&Tag), and single-cell assay for transposase-accessible chromatin using sequencing (ATAC-seq). Advances in multi-omics analyses are expected to provide a comprehensive understanding of the association between 3D genome organization and gene transcription. These sequencing-based approaches have also been adapted to new technologies to investigate the genomic distribution in the 3D space of the nucleus. For example, genome organization using CUT&RUN (cleavage under target & release using nuclease) technology (GO-CaRT) is an application of CUT&RUN for mapping genomic interactions within nuclear compartments and enables profiling of the LAD architecture (Figure 1B; Ahanger et al., 2021).

Second, advances in labeling and imaging technologies have provided novel insights regarding chromatin structure in the nuclear space (Boettiger and Murphy, 2020; Chen et al., 2016; Girelli et al., 2020; Quinodoz et al., 2018; Ricci et al., 2017). New strategies for fluorescence *in situ* hybridization, such as Oligopaints technologies, signal amplification by exchange reaction (SABER) (Jungmann et al., 2014), and labeling using genome editing tools, such as zinc-finger nuclease, transcription activator-like effector nuclease, and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system (Hilton et al., 2015), has enabled innovative chromatin imaging. In particular, the Oligopaint optical reconstruction of chromatin architecture (ORCA) approach allows the visualization of nanoscale DNA paths in single cells and measurement of kilobase-scale interactions among nearby *cis*-regulatory elements (Mateo et al., 2019; Figure 1B). Further improvements in live cell imaging will enable the tracking of dynamic changes in spatial chromatin structures throughout development and in response to stimulation.

Third, various genome engineering technologies have been developed for dynamic manipulation of 3D chromatin structures. Tethering repressed genes to active *cis*-regulatory elements by using artificial zinc fingers substantially activates their expression in both human and mouse cells (Deng et al., 2012). CRISPR-dCas9 (CLOuD9), which uses dCas9 protein in combination with a reversible chemically induced proximity system that utilizes the plant phytohormone S-(+)-abscisic acid (ABA) and modified components of the plant ABA signaling pathway, induces targeted and reversible chromatin looping upon ABA treatment (Hao et al., 2017; Morgan et al., 2017; Figure 1B). CRISPR-genome organizer (CRISPR-GO) is a technology that expands applications of CRISPR into regulation of the spatial positioning of gene loci in the cell nucleus (Wang et al., 2018). CRISPR-GO induces ligand-mediated chemical dimerization of CRISPR-dCas9 and nuclear fraction-specific proteins to artificially rearrange the genome structure, which can be used to elucidate the causal relationship between the macroscale ($\sim\mu$m) spatial structure and cellular functions (Figure 1B). In another method, light-activated dynamic looping enables the formation of long-range contacts between distal genomic loci in response to blue light over short timescales (Kim et al., 2019). Future application of these tools will allow assessment of the causal role of chromatin looping in controlling target gene expression under physiological conditions.

3D genome organization during nervous system development

The nervous system development involves neural patterning, generation of different cell types from neural progenitors, and maturation and integration of various neural cell types. Dynamic physiological changes in the chromatin structure through epigenetic mechanisms enable spatio-temporal regulation of gene expression during these complex processes (Yao et al., 2016; Yoon et al., 2018).

Chromocenter: Compaction of the chromatin state toward neural differentiation

The global nuclear structure undergoes dynamic changes during sequential differentiation from embryonic stem cells (ESCs) to neural progenitor cells (NPCs) and then to terminally differentiated neurons. The 3D positioning of genes in the nucleus plays an important role during brain development (Ito and Takizawa, 2018; Takizawa and Meshorer, 2008). Evidence from several studies has indicated that the chromatin state is globally open in ESCs and becomes increasingly condensed during differentiation into NPCs and neurons (Kishi and Gotoh, 2018). Chromocenters, which are heterochromatin foci that can be stained strongly with DNA-interacting dyes, exhibit considerable diversity in their size, number, and distribution in various differentiation stages and cell types (Billia et al., 1992; Billia and de Boni, 1991; Manuelidis, 1984; Martou and De Boni, 2000; Solovei et al., 2004). Preformed chromocenters in ESCs disperse into smaller foci during differentiation into NPCs (Aoto et al., 2006; Meshorer et al., 2006; Williams et al., 2006). During NPC differentiation into postmitotic neurons, the number of chromocenters decreases, and they converge into larger clusters located in the center of the nucleus. The findings that ESCs have more active chromatin marks and fewer heterochromatin domains suggest that these changes in chromocenters may be associated with the expansion and segregation of heterochromatin and the deposition of active histone marks during cellular differentiation (Hawkins et al., 2010; Kishi et al., 2012; Xie et al., 2013).

Genome-wide analyses of chromatin accessibility using techniques such as assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq), and DNase I hypersensitive site sequencing (DNase-seq) have also revealed dynamic changes in chromatin openness during neural differentiation processes (de la Torre-Ubieta et al., 2018; Frank et al., 2015; Thakurela et al., 2015). Together, these studies provide evidence suggesting that an "open" chromatin structure is crucial for pluripotency during sequential differentiation from ESCs to differentiated neurons.

Compartments and TADs: Fewer chromatin interactions in active domain and more chromatin interactions in inactive domain during neural differentiation

A/B compartments undergo dynamic switching during transitions from ESCs to NPCs and then to neurons. High-resolution Hi-C analyses have revealed the progressive changes in differentiation stage-specific chromatin architecture both in mouse

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and human cultured cells. Interactions between active TADs become weaker, whereas interactions in inactive TADs become stronger as mouse ESCs differentiate into NPCs and then to neurons (Bonev et al., 2017). Similarly, progressive changes in chromatin architecture have been observed during neuron and astrocyte differentiation from human induced pluripotent stem cells (iPSCs) (Rajarajan et al., 2018). About 36% of A and B compartments throughout the genome switch during ESC differentiation (Dixon et al., 2015). Intriguingly, compartment switching affects the expression of only a certain subset of genes, suggesting that compartmental status may influence the accessibility of genomic regions for only limited subsets of genes. Consistent with global compaction of the chromatin structure during ESC differentiation into NPCs, the total size of A compartments in differentiated cells, including NPCs, was reduced by 5% compared with that in ESCs. Collectively, the compaction of nuclear chromatin domains seems to be a general feature of neural differentiation and may contribute to the stable silencing of genes unnecessary for differentiated neurons.

Phase separation, which describes the concept of un-mixing of a liquid into two different liquid phases, has recently emerged as an important phenomenon in the formation of biomolecular condensates. Phase-separated multi-molecular assemblies have been suggested as a general regulatory mechanism to compartmentalize biochemical reactions within cells (Hnisz et al., 2017). Analysis of A/B compartments at a finer resolution of 500 base pair demonstrated the likelihood of a much finer A/B compartment organization emerging with phase separation at the scale of small regulatory sequences, contrary to the conventional findings obtained as large (Mb-scale) blocks of sequence by coarse-resolution compartment profiles (Gu et al., 2021). This study also distinguished between loops that are formed by extrusion and those that are formed through a non-extrusion mechanism.

Compared with A/B compartments, TADs appear to be relatively more stable during neural differentiation. TAD boundaries are stable during cell divisions and conserved among various cell types or lineages (Dixon et al., 2012; Ho et al., 2014), although inter-TAD interactions and chromatin interactions within TADs can occur during cell differentiation (Dixon et al., 2015, 2016; Fraser et al., 2015; Hansen et al., 2018; Nora et al., 2012; Rao et al., 2014; Schmitt et al., 2016). These findings suggest that chromatin is regionalized at somewhat stable boundaries, producing restricted regions in which chromatin interactions occur more often.

Loops and local interactions

Within TADs, chromatin structures seem to be more extensively reorganized locally thorough chromatin looping, including interactions between promoters and enhancers, which often occurs in a developmental stage- and cell type-specific manner.

Chromatin interaction analysis with paired-end tag sequencing has revealed local chromosomal structures linked to the control of cell identity in ESCs (Dowen et al., 2014). Super enhancers, which are cell type-specific and play key roles in cell fate determination, are marked by high levels of H3K27ac (Hnisz et al., 2013; Parker et al., 2013). Super enhancer-driven cell identity genes are generally localized within chromatin loops formed by CTCF and cohesin. These looped structures form insulated neighborhoods whose integrity is important for the proper expression of local genes. The phase separation model was recently used to explain the features of transcriptional control, including the formation of super enhancers and heterochromatin domains (Hnisz et al., 2017).

During the course of neural differentiation, dynamic alterations of CTCF-mediated loops occur in both mouse and human developing brains (Bonev et al., 2017; de la Torre-Ubieta et al., 2018; Lu et al., 2020; Rajarajan et al., 2018). During the NPC to neuron transition, loops associated with genes related to cell proliferation, morphogenesis, and neurogenesis are lost, which is consistent with the commitment to a lineage change from the precursor stage toward a postmitotic neuronal identity (Bonev et al., 2017; Figure 3A). Similarly, during the NPC to glia transition, loops associated with genes related to neuron-specific functions are lost, which is consistent with a non-neuronal lineage commitment (Rajarajan et al., 2018). In addition, the loss of many shorter-range contacts and loops during NPC differentiation into neurons is associated with concomitant increases in longer range (>100–200 kb) contacts in both humans and mice. Together, these results provide insights into the relationship between transcriptional control of cell identity and local chromosome structures mediated by chromatin looping.

Proximity ligation-assisted chromatin immunoprecipitation with sequencing (PLAC-seq) and HiChIP are both powerful tools for studying long-range chromatin contacts anchored at genomic regions bound by specific proteins or histone modifications. Promoter-enriched trimethylation of lysine 4 on histone H3 (H3K4me3) mark-based PLAC-seq analysis of purified radial glia neural stem cells, intermediate progenitor cells, excitatory neurons, and interneurons from the developing human cortex has shown that a subset of promoters with increased levels of chromatin interactivity are enriched for cell type-specific genes (Song et al., 2020), suggesting that interactions at these loci may drive cell type-specific differences during human corticogenesis. Furthermore, during differentiation of human iPSCs into neurons, the number of enhancer and promoter interactions increases in NPCs and neurons compared with those in iPSCs. The genes involved in NPC and neuron chromatin loops are strongly associated with neuronal differentiation functions (Lu et al., 2020). Many neural enhancer-promoter aggregates are substantially strengthened in the primary brain tissue (Lu et al., 2020). In addition, multi-omics analysis among human, macaque, and mouse uncovered a human-specific loop and showed that enhancer-promoter interactions and regulation of EPHA7 affect neuronal dendrite development and spine maturation (Luo et al., 2021). Single-cell analyses have shown that both the 3D genome structural types and transcriptome are fundamentally changed between postnatal (P) 7 and P28 in the mouse brain, and furthermore, adult neurons, but not other adult neural cell types such as astrocytes and oligodendrocytes, undergo large-scale gene positioning changes (Tan et al., 2021). These observations suggest that enhancers and promoters continue to aggregate and stabilize, even after differentiation into neurons, which can regulate neuronal maturation.

LADs

Gene expression is often associated with lamina-genome interactions. In general, genes that move away from the lamina are concomitantly activated, whereas genes that are located within LADs are transcriptionally inactive (van Steensel and Belmont,

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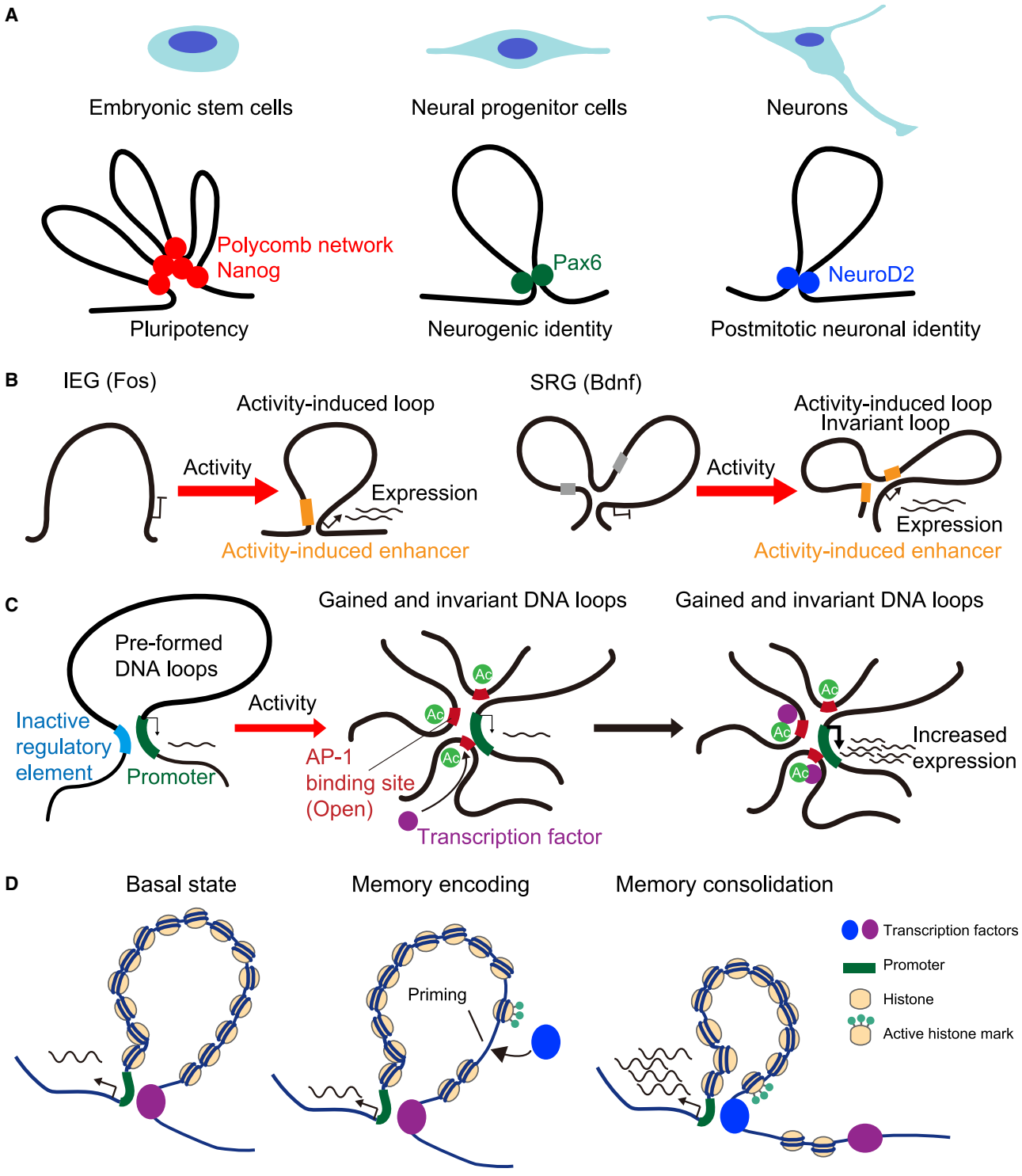

Figure 3. Dynamic 3D genome reorganization during development and in response to stimulation in neurons

(A) Features of 3D genome reorganization during embryonic stem cell—neural progenitor—neuron/glia differentiation.

(B) Dynamic chromatin looping in response to neuronal activation, a model of signal integration for immediate early gene (IEG) and secondary response genes (SRGs).

(C) A potential model for multiple inputs/activity-inducible enhancers to regulate the same downstream gene. Invariant and activity-induced loops form the hub and enable multiple enhancers to contact and regulate the same downstream gene. This process may precede transcriptional activation.

(D) A model of epigenetic priming during sequential phases of learning and memory.

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2017). The 3D positioning of genes in the nucleus plays an important role in neuronal function during brain development (Ito and Takizawa, 2018; Takizawa and Meshorer, 2008). A study on DNA adenine methyltransferase identification (DamID) of lamin B1 to map LADs revealed that in at least four cell types, ESCs, NPCs, terminally differentiated astrocytes, and mouse 3T3 embryonic fibroblasts, LADs are mostly repressive chromatin features (Peric-Hupkes et al., 2010). At a finer level, the dynamic reshaping of nuclear lamina-chromatin interactions of hundreds of genes over ESC to NPC and NPC to astrocyte differentiation has been detected. Associations with nuclear lamina are tightly linked to gene repression, and reorganization of these interactions during differentiation involves many genes that are important for cellular identity. Furthermore, promoter transplantation experiments showed that promoters become active when they are systematically moved from their native LAD location to a more neutral chromatin environment, suggesting a causal relationship between local chromatin state and gene activity (Leemans et al., 2019).

However, other studies over the last decade have demonstrated that some chromatins in proximity to the lamina, especially those portions in proximity to nuclear pores, are euchromatic with some highly expressed genes (Fišerová et al., 2017; Krull et al., 2010). Moreover, a recent study with GO-CaRT has found some highly transcribed genes in LADs characterized by H3K9me2 depletion that are localized close to the repressive nuclear lamina, suggesting that even in the repressive context of genome-lamina interactions, depletion of H3K9me2 may promote transcriptional activation (Ahanger et al., 2021). Through comparison among the genomic interactions with nuclear lamina in the developing mouse, macaque, and human brain, it was found that evolutionarily conserved LADs are enriched for transcriptionally active neuronal genes associated with synapse function. Interestingly, LAD architecture observed in NPCs varies *in vitro* and in the brain *in vivo*. Moreover, region-specific differences of LAD architecture correspond to region-specific gene expression in both the developing human and mouse brain, suggesting that the variety of LAD architecture can control the regional identity of brain cells.

Although it has become clear that dynamic changes in chromosome architecture at various scales play critical roles in brain development, many questions remain to be answered. For example, how is chromosome architecture regulated in space and time throughout the transition from proliferation to differentiation and in postmitotic cells? What exactly are the causal and non-causal roles of 3D genome alterations in neural development? What are the mechanisms underlying transcriptional regulation by chromatin architecture? For instance, several studies reported that transcription can occur without obvious TADs and CTCF-mediated loops at particular stages of early embryogenesis (Du et al., 2017; Ke et al., 2017). Elucidating the detailed mechanisms involved would be a milestone in addressing fundamental questions in neural development.

Dynamic changes in 3D genome organization upon stimulation

Neuronal activation and resulting synaptic activities drive transcriptional programs involving genes critical for proper neuronal circuit formation, maturation, and plasticity. Many immediate early genes (IEGs) are upregulated by neuronal activity and are defined as genes that are rapidly and transiently induced (within 5–10 min of stimulation) by extracellular stimuli without the requirement for *de novo* protein synthesis (Yap and Greenberg, 2018). Since IEGs often encode transcription factors, they can regulate a set of secondary response genes (SRGs), which are expressed in the order of hours in response to signaling and require new protein synthesis. Although there are probably several hundred IEGs, SRGs are far more numerous and are involved in various and cell-type-specific functions in neurons. c-Fos, an neuronal activity-induced IEG, can serve as a pioneering factor in opening chromatin accessibility across the genome at c-Fos-binding sites to regulate SRG expression in the adult mouse hippocampus *in vivo* (Su et al., 2017). In addition, recent studies used high-resolution chromosome conformation capture carbon copy (5C), and Hi-C methods have revealed how activity-dependent enhancers are temporally connected via long-range chromatin loops to regulate gene expression during a wide range of neuronal activity paradigms (Beagan et al., 2020; Fernandez-Albert et al., 2019). IEGs, including *Fos* and *Arc*, connect to activity-dependent enhancers via singular short-range loops that form within 20 min of neuronal stimulation, whereas the SRG *Bdnf* engages with both preexisting and activity-induced long-range loops that form within 1–6 h (Beagan et al., 2020; Figure 3B). Moreover, activity-dependent loops form prior to the peak of mRNA levels of IEGs, suggesting a mechanism to parse multiple inputs for gene expression regulation (Joo et al., 2016). However, the precise mechanism of this delayed peak of transcription after activity-induced loop formation remains unclear. One potential mechanism is the recruitment of a transcriptional activator after epigenetic priming as proposed in the study discussed below (Figure 3C; Marco et al., 2020). The dynamics of the 3D genome structure might allow the sequential control of neuronal activity-induced gene expression, induction of IEGs following by SRGs, resulting in changes in cellular function.

A recent genetic study has shown that chromatin loop formation can be causally linked to activity-dependent gene expression, and such regulation can impact brain function. PLAC-seq identified long-distance interactions between activity-dependent gene promoters and enhancers upon neuronal stimulation (Yamada et al., 2019). The core cohesin subunit Rad21 is required for activity-dependent transcription, and the occupancy of Rad21 at enhancers and promoters is correlated with changes in H3K27ac upon neural stimulation. Furthermore, conditional knockout of Rad21 in adult mouse cerebellar granule neurons significantly reduces enhancer-promoter interactions and delays motor learning in a behavioral test.

Memory formation and recall involve coordinated neuronal activity, and the role of 3D genome organization has been examined in the context of engram ensembles. A multi-omics study, including RNA sequencing (RNA-seq), ATAC-seq, and Hi-C in neurons that were activated during memory encoding, consolidation, or recall in the adult mouse brain has shown sequential changes of 3D genome architecture during different phases of learning and memory (Figure 3D; Marco et al., 2020). First, memory encoding via neuronal activation increases chromatin accessibility, predominantly on enhancers. At this phase, these primed

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loci lack the interaction with promoters and corresponding transcriptional changes, but they engage in *de novo* functional enhancer-promoter interactions during later phases of memory formation, suggesting epigenetic priming. After memory encoding, memory consolidation corresponds with the reorganization of large chromatin segments and increased enhancer-promoter interactions, which enables increased gene expression that presumably allows stabilization of the memory. Furthermore, in the context of memory recall, reactivated engram neurons use a subset of *de novo* long-range enhancer-promoter interactions where primed enhancers are linked to their respective promoters to upregulate genes involved in mRNA transport and local protein translation in synaptic compartments. This study illustrates how the dynamics of 3D genome architecture can support engram formation.

Taken together, the alteration of spatial chromosome structure can be an epigenetic priming event in response to external stimulation, and these changes in chromatin structure potentially control downstream functions, such as gene expression changes, the maintenance of neuronal activity, and plasticity. This epigenetic priming can contribute to the link between environmental changes and brain functions. Since chromatin architecture is cell-type specific and context dependent, it will be interesting in the future to investigate how stimulation-induced changes in the 3D genome may regulate gene expression in other neural cell types, such as glia and microglia, and during other biological processes, such as injury and aging (Fujita and Yamashita, 2020).

Dysregulation of spatial chromatin architecture in brain disorders

Dysregulation of chromatin architecture has been frequently linked to diseases. For example, deleterious mutations in the genes encoding chromatin architectural proteins, such as CTCF and cohesion, cause various developmental abnormalities. Deleterious mutations in cohesin core subunit- or cohesin-related genes cause the multisystem developmental disorder Cornelia de Lange syndrome (CdLS). Mutations in the gene for cohesin loader, NIPBL, were first identified in CdLS, followed by mutations in the genes for the cohesin subunits SMC1A, SMC3, and RAD21. Mutations in the regulatory factors BRD4, ANKRD11, and HDAC8, which regulate SMC3 acetylation and cohesion cycling (Deardorff et al., 2012), were also detected for CdLS (Kline et al., 2018). Individuals with CdLS show diverse symptoms, including intellectual disabilities, anxiety, attention deficit hyperactivity disorder, and autism-like characteristics. In animal models, Smc3-deficient heterozygous mice show increased dendritic complexity and decreased spine density in cortical neurons (Fujita et al., 2017). Neuron-specific Smc3 deletion mice exhibit the same phenotype as the global heterozygous knockout mice, indicating that cohesin function in postmitotic neurons is required for proper neuronal functions (Figure 2B). These mice also demonstrate increased anxiety-related behaviors that are consistent with the symptoms of CdLS. Since Smc3 is an important subunit of cohesin for its recycling and function, these observations suggest that 3D genome organization through cohesin is required to establish neural circuits and expression of specific brain functions.

Causal mutations for some brain diseases have also been shown to impact the spatial genome architecture, such as in Fragile X syndrome (Sun et al., 2018). 5C in B-lymphocytes and brain tissue from individuals with Fragile X syndrome showed severe disruption of TAD boundaries and loss of CTCF occupancy at the specific site adjacent to FMR1 disease-associated short tandem repeats. Future studies will address whether other disease-related large copy-number variants (CNVs) and specific genomic variants located in critical chromatin architecture regulatory regions, such as CTCF binding sites and TAD boundaries, may impact chromatin structure, leading to dysregulation of gene expression.

Recent studies have also revealed spatial chromatin alterations in neurological diseases including schizophrenia (SCZ), bipolar disorder (BD), and Alzheimer's disease (AD) (Bendl et al., 2021; Girdhar et al., 2022; Nott et al., 2019). SCZ and BD risk loci have been reported to be enriched within active neuronal promoters and enhancers in the adult human frontal lobe (Fullard et al., 2018; Girdhar et al., 2018; Hauberg et al., 2020; Roussos et al., 2014). Promoters and enhancers are enriched with trimethyl-H3K4 (H3K4me3) and acetyl-H3K27 (H3K27ac), and these histone modifications are tightly associated with higher-order chromatin structures, including megabase-scale TADs and A/B compartments. Analysis of histone modification landscapes from SCZ, BD, and control brains revealed that widespread disease-associated alterations affect the neuronal H3K27ac acetylome, but not the H3K4me3 methylome (Girdhar et al., 2022). In addition, the authors examined chromosomal domains (known as mini-TADs) as *cis*-regulatory domains (CRDs), which span the spatial clustering of chromatin peaks that extend across 10^4–10^6 base pair chromosomal organization, and showed that CRDs are tightly linked to the structures of self-interacting domains. Furthermore, dysregulated acetylation of mini TAD-level chromosomal domains in the prefrontal cortex of SCZ and BD subjects shows cell-type specificity, with domain hyperacetylation consistently linked to regulatory sequences that are specifically important for glutamatergic neurons, and hypoacetylation to GABAergic inhibitory interneurons and oligodendrocytes and myelination. Similar approaches have been successfully applied to examine open chromatin regions in the postmortem brains of AD patients (Bendl et al., 2021). AD-associated changes in chromatin accessibility varied considerably according to the cell type and brain region. Interactome maps from PLAC-seq identified several parameters, such as AD-risk variants that were linked to more distal active promoters rather than the closest promoter, enhancers harboring AD-risk variants that were PLAC-linked to active promoters of both genome-wide association studies (GWAS)-assigned genes and an extended subset of genes not assigned to GWAS loci, and microglia-specific enhancers harboring AD-risk variants linked to genes expressed in multiple cell types, suggesting cell type-specific disease susceptibility (Gosselin et al., 2017; Nott et al., 2019). Together, these studies suggest that histone modifications and spatial chromatin organization in neurological diseases track the underlying genetic risk architecture and the possible cell types in which they function.

Linking disease-associated genetic variants to target gene regulation via the 3D genome

Over the past decade, a large number of GWAS have identified many genetic variants associated with different brain disorders.

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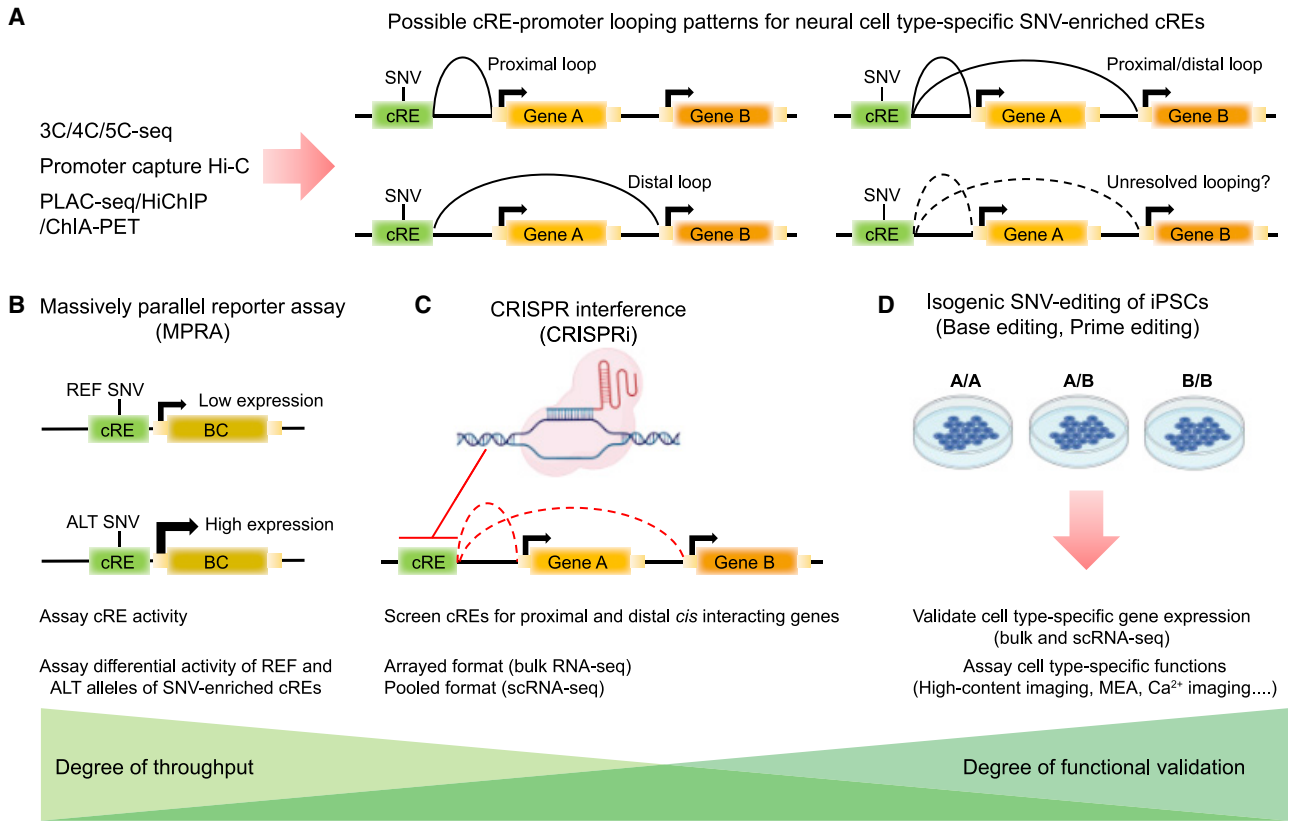

Figure 4. Linking brain-disorder-associated non-coding genomic variants to target genes via chromatin looping and testing their functional impacts

(A) Various methods to profile the 3D genome allow for the high-throughput prioritization of GWAS disease-associated single nucleotide variants (SNVs) in neural-cell-type-specific contexts. SNV-enriched candidate regulatory elements (cREs) defined with biochemical epigenetic marks and accessible chromatin integrated with cRE-promoter looping patterns identity putative SNV-target gene pairs dysregulated in brain disorders. Proximal, distal, proximal/distal, and unresolved cRE-promoter looping patterns are four possible mechanisms of SNV-mediated dysregulation of gene expression via the 3D genome.

(B) Massively parallel reporter assays (MPRAs) are high-throughput screening techniques to assess the differential activity of SNV-enriched cREs between reference (REF) and alternate (ALT) disease-associated alleles in cell-type-specific contexts. Pooled MPRA libraries are assembled with SNV-enriched cREs cloned in front of an expressed barcode (BC) for high-throughput analysis.

(C) CRISPR interference (CRISPRi) targeted to non-coding cREs allow for medium-throughput screening of cRE-target gene pairs. The assay will screen cREs for proximal and distal *cis*-interacting genes and can include SNV-enriched cREs identified in (A). The assay can be performed in an arrayed format with bulk RNA-seq readout or a pooled format with single-cell RNA sequencing (scRNA-seq) readout.

(D) Isogenic SNV-editing of iPSCs via CRISPR base editing or prime editing coupled with functional assays are low-throughput methods to validate the function of disease-associated SNVs in cell-type-specific contexts. Isogenic SNV-edited iPSCs with three possible genotypes (A/A, A/B, and B/B) can be differentiated to desired cell types to assay transcriptomic consequences of SNVs via bulk RNA-seq and scRNA-seq and functional consequences of SNVs such as neural morphology via high-content imaging, electrophysiology via multi-electrode arrays (MEAs), or neuronal signaling via calcium imaging.

In comparison to causal genetic variants for Mendelian disorders that lie mostly within protein-coding regions of the genome, human neurological and neuropsychiatric disease-associated genetic variants are largely found within non-coding regions of the genome that could serve as regulatory elements (Lu et al., 2020; Song et al., 2019, 2020). A challenge for the field is to interpret the functional consequence of these disease-associated genetic variants on disease risk. These non-coding variants are hypothesized to regulate the level of gene expression in the developing and adult human brain in cell type-specific epigenomic contexts, given the unique composition of DNA-binding machinery present within spatiotemporally defined and heterogeneous cell states, such as basal and activity-dependent states in neurons, but the direct target genes and the impact on their expression are not always clear.

Expression quantitative trait locus (eQTL) mapping has provided a powerful *cis*-regulatory logic to link disease-associated genetic variants to target genes in cell type-specific contexts (Consortium, 2020). However, a reliance on large human population sample sizes to identify statistically significant variants or *cis*-eQTLs associated with target gene expression differences is a primary limitation of such eQTL studies. Cell type-specific 3D genome maps provide an alternative framework to link disease-associated genetic variants residing within candidate regulatory elements (cREs) to target genes (Figure 4A). Mapping disease-associated genetic variants to cell type-specific 3D genomes obtained from healthy control samples, such as primary human brain tissue or human iPSC-derived neural cell types, allows for the creation of a predictive framework to link disease-associated genetic variants to candidate target genes via

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enhancer-promoter chromatin contacts in the absence of acquiring primary tissue from patients with neurological or neuropsychiatric disease or creating human disease-specific patient iPSC lines (Rajarajan et al., 2019; Won et al., 2016). Development of computational tools such as H-MAGMA to predict the target genes of disease-associated genetic variants have leveraged Hi-C datasets from primary human developing cortex and adult dorsolateral prefrontal cortex to show that non-coding variants often interact with distal genes, and assigning variants simply to the nearest gene may miss important neurobiological insights into the genetic architecture of neurological and neuropsychiatric diseases (Sey et al., 2020). Simultaneously mapping variants in linkage disequilibrium with GWAS associations to cell type-specific 3D genomes allows for the creation of a more comprehensive predictive framework to identify putative genetic variation conferring disease risk. Incorporation of larger cohorts of human population genetic variation (1000 Genomes Project Consortium et al., 2015), including single nucleotide variants (SNVs) from the recent Genome Aggregation Database (gnomAD) consortium (Karczewski et al., 2020), expands the scope of such a 3D genome predictive framework. Indeed, similar to analyses conducted using the gnomAD datasets (Karczewski et al., 2020) suggesting protein-coding genes intolerant to inactivation, large-scale cohorts of whole genomes could be explored to identify critical non-coding regions of the human genome intolerant to inactivation, which may be important for human neurobiological phenotypes.

Although the 3D genome can be used to link disease-associated variants to candidate target genes, these approaches are largely descriptive and correlative and require further validation via *in situ* and synthetic perturbational approaches to provide deeper neurobiological mechanistic insights into how disease-associated variants drive disease phenotypes. Enhancer-promoter proximity is hypothesized to regulate gene expression, but proximity alone may not explain all cases of gene expression regulation. Proximity may be maintained even for inactive genes or enhancers (Ghavi-Helm et al., 2014), and it is also possible that enhancer transcription in neurons may allow enhancer sequences to exert functional consequences in the absence of direct enhancer-promoter proximity (Kim et al., 2010). Therefore, a variety of synthetic approaches differing in their degree of throughput and functional validation are required to validate how SNVs may exert their impact on disease-relevant phenotypes. As a high-throughput approach with a low degree of functional validation, massively parallel reporter assays (MPRAs) can assess the differential activity of SNV-enriched cREs between reference (REF) and alternate (ALT) disease-associated alleles on activating gene expression in cell type-specific contexts (Figure 4B). A medium-throughput *in situ* perturbational approach with a medium degree of functional validation employs CRISPR interference (CRISPRi) to epigenetically silence cREs to measure the downstream consequences on gene expression in the local genomic area (proximal and distal *cis*-interacting genes) or genome wide (Figure 4C). A low-throughput approach with the highest degree of functional validation involves directly installing SNVs into the genome with techniques, such as CRISPR base editing or prime editing, to directly assess the functional consequence of disease-associated genetic variants on cell type-specific target gene expression and cell type-specific neurobiological functions through comparisons between isogenic conditions (Figure 4D). The advances of human iPSC-derived 2D neural cultures and 3D brain organoids (Qian et al., 2019) provide platforms for these analyses and furthermore for downstream functional analyses of cellular functions (Figure 4D). For further review of these approaches, readers are directed to an elegant recent review (Townsley et al., 2020).

Future perspectives

Our understanding of the role and regulatory mechanisms of spatial chromatin structure and its effects on gene expression and biological functions has progressed significantly. However, as new findings emerge, they raise further questions regarding the mechanism and function of the 3D genome organization in gene expression and physiological functions.

First, we need technological advances for more detailed profiling of 3D genome organization in different cell types and especially from different *in vivo* conditions, such as different developmental stages and basal/stimulated states. Single-cell analysis has led to deeper and more robust classification of brain cell types and advances in technical approaches to understand 3D genome organization at the single-cell level, such as single-cell Hi-C, CUT&Tag, single-cell ATAC-seq, and Oligopaint imaging approaches, will help to specifically address how 3D genome organization affects neural circuit formation and disease pathogenesis in distinct cell types. Future studies to allow for increased resolution, even at the single-cell level, in *in vivo* conditions will help to decipher how chromatin alterations in different contexts link to gene expression changes and functional outcomes.

Second, discerning cause and consequence of the spatial alteration of chromatin architecture is of particular importance. Several studies have shown that the ablation of CTCF or cohesin has limited effects on gene expression and histone modifications, although it causes TAD loss, suggesting that transcriptional regulation might be preserved even if TADs are almost completely disrupted (Nora et al., 2017; Schwarzer et al., 2017). However, deletion of the genes encoding for CTCF or cohesin complex components causes severe deficits in neural circuit formation and animal behavior. The reasons why behavioral changes occur with minimal differences in gene expression remain unclear. It is possible that disorganization of chromatin domain interactions may not affect the basal level of gene expression but impact stimulation-induced gene expression. It is also possible that defects in gene expression is cell-type specific. Indeed, in a single-cell ATAC-seq study of the developing human forebrain, cell-type-specific changes in chromatin accessibility during corticogenesis were detected, and cell type distinctions beyond transcriptional definitions could be resolved (Ziffra et al., 2020). The recent technical advance of simultaneous high-throughput ATAC and RNA expression with sequencing (SHARE-seq) enabled combinatorial measurements of chromatin accessibility and gene expression within the same individual cell, which showed that chromatin accessibility at domains of regulatory chromatin precedes gene expression during lineage commitment (Ma et al., 2020). These studies highlight the importance of investigating the temporal order and casual

relationships among spatial chromatin organization, gene expression, and functional outcomes. Moreover, functional and casual relationship analyses may reveal the fundamental logic underlying the regulation of gene expression by spatial genome organization. Several studies have investigated the alteration of spatial chromosome architecture in different contexts (Marco et al., 2020; Yamada et al., 2019) and demonstrated that spatial chromatin changes serve as a platform for parsing multiple inputs for gene expression regulation and epigenetic priming during learning and memory. Future studies will reveal more basic principles.

Finally, aberrant 3D genome organization has been detected in neurological and psychiatric disorders such as SCZ, BP, and autistic spectrum disorders (Girdhar et al., 2022; Rajarajan et al., 2018). In addition, mutations in the chromosomal architectural proteins, such as CTCF and cohesion, impair neural development and brain functions. The majority of disease-associated SNVs (~93%) are localized within non-coding sequences, suggesting the importance of regulatory elements such as promoters and enhancers in normal development and risk for diseases (Maurano et al., 2012). Addressing the functional link between GWAS variants concerning regulatory DNA variation in common human disease to target genes will contribute to a better understanding of the etiology and pathogenesis of neurological and psychiatric disorders. Furthermore, techniques for the manipulation of 3D genome organization, such as forced chromatin looping, can help to unveil the principles of 3D genome organization and its functions and assist in the development of novel approaches to repair 3D genome disorganization in disease states.

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DECLARATION OF INTERESTS

H.S. is on the advisory board of *Neuron*.

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