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THE GENOME IN 3D: A NEW FRONTIER IN HUMAN BRAIN RESEARCH

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Abstract

Less than 1.5% of the human genome encodes protein. However, vast portions of the human genome are subject to transcriptional and epigenetic regulation and many non-coding regulatory DNA elements are thought to regulate the spatial organization of interphase chromosomes. For example, chromosomal ‘loopings’ are pivotal for the orderly process of gene expression, by enabling distal regulatory enhancer or silencer elements to directly interact with proximal promoter and transcription start sites, potentially bypassing hundreds of kilobases of interspersed sequence on the linear genome. To date, however, epigenetic studies in the human brain are mostly limited to the exploration of DNA methylation and posttranslational modifications of the nucleosome core histones. In contrast, very little is known about the regulation of supranucleosomal structures in brain nuclei. Here, we show that chromosome conformation capture (3C), a widely used approach to study higher order chromatin, is applicable to tissue collected postmortem, thereby informing about genome organization in the human brain. We introduce 3C protocols for brain, and compare higher order chromatin structures at the chromosome 6p22.2–22.1 schizophrenia and bipolar susceptibility locus and neurodevelopmental risk genes (*DPP10*, *MCPHI*) in adult prefrontal cortex and various cell culture systems, including neurons derived from reprogrammed skin cells. We predict that the exploration of three-dimensional genome architectures and function will open up new frontiers in human brain

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research and psychiatric genetics, and provide novel insights into the epigenetic risk architectures of regulatory non-coding DNA.

Keywords

higher order chromatin; chromosome conformation capture; chromatin fiber; chromosomal looping; human brain; genome in 3D

Introduction

A comprehensive exploration of the human genome will have to extend far beyond the fine mapping of its linear sequence, which is comprised of 6 billion basepairs in diploid cells. Importantly, there is a rapidly increasing number of studies that explore, in the human brain, the regulation of various epigenetic markings including DNA cytosine methylation and hydroxymethylation, and many of the (estimated more than 100) residue-specific post-translational modifications such as acetylation, phosphorylation and methylation of the nucleosome core histones (a nucleosome is the elementary unit of chromatin, with 146 bp of DNA wrapped around a histone H2A/H2B/H3/H4 octamer)(1). These epigenetic markings, in various combinations, show a complex sequence-specific distribution in brain and many other tissues, resulting in specific epigenetic signatures of proximal promoters, enhancers, actively expressed genes, silenced genes, facultative or constitutive heterochromatin (such as the pericentric or telomeric repeat sequences at the extreme end of chromosomes)(2–11). However, there is also evidence for coordinated regulation of the epigenome, which includes a non-randomness in the spatial organization of interphase chromosomes, often referred to as ‘loopings’, ‘tetherings’ and ‘globules’. These structures enable non-contiguous DNA sequences, which on the linear genome are separated by various amounts of interspersed sequence, to move into close physical contact with each other (12). Chromosomal ‘loopings’, in particular, are pivotal for the orderly process of gene expression, by enabling distal regulatory enhancer or silencer elements positioned on the same chromosome to interact directly with that specific proximal promoter and the gene’s transcription start site (13, 14). Furthermore, actively expressed genes are more likely to be clustered together and positioned towards a central position within the nucleus, while heterochromatin and silenced loci tend to locate towards the nuclear periphery (15, 16).

Unsurprisingly, therefore, in recent years an increasing number of studies explored three-dimensional genome architectures both in vertebrate(17) and invertebrate(18) model systems and there is little doubt that the regulation of chromosomal conformations is of critical importance for human health, including orderly brain development and function. One well known example relates to Cornelia de Lange Syndrome (CdLS) with an estimated incidence of 1:10 – 30,000 live births among the more frequent genetic disorders (source <http://ghr.nlm.nih.gov>). Of note, CdLS often is associated with severe developmental delay and a range of neuropsychiatric symptoms, including autism spectrum disorder and psychosis(19). The CdLS syndrome (including *Online Mendelian Inheritance of Man (OMIM)* 122470 and 300590) is linked to mutations in the cohesin complex, a multisubunit protein that includes, among others, nipped B-like protein (NIPBL), structural maintenance of chromosomes

proteins SMC1A and SMC3, and histone deacetylase HDAC8 (20, 21). Cohesin is thought to form ring-like structures bringing together DNA segments from different locations, and by interaction with transcriptional co-activators such as Mediator, the complex is thought to promote promoter-enhancer loopings that define cell-type specific gene expression programs(22).

However, despite of the growing realization of the importance of 3-dimensional chromatin architectures for transcriptional regulation, very little is known about their role in the nervous system. Until recently, to the best of our knowledge, only three studies in the literature reported on loop formations in brain tissue(23–25). Other studies used brain as negative control for studies that were primarily focused on the sensory epithelium of the nose (26) or the hematopoietic system(27). Interestingly, there is evidence that at least some of the higher order chromatin structures, including physical interactions of non-contiguous DNA elements and chromosomal loopings, are preserved in human brain collected after death (28) and, furthermore, DNA-histone nucleosomal structures are largely maintained even after 35 hours of autolysis(29). Therefore, it appears feasible to study not only the post-translational decoration of nucleosome core histones but also more complex layers of epigenetic regulation. Recently, for example, chromosome conformation capture (3C) assays and genome-wide histone methylation mappings were employed to the prefrontal cortex of the human and non-human primate brain, revealing multiple physical interactions of DNA elements separated by hundreds of kilobases sites within several neurodevelopmental risk loci (incl. 2q14.1/*DPP10* and 16p11.2) (28).

Based on the above findings then, the ‘genome in 3D’ should be open for investigation in the human brain, including its surrogates such as cultured neurons and glia. Here, we provide an overview on chromosome conformation capture (commonly referred to as 3C) technique, which to date is considered the optimal approach to map and quantify 3-dimensional genome structures (30). We provide illustrative examples to highlight the advantages and limitations of 3C. Specifically, we present chromosome conformation data for the major histocompatibility complex II (MHC II) locus on chromosome 6, long implicated in schizophrenia and other psychiatric disease and recently confirmed by large genome-wide association (GWAS) (31–33).

The 3C technique involves mild crosslinking of nuclei followed by chromatin extraction and restriction enzyme digest, followed by religation of cut DNA fragments with DNA ligase, followed by polymerase chain reaction (PCR)-based quantification of ligation product. The central tenet of 3C is that during the critical religation step, the fusion of the digested DNA ends at the site of chromosomal loopings will result in an artificial DNA sequence that normally is not encountered in the linear genome (Figure 1) (34–39). 3C does not require special reagents such as antibodies and is easily quantifiable and scalable from locus/candidate sequence-specific assays to genome-scale chromosomal interaction maps (39, 40).

Methods

3C. See Supplemental Material for detailed, step-by-step description of protocol

Postmortem tissue—Cerebral cortex from four adult specimens without known neurological or psychiatric disease, ranging in autolysis time from 12–27 hours (Supplemental Material), was dissected from the rostral portions of the frontal lobe (300–500mg/specimen) and homogenized in 1× PBS-buffered, 1.5% formaldehyde to cross-link higher-order chromatin. After 15 minutes, cross-linking was stopped by the addition of glycine to 0.125M final concentration) and samples spun in a tabletop centrifuge at 4000 rpm for 10 minutes at 4°C, then the pellet was resuspended in 2mL of cellular lysis buffer (10 mM Tris-HCl pH8.0/10 mM NaCl/0.2% IPECAL CA-630 (Sigma Aldrich) and incubated at RT for 45 minutes. Next, samples were homogenized by constant pipetting and spun at 5000 rpm, the supernatant removed and the pelleted nuclei stored at –80°C.

Neuronal cell culture—For H9 embryonic stem cells (H9ESC) and differentiated neural cultures (see Supplemental Document for details on differentiation protocol), media was aspirated followed by a quick wash with 37°C warm 1X PBS. Then 5 cc of 1×PBS/1.5% formaldehyde was added to the cultures, the cells scrapped and kept in suspension for 10 minutes. Then cross-linking was stopped by the addition of glycine to 0.125M and cells harvested by centrifugation at 1200 rpm/5 minutes. The pellet is treated with 1× cellular lysis buffer after adding 1/10 volume of protease inhibitor cocktail (Sigma Aldrich), resuspended and left at room temperature for 15 minutes, followed by homogenization and nuclei pelleting (5000 rpm for 5mins), and two washes with 1× New England Biolabs2 (NEB2) restriction enzyme buffer. For each 3C assays on cultured cells, approximately 8–12 ×10⁶ stem cells or differentiated cells were used for input. Restriction Digestion, Ligation and Reverse Cross Linking, DNA Purification and Recovery are described in detail in the Supplemental Material.

3C Quantification—We quantified physical interactions of non-contiguous DNA elements using a PCR-based approach. Primers were designed according to 3C guidelines(30) including 40 cycles of PCR, with 30–32 bp in length, positioned within 200 bp of the Hind III restriction enzyme cut site and a melting temperature of 60°C (see Supplemental Document). PCR products were resolved in 2–3% agarose gels and the level of interaction between any two fragments in the 3C libraries measured semi-quantitatively. To this end, amplicons generated by PCR across a single ligation junction were run in triplicate on 2–3% agarose/1×TAE /130V/25min and band intensities measured by UVP Bioimaging system/Labworks 4.5 software and ImageJ, after background subtractions from blank gel regions. Template titrations (1, 1:2, 1:5, 1:10 etc) of 3C libraries were performed to confirm that 3C PCR differences were within a linear range.

Results

Parsing chromosomal conformations at the MHC II psychiatric susceptibility locus

To illustrate the potential use of chromosome conformation capture in the context of psychiatric disease, we present here novel 3C data that we collected for a portion of

chromosome 6p22.2–22.1, harboring the major histocompatibility complex II (MHC II) locus, conferring significant genetic risk to schizophrenia and related disorders as most recently shown in larger GWAS studies (31–33). These studies identified 33 disease-associated SNPs within 6 megabases of the MHC II locus. Importantly, more than fifty percent of these SNPs are not located near genes (31–33). Because many intergenic regions alter expression of up- and downstream genes (41), the functional exploration of MHC II-associated SNPs will have to include a detailed analyses of the spatial organization of the MHC II locus in 3D. To illustrate the potential knowledge to be gained by the study of MHC II higher order chromatin, we employed chromosomal conformation capture (3C) to interrogate a 230 Kb segment within the MHC II locus.

We employed standard 3C methods and protocols, including bacterial artificial chromosome (BAC) control libraries to control for primer efficiencies (42, 43). 3C libraries were made using 300–500 mg postmortem frontal cortex (postmortem intervals ranging from 12–27 hours) and, in addition, BAC 3C libraries were made using 20 ug of BAC. A mixture of Invitrogen BAC Human RP11 clones (911D8 and 138E19) were used to cover chromosome 6: 27,016,000–27,250,000 (HG19). Primers were designed according to 3C guidelines(30), with 30 bp in length and positioned within 60 bp of the restriction enzyme cut site, a melting temperature of 60°C, and with a GC% content of 45% (Supplemental Document). For 3C assays, BAC control libraries are important in order to control for primer efficiency(30, 37), because after restriction enzyme digest, ligation products between the various non-contiguous restriction fragments are expected to occur equally at random (obviously BACs are not associated with chromatin), and differences between products should only reflect primer efficiency.

Specifically, we explored the physical interactions of DNA elements with an anchor primer positioned at the schizophrenia risk SNP rs6904071 that was positioned in a sequence that in the frontal cortex is enriched with the enhancer-associated mark, histone H3 mono-methylated at lysine 4 (H3K4me1)(44) (Figure 2A). We observed interactions with various DNA elements, which including sequences in the histone gene cluster positioned approximately 75 kb from the Anchor, that showed sharp enrichments for promoter and enhancer-associated histone marks, including histone H3 tri- and mono-methylated at lysine 4 (H3K4me3/me1) and H3 acetylated at lysine 27 (H3K27ac)(44) (Figure 2A, B). Of note, 3C assays in which the critical DNA ligase step was omitted did not result in product, which suggests that the 3C-PCR products in the (ligase-treated) samples indeed reflect physical proximity in higher order chromatin. Thus, based on these preliminary experiments, one could conclude that at least a subset of schizophrenia-associated SNPs in the MHC II locus, including rs6904071, are (i) positioned in intergenic sequences many kilobases apart from the surrounding gene(s), and (ii) defined by distinct epigenetic signature associated with open chromatin-associated histone marks associated with enhancers and (iii) engage in physical interactions with DNA elements that are positioned in close proximity to coding sequences (a histone gene cluster in the case of rs6904071, see Figure 2A) and epigenetically decorated with additional promoter and enhancer-associated histone H3K4 methylation and acetylation marks. This is an interesting finding given that both preclinical and genetic findings associate multiple enzymes involved in H3K4 methylation, including MLL1 and MLL2 methyltransferases and JARID1C demethylase in the neurobiology of

cognition and cortical development(45–49). Furthermore, in the mouse, excessive histone deacetylase activity in prefrontal cortex results in altered expression of MHC II genes, in conjunction with cognitive dysfunction and working memory(50). Therefore, it will be interesting to obtain a more comprehensive 3-dimensional chromosome conformation profile for the MHC II locus, including developmental and disease-associated changes in the brain, complemented by additional studies to clarify the potential role of the aforementioned regulators of H3K4 methylation and acetylation. In any case, as shown by the examples provided here, chromosome conformation capture and higher chromatin mapping at neuropsychiatric susceptibility loci is feasible in postmortem brain tissue collected many hours after death. It should be noted, however, that the signal in the postmortem brains was weaker and in some cases less consistent, compared to the BAC positive control (Figure 2C). Furthermore, there was a significant inverse correlation between brain tissue pH (Supplemental Material) and physical interaction frequencies between the anchor and a restriction fragment separated by 175kb (primer H4, Figure 2B), $R = -0.97$, $P = 0.03$). Given the small sample size in the present study ($N=4$ brains), additional studies are required to test the effects of other confounds including age and tissue autolysis time.

Chromosome conformation decay after tissue autolysis

The reproducible interactions of non-contiguous DNA elements in human (postmortem) frontal cortex, as discussed above for the MHC II locus on chromosome 6p22.2–22.1 (Figure 2), in conjunction with similar findings recently reported for 500kb portions of 2q14.1 and 16p11.2(28), leave little doubt that at 3C assays conducted on postmortem brain provide reproducible products across different brains/donors. However, it remains unclear whether the sensitivity of 3C is affected by tissue autolysis, particularly for some of the weaker interactions. To explore this, we prepared 3C libraries across 230kb of mouse chromosome 13, which corresponds to portions of the human MHC II locus on chromosome 6. Altogether eight 3C libraries in the presence or absence of DNA ligase (a critical step in 3C assays, which will fuse the noncontiguous DNA elements that are in physical proximity to each other) were generated from two adult mouse cerebral cortex specimens. Each brain was processed in parallel, after one hemisphere was preserved by freezing immediately after tissue harvest while the other hemisphere was subjected to 15 hours of autolysis at room temperature. Indeed, while 3/6 primers yielded 3C-PCR products in both animals, consistent amplification both at the 0 and 15 hrs time point was limited to 1/6 primers (Figure 3A, B). We conclude that at least some of the 3C interactions that are detectable in fresh (non-autolytic) brain subsequently undergo decay during the course of autolysis. Importantly, omission of DNA ligase from the 3C assays again did not result in detectable 3C PCR product (Figure 3C), further confirming that positive 3C products in with-ligase assays are specific and very likely to result from physical proximity and direct interaction of the non-contiguous DNA elements.

Modeling (brain) 'genomes in 3D' in culture

The non-random configuration of the chromosomal material inside the nucleus is highly specific for cell type, and shared physical interactions of non-contiguous DNA elements between different cell lines and tissues may not exceed approximately one third of the total number of loopings observed in each specific cell line (51). Given the obvious limitations in

studying human brain tissue, including the potential decay in some of the higher order chromatin structures during autolysis, neuronal and glial culture systems differentiated from embryonic or induced pluripotent stem cells, or directly transduced from skin fibroblasts (1, 52–54) could provide reasonable alternatives. Of note, 3C typically require $10^5 - 10^7$ or more cells as input. Using protocols to generate 10^7 MAP2 immunoreactive neurons in a mixed culture of neurons, glia and Nestin-immunoreactive neural precursors over the course of 35–42 days, starting from 5×10^6 pluripotent embryonic stem cells (ESC, line H9) or induced pluripotent stem cells (iPS) as input material (Figure 4A–G and Supplemental Document), we then employed 3C and interrogated four chromosomal loopings in human prefrontal cortex collected postmortem. Parallel studies were conducted in neuronal cultures differentiated from embryonic stem cell line H9, and HEK293 cells which are known to express a significant number of neuronal transcripts (55). The distance between the interacting fragments ranged from 43 to 932Kb. Two of the interaction (*DPP10* locus on chromosome 2 and *LOC100271836 /SMGIP1* on chromosome 16) had been described in a recent study exploring higher order chromatin structures in human as compared to non-human primate prefrontal cortex(28) while the remaining interactions at NMDA receptor subunit *GRID2* (chromosome 4) and Microcephalin *MCPH1* (chromosome 8) were identified as human homologues of histone H3K9 methyltransferase KMT1E/SETDB1/ESET target sites recently implicated in the regulation of higher order chromatin structures in mouse cerebral cortex(25). Comparative 3C analyses revealed that while some interactions, including the chromosome 2 loop at the neurodevelopmental risk locus, *DPP10*, (encoding a dipeptidyl peptidase-related protein regulating potassium channels and neuronal excitability(56)) were specific for the prefrontal cortex, the remainder of the interactions observed in the PFC also existed in the HEK293 cell and in differentiated cultures. In contrast, 3C interactions in the PFC were not representative for the H9 stem cells (Table 1).

Discussion

With vast portions of the human genome engaged in non-random physical interactions with sequences from the same chromosome potentially separated by hundreds of kilobases, or even with sequences from different chromosomes(40, 57), it is obvious that insight into these mechanisms will become an important part of genomic medicine. Furthermore, psychiatric ailments such as schizophrenia and autism, the majority of single nucleotide polymorphisms and other DNA variants associated with disease are located outside of sequences encoding protein, and many are located in intergenic regions (58). In the present study, we interrogated in human prefrontal cortex higher order chromatin at multiple genomic locations and observed reproducible and specific interactions of non-contiguous DNA elements, many of which could were also found in cultured neurons and other cells. Therefore, at least some of the chromosomal conformations are maintained in postmortem tissue.

Limitations of chromosome conformation capture (3C) in postmortem brain

However, the animal work in the present study demonstrates that even a (for postmortem brain standards comparatively short) postmortem interval of 15 hours, there is loss of

detectable 3C PCR signal for some of the interactions that were readily detected in mouse brain collected immediately after death. Other interactions remained stable in autolytic brain. Thus, caution will be required in interpretation of 3C signals in postmortem tissues, especially in cases where signals between different brains could vary. In case of disease studies, it is probably best to closely matched cases and control for autolysis time and other measures of tissue quality, including pH. The lack single cell resolution in 3C assays is another potential challenge for studies in human brain given the considerable degree of cellular heterogeneity in this tissue. However, there are protocols in place for cell-type specific sorting and separation of neuronal and non-neuronal chromatin from brain tissue in quantities sufficient for these types of experiments (59, 60). Furthermore, there are alternative techniques such as dual or multi color DNA in situ hybridization which are more limited than 3C on the scale of cell populations, while providing single cell resolution(26).

Based on the comparative 3C studies presented here (Table 1), neurons and glia differentiated from induced pluripotent (iPS) and other stem cells, or even cell lines such as *Human Embryonic Kidney* 293 (HEK) which express many neuronal transcripts (55) may be a valuable surrogate to model higher order chromatin from brain tissue, at least for some genomic loci, and therefore may overcome some of the aforementioned limitations. Another alternative could be studies on the olfactory epithelium of the nose which recently was suggested as a model system to study epigenetic dysregulation in schizophrenia, including oxidative stress-related pathophysiology that may operate during neurodevelopment (61) and which would be difficult to explore in adult postmortem brain. However, chromosomal conformations are considered to be highly regulated in a cell-type and tissue specific manner (51), and no single cell culture system is expected to model all molecular, cellular and functional aspects that define mature human cerebral cortex. Consistent with this, adult PFC profiles for the five interactions shown in Table 1 matched only partially to the ones encountered in cultured neurons or HEK cells, and showed little, or no overlap with the profile of embryonic stem cells.

Three-dimensional chromosomal architectures and epigenetic landscapes in brain cells

Many chromosomal areas in the interphase nucleus are thought to be partitioned into 'topological domains, extending over hundreds of kilobases or even megabases, which are defined by robust physical interaction of intra-domain sequences carrying the same set of epigenetic decorations (62). In neuronal chromatin from human prefrontal cortex, for example, sequences that are defined by much higher enrichment with trimethyl-H3K4 (H3K4me3) mark (as compared to non-human primates), including two neurodevelopmental disease loci in chromosomes 2q14.1 and 16p11.2, showed evidence for physical contact of sequences that on the linear genome are spaced 0.5–1 Mb apart(28). Additional work is necessary to extrapolate these principles of spatial organization to other types of histone modifications and epigenetic decorations. Furthermore, presently there are no techniques available that allow nucleosome or bp resolution of chromosomal loopings and tetherings, and it remains unclear for the majority of higher order chromatin structures (both in brain or in peripheral tissues) how many nucleosomes are involved in forming physical contact between the non-contiguous interacting fragments.

Regulation of gene expression by a 3-dimensional genome

The regulation of gene expression in a vertebrate cell goes beyond the genetic and epigenetic architectures of proximal promoters and transcription start sites. This is because transcriptional units inside the cell nucleus are organized as complex dynamic 3-dimensional chromosomal loopings via direct physically interactions of distal enhancer and gene proximal promoter elements (63–65). Notably, 90% of annotated exons are expressed in the human brain in a region-specific manner and with dynamic regulation at various stages of development, and often associated with genetic polymorphisms positioned in regulatory sequences separated by many kilobases from coding sequence(66, 67). Therefore, it can be expected that the exploration of enhancer-promoter interactions, including 3C assays in developing or diseased brain tissue, will provide important information to decipher the regulatory networks that orchestrate gene expression across the lifespan. Furthermore, it is very likely that 3C assays, and more broadly genome exploration in 3D, will provide mechanistic evidence and validation for expression Quantitative trait loci (eQTLs) that are hitherto defined by statistical association between DNA polymorphism and gene expression(68). There are plenty of recent examples in the literature that illustrate the potential of such type of integrative approach. For example, skin pigmentation in human is regulated by the skin pigment gene *OCA2*, which in turn is modulated by regulatory sequences positioned 21kb upstream of the *OCA2* transcription start site, which include SNP rs12913832 which has a strong statistical association with pigmentation(69). Of note, this SNP heavily influences physical interaction between the *OCA2* gene proximal promoter and the -21kb sequences further upstream, resulting in altered expression (70). Another early example is the cancer risk SNP rs6983267, which is positioned in an enhancer sequence that loops around 330 kb of chromosomal sequence to interact with the *Myc* oncogene promoter (71). We predict that similar insights will be obtained for various SNPs that show significant risk for psychiatric and neurological disease in genome wide association studies in the absence of a detectable transcript in *cis* at the genome position harboring the SNP. Clearly, exploration of cell-type specific genome architectures in the developing and diseased brain is one of the most exciting research endeavors in the relatively young field of psychiatric epigenetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chromosome conformation capture

(A) For 3C assays, primers (represented as arrows) are designed within 30 base pairs of the restriction fragments (shown as colored rectangles). For 3C interactions in *cis* (same chromosome), primers typically are placed in 5' to 3' orientation along the same DNA strand to detect looping interactions. Typically the first primer is in a fixed position and the 3C assays are anchored to that specific restriction fragment (light blue in A) and interactions are tested with a set of second primers, each from a different fragment positioned at increasing distances from the anchor. (B) (C) Hypothetical chromosome without (B) or with (C) a loop formation. Theoretically, only (C) will result in PCR product from non-contiguous DNA elements with the anchor. (D) Hypothetical outcome from a typical 3C experiment, revealing in the absence of a loop formation ('no interaction') exponentially declining 3C product intensity with increasing distance from the anchor (A, fragment 0). In contrast, there is a robust interaction between the anchor and the more distal fragment (black/brown fragments 5, 6) in chromosome with loop formation.

Figure 2. Human frontal cortex 3C in the chromosome 6 MHC II schizophrenia risk locus (A) 3C interactions were investigated across a 250kb region on human chr6:27,016,918–27,250,000 (human genome assembly hg19), including a histone gene cluster (HIST) and the *PRSS16* gene. Browser tracks show landscapes for frontal cortex (from top to bottom: H3K4me1 Brain 1, H3K4me1 Brain 2, H3K4me3 Brain 1, H3K4me3 Brain 2, H3K27ac Brain 1) (72). 3C primers (H1, H2, H3, and H4) were designed to interrogate interactions with anchor primer (‘ANCHOR’) at schizophrenia risk SNP rs6904071. (B) Interaction frequencies (IF) of non-contiguous DNA elements in frontal cortex (N= 4, mean $\pm$ S.E.M. after normalization to BAC control), showing a strong tendency for site-specific interactions with Anchor primer (One-way ANOVA $P = 0.06$). (C) Representative agarose gel from 3C-PCR, showing 221 bp product of H1:Anchor primers in 4 adult frontal cortex specimens (Brain (BR) no.5, 29, 39 and 40) with postmortem intervals ranging from 12–27 hours, a 3C library from a BAC with (BAC) and without (BAC-) the ligase treatment, and water control.

Figure 3. Stability of 3C signal in postmortem brain

(A) 3C interactions from mouse cerebral cortex were investigated across 230kb of chr13:21,920,000–22,150,000 (mouse genome assembly mm9), which includes the murine homologue to human chromosome 6 MHC II region shown in Figure 2. Adult mouse cortex H3K4me1, H3K4me3, and H3K27ac landscapes are from (17, 62). Mouse 3C primers (M1, M2, M3, M4, M5, and M6) and Anchor primer at *HIST1H4I* in the histone gene cluster (B) Summary table of 3C interactions from cerebral cortex of two mouse brains, with one hemisphere processed at time of brain harvest and one hemisphere subjected to 15 hours autolysis time at room temperature. Notice that consistent amplification both at 0 and 15 hrs postmortem interval is limited to a primer M5. Notice lack of 3C PCR product in all samples when DNA ligase is omitted from 3C assays, confirming that positive product from (ligase-treated) 3C assays reflect physical interaction of the non-contiguous DNA elements. (C, D) Representative 3C-PCR agarose gels showing products (approximately 200bp) between Anchor and (C) M5, (D) M6 primers from cortical hemisphere with (left) 0 and (right) 15

hours PMI, with (+) and without (–) DNA ligase in 3C assay. Notice lack of detectable product in all (–) and water (w) controls. Notice weaker 3C PCR product after 15 hours of tissue autolysis, particularly for M6 primer.

Figure 4. Modeling higher order chromatin of brain cells in the cell culture dish

(A) Time line summarizing the generation of $> 1.5 \times 10^7$ cells with at least 50% MAP2 immunoreactive neurons in a mixed culture over the course of 35–42 days, from 5×10^6 pluripotent embryonic stem cells (ESC) or induced pluripotent stem cell s(iPS) as starting material, with intermediate steps of embryo bodies and cultures of primary, secondary and tertiary neural rosettes. The bar graphs display RNA expression of pluripotency marker Oct4 (left) and neuronal marker (MAP2) (right) normalized to -actin in fibroblasts (FB), embryonic stem cells (SC), neural rosettes (NR), and differentiated neurons (DN). Data shown as mean $\pm$ S.D. (N=5 independent experiments). Octamer binding transcription factor 4 (OCT4) and microtubule associated protein 2 (MAP2) expression were quantified in fibroblasts (FB), embryonic stem cells (SC), neural rosettes (NR), and differentiated neurons (DN). The OCT4 forward primer (OCT4-F), GTATTCAGCCAAACGACCATCT, OCT4 reverse primer CGATACTGGTTCGCTTTCTCTT. The MAP2 forward primer CCACCTGAGATTAAGGATCA, reverse (MAP2-R) GGCTTACTTTGCTTCTCTGA. - actin (ACTB) forward primer ACCATGGATGATGATATCGC reverse (hACTB-R) TCATTGTAGAAGGTGTGGTG. Representative examples of (B) H9 human ESC colony, (C) embryo body grown in neural induction media; (D) neural rosettes forming after 4 days, plated in laminin and grown in neural induction media; (E) neural rosettes expanding after collection and replating on laminin (F) MAP2 immunoreactive neurons (green) intermingled with Nestin positive precursor cells (red) differentiated from H9ESC (G) Representative image of MAP2+ (green) neurons from skin fibroblast-derived iPS, intermingled with

Nestin-positive precursors (red), differentiated from iPS and other cells. Images in (F)(G) taken with 50x (200x) magnification.

Table 1

Interaction frequencies of 5 non-contiguous sequences: Comparison between postmortem PFC and three different cell types.

Interacting Region 1			Interacting Region 2			Distance between Interacting Region 1 and 2 (kb)	PFC (N=3)	Neuronal Culture (N=3)	Stem cells (ESC; N=3)	HEK 293 (N=3)
Location	kb to nearest TSS	nearest Gene	Location	kb to nearest TSS	Nearest Gene					
chr2: 115,414,659	4,999	DPP10	chr2: 115,926,328	-6,328	DPP10	511,669	++	-	-	++
chr4: 93,222,850	2,700	GRID2	chr4: 93,265,808	-40,258	GRID2	42,958	+++	+++	++	++
chr8: 6,270,810	6,697	MCPH1	chr8: 6,404,143	-140,330	MCPH1	133,333	++	++	-	++
chr8: 6,270,810	6,697	MCPH1	chr8: 6,406,040	-141,927	MCPH1	135,230	++	++	-	++
chr16: 21,518,730	-5,000	LOC100271836	chr16: 22,450,542	-2,213	SMGIP1	931,812	+	ND	-	++++

Summary presentation for physical interactions of non-contiguous DNA sequences at 5 different locations in the genome, as indicated. 3C libraries were created from postmortem prefrontal cortex (PFC), from stem cells (ESC line H9), neuronal culture differentiated from H9 ESC, and HEK293 cells. For quantification, amplicons generated by PCR across a single ligation junction run in triplicate on 2% agarose/1xTAE /130V/25min and band intensities measured by UVP Bioimaging system/Labworks 4.5 software and ImageJ, after background subtractions from blank gel regions. Pixel intensities were expressed as 0 = nondetectable; +, 0.01 - 0.2; ++ = 0.21 - 0.4; +++ = 0.41 - 0.6; ++++ = 0.61 - 0.8; +++++ = 0.81 - 0.99. N = 3 for each cell type or tissue.