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Application of CRISPR/Cas9 to the Study of Brain Development and Neuropsychiatric Disease

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Abstract

CRISPR/Cas9 technology has transformed our abilities to manipulate the genome and epigenome, as applications have expanded from efficient genomic editing to include the targeted localization of effectors to specific genomic loci. By facilitating the manipulation of DNA- and histone-modifying enzyme activities, activation or repression of gene expression, and targeting of transcriptional regulators to defined loci, it is now possible to directly probe the role of gene-regulatory and epigenetic pathways in order to examine their roles in basic biology and disease processes. Here we discuss these emerging CRISPR-based methodologies, with specific consideration of neurobiological applications using human induced pluripotent stem cell (hiPSC)-based models.

I. Epigenetics in Neuropsychiatric Disease

Increasing evidence supports a strong role for transcriptional and epigenetic abnormalities in the development of neurological (Jakovcevski and Akbarian, 2014) and psychiatric (Peña et al, 2014) diseases. Epigenetic mechanisms, such as DNA and histone post-translational modifications, ncRNA-mediated processes, and higher order chromatin structure dynamics regulate a wide variety of neuronal processes and modulate susceptibility to neuropsychiatric illnesses (McGill and Zoghbi, 2014; Rajarajan et al., 2016). Current strategies to study epigenetic processes in disease biology include the manipulation of gene-expression regulatory factors, alteration of non-coding regulatory regions, and pharmacological inhibition of regulatory cascades. Here we discuss application of CRISPR/Cas9 technologies to advance our understanding of the gene-regulatory and epigenetic

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mechanisms contributing to neuropsychiatric diseases. First, we will provide an overview of CRISPR/Cas9 technologies and their reported applications to the study of neurodevelopment and neuropsychiatric disease (particularly Glioblastoma multiforme, schizophrenia, and autism spectrum disorder (ASD)). Second, given the utility of applying CRISPR-based approaches to human induced pluripotent stem cell (hiPSC)-based models of neurodevelopment and disease, we highlight current applications and future challenges in combining CRISPR/Cas9 and hiPSCs-based approaches for the study of a variety of conditions, including Parkinson's disease (PD), ASD, and schizophrenia. Finally, we conclude with a discussion of advancements necessary to facilitate further studies of the aberrant transcriptional and epigenetic mechanisms underlying neuropsychiatric disease.

II. Introduction to CRISPR/Cas9

The clustered, regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR-associated protein) system allows targeted and specific genetic manipulation. It was initially discovered as a key defense mechanism against invading viruses and plasmids in several bacterial species and Archaea (well reviewed in Wang et al., 2016). When microorganisms incorporate invading DNA sequences into their genomes, these sequences are transcribed into CRISPR RNAs (crRNAs) (Ishino et al., 1987) that, when expressed with Cas endonucleases, enables targeting of the endonuclease to the invading DNA (Jinek et al., 2012). In type II CRISPR/Cas9 systems, a noncoding *trans*-activating RNA (tracrRNA) mediates the binding of Cas9-crRNA complexes to target loci (Deltcheva et al., 2011). Provided that the target genetic locus contains a protospacer adjacent motif (PAM) that is compatible with Cas endonuclease binding, the Cas9-crRNA-tracrRNA complex creates double stranded breaks (DSBs) in the target locus (Bolotin et al., 2005; Mojica et al., 2009; Shah et al., 2013). In this way, CRISPR/Cas9 serves as a key component of microbial immune defenses.

This process has been adapted to achieve targeted genome editing in experimental systems and has been simplified through technical advances since its original discovery. Great experimental simplification was achieved with the demonstration that crRNA and tracrRNA can be engineered into a single guide RNA (sgRNA) (Jinek et al., 2012). Furthermore, a variety of Cas9 endonucleases have now been isolated from multiple organisms; each orthologue has different properties that enable adaptive fine-tuning of experimental approaches. The *Streptococcus pyogenes* Cas9 (Sp Cas9) is the endonuclease used most frequently, largely because its PAM consists of NGG, which occurs approximately every 8 base pairs in the human genome (Sander and Joung, 2014), enabling the targeting of Sp Cas9 to virtually any locus. An additional Cas9 endonuclease was derived from *Staphylococcus aureus* (Sa Cas9), whose small size of 1,053 amino acids (Ran et al., 2015), compared to that of 1,368 amino acids for Sp Cas9 (Chylinski et al., 2014), offers an advantage over other Cas9s in situations in which delivery of large constructs may be problematic. Beyond the available native Cas9 orthologues, several endonucleases have been further engineered to confer additional properties. Mutation of one of the two Cas9 nuclease domains creates a “nickase Cas9 (nCas9)” that cleaves only one of the target DNA strands (Mali et al., 2013), and thus enhances target specificity, as only adjacent nCas9-mediated cuts will bring about the desired DSB (Mali et al., 2013; Ran et al., 2013; Shen et al., 2014).

Finally, mutating both nuclease domains generates a “dead” or “deactivated” Cas9 (dCas9) (Qi et al., 2013) to which a variety of proteins can be fused for numerous applications (e.g., Kiani et al., 2015) (Table 1).

Current CRISPR/Cas9 technologies enable a variety of gene-editing approaches. Following the generation of a DSB by Cas9, the break is repaired through one of two endogenous processes. In the first, non-homologous end-joining (NHEJ) attempts to correct the break, but often creates random insertion or deletion mutations at the DSB site, which in turn can lead to gene-knockout (Lieber, 2010). Second, the DSB repair process known as homology-directed repair (HDR) can be harnessed to generate the replacement of DNA sequence (knock-in) at the site of the DSB using an exogenous DNA template (Doudna and Charpentier, 2014). Because Cas9 endonucleases can manipulate several sites when used with multiple sgRNAs, this approach permits chromosomal inversions, deletions, and translocations (Blasco et al., 2014; Maddalo et al., 2014; Torres et al., 2014). Therefore, CRISPR/Cas9 serves as an improved strategy for DNA deletion, insertion, site-directed mutagenesis, and chromosomal manipulation (see Figure 1 for an illustration of these processes).

III. Applications of CRISPR/Cas9 to Study Transcriptional and Epigenetic Mechanisms Underlying Disease Processes

CRISPR/Cas9 can be used to modulate expression of histone- and DNA-modifying enzymes, alter DNA-binding sites of factors that regulate transcription, perturb non-coding RNA expression, modify non-coding gene-regulatory regions, and manipulate higher-order chromatin structure. In this section, we focus on the application of CRISPR/Cas9 to study gene regulatory and epigenetic pathways in neurobiology contexts.

Applying CRISPR to Understand Developmental Neurobiology

The development of the nervous system requires the orchestration of complex gene-regulatory pathways, activating those that promote differentiation and suppressing those involved in maintaining the undifferentiated state, and various groups have used CRISPR/Cas9 approaches to study the roles of transcription factors in directing neurodevelopment.

Deletion of key transcription factors can help elucidate their critical functions. For example, the transcription factor *Satb2* is necessary for the proper maturation of axonal projections from layers 2 and 3 of the cerebral cortex to the contralateral side of cortex (Alcamo et al., 2008; Britanova et al., 2015); deletion of *Satb2* with CRISPR/Cas9 in mice at embryonic day 15.5 ablated proper development of cortical projections (Shinmyo et al., 2016). Additionally, CRISPR/Cas9-mediated knockout of the bHLH transcription factor NeuroD demonstrated its requirement in retinal progenitors cell cycle exit and photoreceptor maturation (Taylor et al., 2015).

In addition to deleting transcription factors, CRISPR/Cas9 can be used to remove specific enhancer regions, thereby disrupting their gene-regulatory functions. For example, Endothelin signaling is essential to the proper differentiation of neural crest cells, promoting differentiation of key craniofacial structures (Pla and Larue, 2003; Ruest et al., 2004).

CRISPR/Cas9-mediated deletion of an endothelin-responsive enhancer (*Mef2C-F1*) in mice resulted in decreased *Mef2C* expression and embryonic lethality due to palate and craniofacial obstruction (Hu et al., 2015). Moreover, *Blimp1* is a zing-finger transcription factor that regulates the development of the proper ratio of bipolar cells and rods in the developing mouse retina; CRISPR/Cas9-mediated deletion of an enhancer ~12kb upstream *Blimp1* phenocopied the effects of *Blimp1* knockout in the retina (Wang et al., 2014).

By recruiting activators or repressors of transcription, dCas9 fusion proteins can be used to directly increase or decrease gene expression. For example, Frank et al (2015) focused on the role of *Grin2c* enhancer elements in the maturation of cerebellar granular neurons (CGNs) at three different stages of post-natal development, finding that dCas9-VP64-mediated activation of a specific enhancer region of *Grin2c* was a key driver of CGN maturation (Frank et al., 2015).

Finally, CRISPR/Cas9-mediated gene-editing can introduce protein tags to target loci, facilitating biochemical and epigenetic studies of endogenous gene products. The transcriptional targets of the zinc-finger transcription factor Rotund have been poorly defined due to a lack of ChIP-validated antibodies, and Li et al. 2015 employed CRISPR/Cas9 to incorporate protein tags in the *Rotund* gene, finding that Rotund preferentially interacts in heterochromatic regions, particularly at a specific *bric-a-bric* Rn target locus (Li et al., 2015).

Altogether, these examples highlight the utility of CRISPR/Cas9 approaches to study the roles of gene regulatory processes in neurodevelopment.

Using CRISPR/Cas9 to Study Diseases of the Central Nervous System

Glioblastoma multiforme—The chromatin assembly factor 1 subunit A (CHAF1A) is involved in DNA replication and repair and epigenetic silencing (Kaufman et al., 1995; Moggs et al., 2000; Ridgway and Almouzni, 2000; Dong et al., 2001; Quivy et al., 2001) and has been linked to several cancers (Barbiere et al., 2013; Barbiere et al., 2014; Wu et al., 2014). Confirming the direct role of CHAF1A in the pathogenesis of Glioblastoma multiforme, CRISPR/Cas9-mediated knockout of *CHAF1A* in two glioblastoma cell lines suppressed proliferation and increased levels of apoptosis (Peng et al., 2016). Similar effects were obtained by removing regulatory regions rather than genes: CRISPR/Cas9-mediated deletion in a patient-derived gliomasphere line of one downstream locus, a CTCF-bound insulator region of platelet-derived growth factor receptor, alpha polypeptide (*PDGFRA*), resulted in increased *PDGFRA* mRNA and protein levels and conferred a growth advantage (Flavahan et al., 2016). Owing to straightforward phenotypic effects such as altered rates of cellular proliferation and/or death, we foresee that CRISPR-based genome-wide screens could soon be applied towards the unbiased study of the molecular mechanisms underlying Glioblastoma multiforme and other malignancies of the nervous system.

Neuropsychiatric conditions—Genes implicated in Autism Spectrum Disorder (ASD) are predominantly involved in synapse formation, chromatin remodeling, and transcriptional regulation (De Rubeis et al., 2014). Loss-of-function studies of several ASD-associated genes have now been conducted via CRISPR/Cas9 editing. First, deletion in mouse

embryonic stem cells (mESCs) of *CHD5*, a chromatin helicase DNA binding protein that is essential for proper neuronal differentiation (Egan et al., 2013), led to increased expression of the retrotransposon *MERVL*, with a corresponding decrease in H3K27me3 and increase in histone variant H3.1 and H3.2 deposition at the *MERVL* locus (Hayashi et al., 2016). Second, introduction of nonsense mutations and deletions of the ASD-associated genes *Pten* (Goffin et al., 2001) and Katanin P60 subunit A-like 2 (*Katnal2*) (Neale et al., 2012; O’Roak et al., 2012), led to changes in neuronal cell body size and dendritic morphology in mouse neurons (Williams et al., 2016).

Variants in a locus containing *transcription factor 4* (*TCF4*) have been repeatedly associated with Schizophrenia (Schizophrenia Psychiatric Genome-Wide Association Study Consortium 2011; Schizophrenia Working Group of the Psychiatric Genomic Consortium, 2014). *In utero* CRISPR/Cas9 knockout of *TCF4* led to decreased neuronal firing and hyperpolarization of cortical neurons (Rannals et al., 2016). Translating ribosomal affinity purification (TRAP) revealed increased expression of *Kcnq1* and *Scn10* in *TCF4*-knockout neurons, and CRISPR/Cas9-mediated deletion of these two genes rescued the hypoexcitable phenotype of *TCF4* knockout neurons (Rannals et al., 2016).

Although ASD and schizophrenia are both complex genetic disorders arising through the interplay of highly penetrant rare mutations and common variants of small effect (De Rubeis et al., 2014; Neale et al., 2014; O’Roak et al., 2012; Schizophrenia Psychiatric Genome-Wide Association Study Consortium 2011; Schizophrenia Working Group of the Psychiatric Genomic Consortium 2014), CRISPR-Cas9 mediated gene editing or regulation of expression will facilitate isogenic molecular and phenotypic characterizations, improving our understanding of the impact of each of these disease-relevant mutations.

IV. Applications of CRISPR/Cas9 Techniques to Human Induced Pluripotent Stem Cell Models

In this section, we provide a brief overview of human induced pluripotent stem cell (hiPSC)-based models and highlight recent advances in the field that have used CRISPR/Cas9 strategies to study gene-regulatory processes in neuropsychiatric disease.

Overview of hiPSC-based models

hiPSC-based models facilitate the study of normal developmental biology and disease mechanisms, and can serve as a component of drug-discovery platforms (Mertens et al., 2015; Brennand et al., 2015; Hartley and Brennand 2016; Xu et al., 2016). Expression of the four Yamanaka factors (*C-MYC*, *KLF4*, *OCT4*, and *SOX2*) reprograms adult somatic cells into hiPSCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007), from which neurons can be differentiated by sequential treatment with growth factors or small molecules in a manner recapitulating neuronal development *in vivo* (Watanabe et al., 2005). Through a related strategy, induced neurons (“iNeurons”) can be directly converted from fibroblasts via overexpression of the transcription factors *NEUROD*, *ASCL1*, *BRN2*, and *MYT1L* (Vierbuchen et al., 2010; Pang et al., 2011), or through overexpression *Ngn2* in hiPSCs (Zhang et al., 2013) or neural progenitor cells (NPCs) (Ho et al., 2016). Because

many subtypes of neurons and glia have been implicated in neuropsychiatric disease (Gotter et al., 2001), it is crucial to study defined cell types, both alone and in combination, in order to understand how they function in health and disease. While discussed in great detail by others (reviewed in Ho et al., 2015), a variety of protocols have reported differentiation or induction of glutamatergic (Zhang et al., 2013, Ho et al., 2016), GABAergic (Sun et al., 2016), dopaminergic (Theka et al., 2013), and serotonergic (Lu et al., 2016) neurons, as well as astrocytes (Emdad et al., 2012), oligodendrocytes (Espinosa-Jeffrey et al., 2016; Thiruvalluvan et al., 2016), and microglia-like cells (Muffat et al., 2016).

Limitations of hiPSCs-based models

There remain several limitations of hiPSC-based models. First, current diagnostic criteria for psychiatric disorders are based upon symptomatology alone and do not classify patients according to underlying disease biology (*Diagnostic and statistical manual of mental disorders* (5th edition), 2013); for this reason, recruitment and selection of patients with psychiatric diagnoses frequently yields heterogeneous samples lacking shared disease etiology. Although this problem may be overcome by studying hiPSCs only from individuals with well-defined genetic lesions and/or disease-relevant endophenotypes (e.g., Wen et al., 2014; Lee et al., 2015; Chailangkarn et al., 2016), such an approach necessarily limits the generalizability of any findings to the broader disorder. Second, because hiPSC-derived neurons most resemble fetal brain cells in temporal and spatial patterning (Mariani et al., 2012; Lancaster et al., 2013; Miller et al., 2013; Vera and Studer, 2015), they better model aspects of disease predisposition than the disease-state itself (Brennand et al., 2015). Even neural organoids (“brain balls”), which provide some three dimensional context to *in vitro* models (reviewed in Hartley and Brennand, 2016), still most resemble fetal, rather than adult, brain tissue (Pasca et al., 2015). Third, somatic mosaicism - differences in genomic content between cells from the same organism – occurs both *in vivo* and in hiPSC-based models (McConnell et al., 2013), and the extent to which cell-based models recapitulate the diversity of mosaicism detected in the human brain remains unclear. In fact, reprogramming of fibroblasts and subsequent differentiation to neurons may induce substantial copy number variants (McConnell et al., 2013) not found in the source somatic cells. Because single-cell resolution studies have demonstrated somatic mosaicism in both adult human brain (McConnell et al., 2013) and hiPSCs neurons (Erwin et al., 2016), single-cell approaches may be increasingly necessary to query intercellular variability in genomic content and phenotypes (Bardy et al., 2016). Nevertheless, despite these limitations, many groups have already successfully employed hiPSC-based models for the study of neuropsychiatric disorders (e.g., Brennand et al., 2011; Brennand et al., 2015; Chailangkarn et al., 2016; Balachandar et al., 2016).

Using CRISPR/Cas9 to probe gene-regulatory pathways in hiPSCs models of neuropsychiatric disease

Parkinson’s Disease (PD)—CRISPR/Cas9-mediated genome editing introduced a PD-associated SNP in the enhancer region of alpha-synuclein (*SNCA*), which resulted in increased H3K27ac at this enhancer region and increased *SNCA* expression in hiPSCs-derived neurons (Soldner et al., 2016). Binding of two transcription factors, NKX6-1 and EMX2, was SNP-dependent; both showed increased binding to the protective allele.

Altogether, these experiments demonstrate that a PD risk allele in the *SNCA* enhancer affects the regulatory actions of two transcription factors, leading to over-expressed *SNCA*, a key pathological hallmark of PD (Soldner et al., 2016).

Fragile X Syndrome and Autism Spectrum Disorder (ASD)—Fragile X Syndrome (FXS), caused by a CGG trinucleotide repeat expansion in the 5'-UTR of the *FMR1* gene (Verkerk et al 1991), is the most common form of inherited intellectual disability (Folstein and Piven, 1991), and approximately 20% of people with FXS also exhibit ASD-like behaviors (Crawford et al 2001; O'Donnell and Warren 2002). The CGG expansion is associated with suppressed expression of *FMR1* via abnormal epigenetic marks at the *FMR1* promoter, such as increased DNA cytosine methylation (Coffee et al., 1999; Urbach et al 2010), decreased histone tail acetylation (Urbach et al 2010), increased H3K9me3 methylation (Avitzour et al, 2014), and loss of H3K4me2 (Avitzour et al, 2014). CRISPR/Cas9-mediated deletion of the CGG repeat in FXS hiPSCs resulted in normalized expression of *FMR1*, a change that was maintained through subsequent differentiation to NPCs and mature neurons; moreover, hypermethylation of the CpG sites upstream of *FMR1* was reversed with CGG deletion (Park et al., 2015). Chromatin immunoprecipitation revealed that CGG repeat deletion led to increased acetylation of the histone 3 tail and H3K4me2 (both transcriptionally activating) and decreased H3K9me2 (transcriptionally repressive). This was the first causal demonstration that removal of CGG expansion was sufficient to reverse the transcriptionally repressed chromatin state of *FMR1* in FXS patient-derived cells (Park et al., 2015).

Genetic studies have identified the chromodomain helicase DNA binding protein 8 (CHD8) to be significantly enriched in ASD-associated *de novo* mutations (Neale et al., 2012; O'Roak et al., 2012a; O'Roak et al., 2012b). The generation of heterozygous *CHD8*^{+/-} hiPSCs via CRISPR/Cas9 editing and their subsequent differentiation into NPCs and neurons, revealed differential expression of genes involved in neural development and Wnt signaling (Wang et al., 2015), consistent with hiPSC-based models of idiopathic ASD, which have reported similar deficits (Marchetto et al., 2016). While CRISPR/Cas9 hiPSC-based studies of ASD have focused on rare mutations to date, they have unequivocally demonstrated that these highly penetrant mutations are sufficient to produce molecular and functional phenotypes in human neurons *in vitro*.

Schizophrenia—Many loci have been implicated in the risk architecture of schizophrenia, but only one schizophrenia-linked gene has been manipulated by CRISPR-based methods in hiPSCs thus far. Although not significant in genome wide associate studies, the disrupted in schizophrenia 1 (*DISC1*) gene was identified in a Scottish family in which a chromosomal 1:11 translocation co-segregated with incidence of schizophrenia and major depressive disorder (Millar et al 2000a; Millar et al. 2000b). *DISC1* function has been implicated in neurite outgrowth, neuronal development and migration, synaptic maturation, and cell proliferation (Bradshaw and Porteous, 2012). Srikanth et al., 2015 employed TALENs and CRISPR/Cas9 to delete a critical *DISC1* exon in control hiPSCs and showed increased *DISC1* nonsense-mediated decay of splice transcripts and altered Wnt signaling (Srikanth et al., 2015). Additionally, Wen et al., 2015 used TALENs to both reconstruct a disease-

associated mutation in controls and repair it in *DISC1*-patient derived hiPSCs (Wen et al., 2015). Across isogenic comparisons, changes in *DISC1* genotype produced altered expression of key synaptic genes in hiPSCs-derived neurons and perturbations in synaptic activity (Wen et al., 2015). Altogether, these results convincingly demonstrated a causal role for *DISC1* in neural development and synaptic function. Isogenic comparisons made possible via CRISPR/Cas9 gene-editing approaches have helped to elucidate disease mechanisms in hiPSC-derived neurons.

V. Future Directions

In the next section, we discuss ways in which advances in CRISPR/Cas9 technology may further expand our understanding of neuropsychiatric disease. See Figure 2 for an illustration of these processes.

Advances in chromatin biology

An ongoing challenge of using chromatin immunoprecipitation (ChIP) to study protein-DNA interactions is the paucity of suitable antibodies. To overcome this limitation, it is now possible to use CRISPR/dCas9 to tag a putative DNA-binding protein with a ChIP-compatible epitope, enabling interrogation of DNA-binding patterns of any protein of interest (Savic et al., 2015). By integrating epitope tags in different exons of alternatively spliced genes of interest, this method can also be employed to study DNA-binding patterns of specific splice isoforms of a transcription factor (Li et al., 2015). Even more scalable, as it does not require genetic editing, is the use of sgRNAs to target dCas9 to a locus of interest, and then performing ChIP experiments to dCas9 itself or a protein-tagged dCas9 (Fujii and Fujita, 2015). This approach enables profiling of all proteins interacting at single genomic locus, and has already been used to characterize site-specific proteomic binding patterns by combining dCas9-ChIP with by mass spectrometry (Zhang et al., 2016). The incorporation of selective isotopic labeling of amino acids in cell culture (SILAC) with dCas9-ChIP would further allow investigation of protein turnover rates, at any locus, and over defined periods of time (Fujita and Fujii, 2014).

Moving forward, we expect further development of dCas-epigenetic regulator fusion proteins beyond those that are currently available (see Table 1 for current options). Presently, fusion with VP64, VIPR, and p300a increase gene expression, whereas fusion to KRAB decreases gene expression (reviewed in Vora et al., 2016). In theory, it should be possible to target any known histone- or DNA-modifying enzyme to any specific loci. Already, targeted DNA methylation via dCas9-DNMT3A (DNA methyltransferase 3A) (Vojta et al., 2016; Liu et al., 2016; McDonald et al, 2016) and DNA demethylation via dCas9-TET1 (ten-eleven translocation 1 dioxygenase) (Liu et al., 2016) has been demonstrated. Gene expression can be altered by the addition and removal of a variety of histone post-translational modifications including acetylation, methylation, phosphorylation, sumoylation, and ribosylation, each with different effects depending on the number of specific histone residues modified, and with new “epigenetic editing” tools we expect further elucidation of the function of gene-regulatory complexes as well as targeted exploration of epigenetic modifiers.

Finally, the localizability of dCas9 and amenability to fusion to numerous proteins allows visualization of specific genomic loci, which is a promising approach for further investigation of chromatin dynamics. Already, some groups have tagged TALEs with fluorescent proteins to visualize repetitive elements in living cells (Miyazari et al., 2013; Ma et al., 2013; Thanisch et al., 2013). One report demonstrated imaging of coding and non-coding loci dynamics in living cells using Sp dCas9 fused to GFP (Chen et al., 2013). Strikingly, a different group (Ma et al., 2015), demonstrated multi-color dynamic genome imaging by employing dCas9s from different bacterial species fused to varying fluorescent proteins. Based upon these initial and promising studies, we propose that these and similar approaches will soon permit tracking of the spatial and temporal dynamics of 3D chromatin structures.

Investigation of non-coding gene regulatory regions in neurological and psychiatric disease

It is critical to establish causal roles of non-coding gene-regulatory regions in neuropsychiatric disease. For example, of the 108 loci that are significantly associated with schizophrenia risk (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), many occur in non-protein coding regions (Ripke et al., 2013) and several are enriched in brain-specific promoter and enhancer regions (Roussos et al., 2014), where they likely regulate gene expression (Fromer et al., 2016). CRISPR/Cas9 can be used to perform tiling screens to identify enhancers (Korkmaz et al., 2016) and non-enhancer unmarked regulatory elements (Rajagopal et al., 2016), and to systemically map promoter-enhancer interactions (Fulco et al., 2016). We propose that these kinds of CRISPR/Cas9 techniques will enable identification and investigation of non-coding gene-regulatory regions and their roles in neuropsychiatric disease.

Growing evidence that loci found within insulators, enhancers, and promoter regions contribute to the risk of developing neuropsychiatric diseases implicate a potential role for higher-order chromatin structure in these conditions. In fact, higher-order chromatin structures are key regulators of genome function and gene expression (Bickmore and Van Steensel 2013). CRISPR/Cas9 has been employed to delete, invert, and duplicate sites involved in chromatin looping and to study the effects of such manipulations on gene expression and function (Guo et al., 2015; Lupiáñez et al., 2015; Yang et al., 2016). Increasing evidence implicates higher-order chromatin structure in brain function and cognition (reviewed in Rajarajan et al., 2016); for example, Bharadwaj et al (2014) identified enhancer regions of the NMDA receptor component *GRIN2B* and demonstrated a neuronal activity-dependent alteration in enhancer-transcription start site interactions. Additionally, CRISPR/Cas9-mediated deletion of loop-interacting regions (including a schizophrenia risk allele in the *GRIN2* locus), were associated with altered looping interactions and decreased *GRIN2B* expression (Bharadwaj et al., 2014). For these reasons, CRISPR/Cas9 editing is an exciting approach for targeting perturbations of chromatin looping and studying the effects of these manipulations on gene expression and neuronal function. Future investigations must apply these approaches to hiPSCs-derived neurons and animal models in order to explore the role of disease-associated gene-regulatory regions in neuropsychiatric conditions.

Investigation of long non-coding RNA function

Long non-coding RNAs (lncRNAs) have important gene-regulatory functions (Quinn and Chang, 2016) and have been implicated in psychiatric (Chubb et al., 2008; Sartor et al., 2012; Le-Niculescu et al., 2013; Liu et al., 2014; Spadaro et al., 2015;), neurodevelopmental (van de Vondervoort II et al., 2013), and neurodegenerative disorders (Faghihi et al., 2008; Szafranski et al., 2015). CRISPR/Cas9-mediated genomic deletion screening has already identified 51 lncRNAs that regulate cancer growth (Zhu et al., 2016), and this approach could be applied to identify lncRNAs with important roles in the regulation of gene expression in hiPSCs-derived neurons. In order to investigate the mechanisms by which individual lncRNAs regulate gene expression, further advancements would need to enable targeting of lncRNAs to specific loci. A recently developed technique, termed “CRISP-Disp,” allows targeting of large, putative regulatory RNAs to genomic loci of interest (Shechner et al., 2015). Using this technique, Shechner et al, 2015 complexed *Streptococcus pyogenes* (Sp) dCas9-VP64 to increasingly larger RNA cargos, demonstrating that these complexes maintained their transcriptionally activating effects at defined reporter loci. Having established that this was the case for artificial RNA cargos, the investigators next loaded lncRNAs to dCas9-sgRNA complexes, finding that expression of genes at the targeted loci changed in a manner consistent with previously defined gene-regulatory effects of the lncRNAs chosen (Shechner et al., 2015). Such CRISPR-based approaches could theoretically explore functions of regulatory RNAs, providing the means to directly study the consequences of regulatory RNA targeting at any locus of interest.

VI. Summary

Neurological and psychiatric diseases frequently involve pathogenic alterations in gene-regulatory and epigenetic processes. With the advent of CRISPR/Cas9, targeted manipulation of a variety of regulatory factors, non-protein-coding sequences, and non-coding RNAs can now explore the role of these pathways in neuropsychiatric conditions. Future approaches will expand the ability of CRISPR/Cas9 to effect specific perturbations in epigenetic pathways. By applying these new tools to a variety of hiPSC- and animal-based models, CRISPR/Cas9 techniques will help reveal new epigenetic mechanisms underlying the biology of neuropsychiatric disease.

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Highlights

- An overview of CRISPR/Cas9 techniques and their applications to the study of neurodevelopment and neuropsychiatric disease.
- An introduction to hiPSC-based models of neuropsychiatric disease and CRISPR/Cas9 applications to these models.
- The potential for CRISPR/Cas9 approaches in a variety of models, including hiPSCs, to advance understanding of psychiatric illnesses

Figure 1. General Applications of CRISPR/Cas9

A. sgRNA guides Cas9 endonuclease to target locus of interest. A double stranded break (DSB) is made and is repaired in one of two ways. In non-homologous end-joining (NHEJ), DNA is deleted or inserted at the site of the DSB and the gene is knocked out. In homology-directed repair (HDR), a template strand is supplied with homology to the cut region; template DNA is then inserted at the site of the DSB.

B. “nickase” Cas9. Cleavage of one of the two nuclease domains of Cas9 results in nickase Cas9 (nCas9). DSBs are made only at a site in which adjacent single-strand cuts are made by nCas9, each guided by their own sgRNA. A double stranded break is made when single-strand cuts are made at adjacent sites. The DSB can then be repaired with either HDR or NHEJ. This allows for improved specificity of endonuclease-mediated cuts.

C. CRISPR/Cas9 effectors. Fusion of “deactivated” or “dead” (dCas9) to a transcriptional effector allows targeted increase or decrease in the expression of a gene of interest

D. CRISPR/Cas9-mediated genomic rearrangements. DSBs at two sites by two separate sgRNAs can lead to inversion of the region in between the two DSBs.

Fig. 2a

Fig. 2B-D

Figure 2. Future applications of CRISPR/Cas9

A. **(Left).** Inserting ChIP-compatible epitopes into a DNA-binding protein of interest. A DSB is made adjacent to a gene for a DNA-binding protein of interest and is repaired by HDR using a template strand for a ChIP-compatible epitope. Expression of the fusion protein allows chromatin immunoprecipitation (ChIP) experiments for proteins without

ChIP-compatible antibodies. Here, a transcription factor binds to its target regions. The chromatin is cross-linked and sheared. Antibodies to the epitope are then applied to precipitate out fragments of DNA bound to the transcription factor. **(Right)**. Unbiased CRISPR/Cas9 ChIP using dCas9; allows for immunoprecipitation and proteomic characterization of a specific chromatin region. sgRNA guides dCas9 or an epitope-tagged dCas9 to a region of interest. The protein/DNA complex is cross-linked and sheared. An antibody to either dCas9 or the epitope-tagged dCas9 is used to immunoprecipitate the target chromatin fragment. After reversing cross-links, all of the proteins at that particular region can be analyzed.

B. Fusion of dCas9 to histone and DNA-modifying enzymes; allows such an enzyme to be targeted to a specific region and transcription to be effected accordingly

C. Chromatin dynamics. dCas9 is fused to fluorescent proteins and targeted to two regions of interest to see if they interact with each other (image for both fluorescent proteins). Sequential imaging with different sgRNA-guided fluorescent proteins (FPs) allows tracking of live chromatin interactions

D. A ncRNA can be delivered via dCas9 and its effects can be studied at the target locus.

Table 1

Current applications of CRISPR/dCas9 technologies

Effector Description	Effect on	Transcription
VP64	4 tandem repeats of herpes simplex virus protein 16, VP 16	Activation
VP160	10 tandem repeats of herpes simplex virus protein 16, VP16	Activation
VP192	12 tandem repeats of herpes simplex virus protein 16, VP16	Activation
VP64-dSpCas9-BFP-VP64	4 tandem repeats of herpes simplex virus protein 16, VP 16 at N-terminus of dCas9, and blue fluorescent protein with 4 tandem repeats of VP16 at the N-terminus	Activation
SunTag	Repeating peptide array that recruits transcription effector proteins	Activation
MCP-VP64	RNA hairpins from male-specific bacteriophage 2 (MS2) along with MS2 coat protein (MCP) fused to VP64 activation domain	Activation
Synergistic activation mediator (SAM)	2 MS2 hairpins and MCP fused to p65 -HSF1; MCP-p65-HSF1 activators bind the MS2 hairpins as homodimers, resulting in 4 copies of MCP-p65-HSF1 scaffolding onto the SAM sgRNA, which is bound to dSpCas9-VP64	Activation
dSpCas9-VP64-p65-Rta	VP64, p65, and Rta activators fused to dCas9	Activation
dCas9-p300	dCas9 fused to the histone acetyltransferase p300 to add H3K27ac to target loci	Activation
KRAB	Kruppel-associated box domain of Kox1 fused to dCas9	Inhibition
SID4X	4 tandem repeats of mSin3 domain (SID4X) fused to dCas9 scaffold	Inhibition
LSD1-dNmCas9	dCas9 fusion to histone demethylase LSD1 removes transcriptionally activated H3K4 methylation at target loci	Inhibition