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Neuroepigenetic Editing

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Abstract

Studies of the mammalian nervous system have revealed widespread epigenetic regulation underlying gene expression intrinsic to basic neurobiological function as well as neurological disease. Over the past decade, a critical role has emerged for the neural regulation of chromatin-modifying enzymes during both development and adulthood, and in response to external stimuli. These biochemical data are complemented by numerous next generation sequencing (NGS) studies that quantify the extent of chromatin and DNA modifications in neurons. Neuroepigenetic editing tools can be applied to distinguish between the mere presence and functional relevance of such modifications to neural transcription and animal behavior. This review discusses current advances in neuroepigenetic editing, highlighting methodological considerations pertinent to neuroscience, such as delivery methods and the spatiotemporal specificity of editing. Although neuroepigenetic editing is a nascent field, the studies presented in this review demonstrate the enormous potential of this approach for basic neurobiological research and therapeutic application.

Keywords

Epigenetic editing; Chromatin; Neuroscience; Psychiatric disease

1 Introduction

Regulation of gene expression is a fundamental mechanism of nervous system development and plasticity. Like other cells, neurons possess a genome capable of expressing programmed output. However, neurons are unique in the degree to which they respond and adapt to external stimuli. Epigenetic regulation of neuronal gene expression is a fundamental mechanism by which environmental stimuli are transmitted into neurobiological substrates, capable of driving behavior in both health and disease. This review focuses on the emerging field of neuroepigenetic editing, a technique by which specific epigenetic biochemical modifications can be targeted to a single genomic locus in order to causally link these events to altered gene expression and consequent effects on neural function and behavior.

Epigenetic mechanisms of gene regulation include the expression and activity of histone-modifying enzymes, global regulation of histone posttranslational modifications (hPTMs) and DNA methylation (DNAm), as well as regulation of noncoding small and long RNAs [1, 2]. These mechanisms elicit defined programs of gene expression during development and throughout life, as well as respond to sensory experience. Stable biochemical modifications to chromatin and DNA are capable of eliciting long-term changes in gene expression that underlie various forms of plasticity and memory. The nervous system is continually responsive to environmental stimuli throughout life since the integration of such

signals is intrinsic to the proper function of the organism. The field of neuroepigenetics has demonstrated that experience-induced changes to the neuronal epigenome can be persistent and act, in part, to regulate transcriptional responses. These mechanisms underlie the transcriptional memory necessary for the nervous system to develop and adapt.

Over the past decade, a critical role has emerged for the neural regulation of chromatin and DNA-modifying enzymes both during development and adulthood and both basally and in response to external stimuli [3–10]. These biochemical data are complemented by numerous next generation sequencing (NGS) ChIP studies that quantify the extent of hPTMs in neurons and other cell types in the nervous system in the contexts of mammalian development [11, 12], learning paradigms [13–15], and neurological and psychiatric disease [14, 16–18]. In addition, whole genome bisulfite-sequencing studies, as well as methylation-sensitive immunoprecipitation-based sequencing approaches, have revealed a critical role of DNA methylation and hydroxymethylation in regulating neuronal function in learning and disease models [9, 10, 19–21]. In sum, these studies point to a contribution of aberrant neuroepigenetic events to the pathogenesis of neuropsychiatric disease states.

While NGS and biochemical studies have strongly correlated epigenetic reprogramming to changes in neuronal gene expression, these studies are intrinsically limited in their elucidation of the precise causal relevance of epigenetic modifications to gene expression. Experience-dependent deposition of hPTMs at a given gene of interest is analyzed in the context of global chromatin changes; thus, it is not possible to discern direct action from pleiotropic effects. Epigenetic editing tools, which can exogenously introduce a given chromatin modification at a single target locus [22–24], are necessary to discriminate between the mere presence of hPTMs and causal relevance of such mechanisms to gene expression. Further, such tools can be used to identify new gene regulatory elements, such as distal promoters and enhancers [25, 26], the genetic diversity of which have been linked to aberrant neurobiological function [11, 27–31]. This review focuses on a small but growing number of compelling studies that have applied locus-specific epigenetic editing to the nervous system.

Several unique features of the nervous system underscore the utility of neuroepigenetic editing to elucidate mechanisms of gene regulation. First, prior studies to examine the role of particular gene products have largely relied on their exogenous overexpression, either through virally mediated or transgenic techniques. Both methods drive expression of the gene of interest to a much greater extent than the physiological range of gene activation, which is typically between one- and twofold from basal levels for most regulated genes in postmitotic neurons. By reprogramming endogenous mechanisms of gene activation, neuroepigenetic editing has the capacity to drive biologically relevant gene expression. Second, neurobiological application typically requires the targeting of endogenous genes rather than reporter systems, promoting (1) the elucidation of bona fide mechanisms of experience-dependent gene regulation and (2) the potential therapeutic applications of neuroepigenome engineering. Finally, epigenetic editing lends itself to studies of chronic changes in gene expression, as these tools can potentially deposit long-lasting modifications to histones and DNA. This approach can therefore be used to examine persistent mechanisms of gene regulation that underlie neurobiological function.

2 Epigenetic Editing Tools

There exist several excellent reviews of the various methods of epigenetic editing [22, 24, 32, 33], which cover tools that target reporter systems as well as endogenous loci. This review will focus specifically on epigenetic editing in neurons, particularly applied to endogenous genes. The majority of the published literature utilizes gene expression profiling to assess the efficacy of neuroepigenetic editing tools, but a few assess chromatin modifications as well [31, 34–36]. These latter examples allow conclusions to be drawn on the causal transcriptional relevance of specific histone modifications. Figure 1 illustrates the main tools available for epigenetic editing.

The first study of epigenetic editing at an endogenous locus, exemplifying many of the themes of this review, utilized zinc-finger proteins (ZFPs) targeting the human vascular endothelial growth factor (VEGF) gene in cultured human embryonic kidney (HEK) 293T cells [37]. ZFPs designed to recognize and bind the *VEGF* promoter were fused to a truncated version of the histone methyl-transferase, G9a, to repress VEGFA expression via deposition of H3 lysine 9 dimethylation (H3K9me2). ZFP-G9a activity, as well as that of a catalytic mutant version, was assayed by in vitro methylation of purified histone H3 and quantitative chromatin immunoprecipitation (qChIP) of cellular histones, establishing the utility of this functional domain in epigenetic editing. Importantly, while ZFP-G9a effectively repressed expression of endogenous VEGFA in cell culture, it was not effective in repressing expression from a luciferase reporter plasmid, indicating that the function of this repressor relies on a chromatinized genomic context. Because methylation of H3K9 leads to a spreading of repressive methylation via recruitment of HP1, the authors analyzed H3K9me2 at sites 500 bp up- and down-stream of the ZFP binding site and found that, indeed, ZFP-G9a induced H3K9me2 enrichment at sites both proximal and distal to the ZFP binding site [37]. This result was the first to establish that targeting an exogenous histone methyltransferase to an individual gene can regulate the endogenous transcriptional machinery.

Transcription activator-like effectors (TALEs) are similar to ZFPs in that they rely on a protein-DNA interaction, determined by the primary amino acid structure [38, 39]. TALEs, derived from plant pathogenic bacteria, consist of a DNA-binding domain that contains tandem repeats of 34-aa sequences (termed monomers) that are required for DNA recognition and binding. Each TALE DNA-binding monomer contains a tandem repeat variable domain (RVD) which can bind to one base pair in the DNA [39]. Several groups have published protocols and resource papers on TALE and ZFP design and cloning, as well as open-source platforms for in silico binding assays [38, 40–42].

The most recently developed tool for neuroepigenetic editing is derived from the prokaryotic RNA-guided endonuclease, CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 system [43–46]. Cas9 can be targeted to a specific genomic locus of interest using a rationally designed single guide RNA (sgRNA). A nuclease-deficient Cas9 (dCas9) fused to an effector domain can thus be used as an epigenetic editing tool. The ease of design and synthesis as well as the scalability of the CRISPR/Cas9 system has led to its use

as an endonuclease in neurons [47, 48], but only a relatively few preliminary studies have demonstrated the utility of CRISPR/dCas for locus-specific neuroepigenetic editing [49–51].

With regards to the selection of editing tools, the specificity of target sequence binding is perhaps the first consideration. In this respect, accessibility of the target site is of particular importance with respect to epigenetic editing of chromatinized endogenous loci in neurons. For example, restriction enzyme or DNaseI hypersensitivity mapping of “open chromatin” can be an efficient means to select accessible target sites. One study utilized this approach to design ZFP-Kruppel-associated box (KRAB) and ZFP-p65 proteins to repress and activate the *CDK2* gene, respectively, and demonstrated target gene-specific repression using a microarray [52]. With respect to target specificity, a small number of publications report comparisons of the various tools within a single study [34, 53, 54]. Genome-wide mapping of ZFP, TALE, and CRISPR/Cas9 binding by ChIP-sequencing (ChIP-seq) has revealed substantial off-target localization of DNA-binding domains [54–58]. However, because these proteins rely on genome scanning to find their putative binding sites, ChIP-seq leads to false-positive identification. That is, off-target localization rarely corresponds to changes in gene transcription or chromatin accessibility [56, 57, 59, 60]. For example, ChIP-seq analysis of affinity-tagged NFD (no functional domain)-ZFPs found ~25,000 off-target binding sites, yet less than 2.8% of these target sites correlated with changes in nearby gene expression by RNA-seq [57]. Additional studies of specificity found that increased off-target zinc-finger nuclease (ZFN) activity is correlated with the concentration of transfected ZFN [61], while Cas9 off-target interactions can be computationally predicted based on frequency of highly similar genomic target sites [48]. Further, considering the evidence that these tools function within specific epigenetic contexts [37], off-target effects would require improbable off-target DNA binding to gene regions of similar epigenetic microenvironments to the targeted high-affinity site (i.e., gene promoters). These findings indicate the importance of rational tool design and empirical validation to avoid potential off-target effects.

3 Special Considerations for Epigenetic Editing in Neuroscience

3.1 In Vivo Delivery Methods

Beyond the selection of the appropriate neuroepigenetic editing tool, special consideration must be given to the application of these tools in the brain, particularly with respect to delivery method. While it is possible to introduce such tools to cultured cells by biological particle delivery (i.e., “gene gun”) [62] and conventional transfection [37, 63], these methods have not been commonly used for in vivo brain delivery. As summarized in Fig. 2, the most widely applied in vivo delivery method is stereotaxic injection of viral vectors expressing the tool of interest. Such viruses include adeno-associated virus (AAV) [68, 69], characterized by long-term expression but limited packaging capacity (~4.5 kb); herpes simplex virus (HSV) [70], characterized by its short half-life (~7 days) and large packaging capacity (~14 kb); and lentivirus [69, 71], which allows both long-term expression and large packaging capacity but is varied in its safety in vivo due to genomic integration [69]. Spatial specificity must be considered in selection of delivery method, given the diversity of brain regions and neuronal and non-neuronal cell types. Furthermore, as there is meager published data on the stability of editing-induced chromatin and DNA modifications in neurons [34], it

is crucial to consider the induction and stability of induced modification with respect to developmental time period, potential toxicity of the regulated gene, and the schedule of measured behavioral endpoints.

AAV delivery of neuroepigenetic editing tools has proved promising in preclinical models of neurodegenerative diseases. Briefly, striatal delivery of AAV-ZFP-p65 transcriptional activators targeting the endogenous glial cell line-derived neurotrophic factor (GDNF) gene rescued the deficits in a preclinical Parkinson's disease model [63]. Studies to combat Huntington's disease found that AAV-ZFP-KOX1 fusion was sufficient to repress expression specifically of the mutant huntingtin (*HTT*) gene in mice [72]. In addition to AAV-ZFPs, AAV-TALE-VP64 activators are capable of activating gene expression in prefrontal neurons in vivo [36].

Alternatively, the CRISPR/Cas9 system, while just beginning to be applied for in vivo neuroepigenetic editing [49–51], has been applied to neurons primarily to date as a nuclease [47, 66, 73, 74]. Several in vivo applications of CRISPR/Cas9 rely on a recently developed Cre-dependent Cas9 knock-in mouse model [64], combined with viral or particle-mediated delivery of guide RNAs to neurons. For example, AAV delivery of a guide RNA to Cas9 mice was effective in editing the autism-risk gene *CHD8* [73]. The main limitation of the AAV system in neuroepigenetic editing is its relatively small packaging limit, which is not sufficient to incorporate an expression vector containing a sgRNA and a dCas9 fused to an effector domain. The recent development of smaller Cas9 systems that are compatible with AAV [75] may allow the use of this tool in neuroepigenetic editing approaches, although it still precludes the inclusion of all but the tiniest effector domains.

HSV has emerged as the delivery method for all studies of neuroepigenetic editing in psychiatric models to date, due to its neuronal specificity and large packaging size [70, 76]. HSV-ZFPs targeting the *Fosb* [34, 65] or *Cdk5* [35] gene have been applied to preclinical models of stress and depression. Despite the relatively short expression of 5–7 days, stereotaxic injection of HSV expressing ZFP-G9a fusions was sufficient to regulate both basal and induced gene expression, as well as behavior [34, 35, 65]. In addition, Cre-dependent HSVs harboring a floxed stop codon upstream of the ZFP expression cassette enabled neuroepigenetic editing of the *Fosb* locus in a cell-type-specific manner within an injected region [65], an approach critical to the elucidation of neuronal gene function.

While the application of HSV delivery of CRISPR/dCas9 for neuroepigenetic editing is currently under development, lentiviral delivery of CRISPR/Cas9 has had some traction. One study that applied a dual lentivirus approach to study the efficacy of a split Cas9, reported that split Cas9 had lower expression levels and fewer offtarget effects but still retained 43% of the desired effect at the target site, compared to 92% for full-length Cas9 [77]. Furthermore, a landmark study of dCas9-mediated DNA methylation utilized lentivirus constructs expressing dCas9 fused to ten-eleven translocation-1 (Tet1) or DNA methyltransferase-3 (Dnmt3), to drive DNA demethylation and methylation, respectively, in cultured neurons and in vivo [51]. In this case, a highly innovative lentivirus cassette co-expressed mCherry fluorescent protein, enabling fluorescence-activated cell sorting (FACS)

of sgRNA-expressing cells and specific quantification of DNA methylation at the targeted genomic loci [51].

While the current neuroepigenetic editing literature primarily describes viral transduction of editing tools, in vivo delivery of viral vectors limits their translational application, due to the requirement of stereotaxic injection and the potential infectious nature or immune responses elicited by virus injection. To address these limitations, one group recently explored the direct neuronal delivery of preassembled Cas9 ribonucleoprotein (RNP) complexes [66], in which Cas9 is fused with N-terminal nuclear localization signal (NLS) arrays to enable cellular uptake. Stereotaxic injection of 4xNLS-Cas9-2xNLS RNPs harboring a tdTomato guide RNA into a reporter mouse (Ai9) allowed direct quantification of Cas9 activity as a measure of neural tdTomato expression [66]. Furthermore, a seminal study applied purified zinc fingers targeting the *Ube3* locus in preclinical models of Angelman's syndrome, an imprinting neurodevelopmental disorder [67]. This study, described in further detail below, applied ZFP-VP64 fusions that contained the 10-aa transduction domain of the HIV transactivator protein (TAT, residues 48–5719), to deliver purified protein to the brain. The fusion construct specifically bound its target region as assayed by electromobility shift assays and ChIP assays in N2a cells lines, as well as activated *Ube3* target gene and protein expression in mice in both hippocampus and cerebellum [67]. These encouraging results show that widespread protein activation can be accomplished in the brain in vivo using purified epigenetic editing proteins, with no apparent immunological response, garnering hopes for the eventual clinical application of this approach to neuroepigenetic editing.

3.2 Spatial and Temporal Control

Given the tremendous cellular diversity in the central nervous system, it is crucial to develop applications for cell-type-specific epigenetic editing. Brain tissue consists of thousands of intermingled cell types, each with unique gene expression profiles, connectivity, and roles in behavior. Furthermore, temporal control of epigenetic editing, ideally limited to the extent of observed endogenous effects, is necessary to elucidate the causal function of experience-dependent gene regulation. Because inducible gene expression systems that allow spatiotemporal control of neuroepigenetic editing have been applied only in a limited number of cases in neuroscience to date [36, 65], it is helpful to mine studies in other cellular systems for applications in neurons. For example, one study applied doxycycline-inducible, repressive ZFPs to tumor suppressor genes in a breast cancer cell line [78]. This study compared ZFP-KRAB and ZFP-Dnmt3 transcriptional repressors with respect to promoter DNA methylation status and breast cancer cell colony formation. By examining these phenomena both during doxycycline-induced repressor expression and after a washout period, the authors observed differences in the relative stability of KRAB- and Dnmt3-induced DNA methylation [78]. Thus, chemically inducible expression enables elucidation of the perseverance of exogenous epigenetic modifications beyond expression of the epigenetic editing tool.

Cell-type-specific expression of epigenetic editing tools is also achieved through the incorporation of a loxP-stop (33polyA signal)-loxP (LSL) sequence, rendering expression inducible by Cre recombinase, which can be transgenically or virally co-expressed (Fig. 3a).

In a recent study, transgenic mice expressing Cre recombinase in specific neuronal cell types were injected with HSV that express ZFP-p65 or -G9a fusions in a Cre-dependent manner, revealing cell-type-specific behavioral effects of neuroepigenetic editing of the *Fosb* gene within the targeted brain region [65]. Cas9 nuclease expression can also be regulated by viral Cre delivery to a knock-in mouse containing Cas9 linked via a self-cleaving peptide (P2A) to an enhanced green fluorescent protein (EGFP) [64]. Prefrontal cortex injection of AAV-sgRNA-Crc targeting the *NeuN* gene led to efficient effects 3 weeks after viral transduction [64], suggesting that development of a Cre-dependent dCas9-fusion transgenic mouse may be useful for spatially and temporally controlled neuroepigenetic editing in vivo.

Beyond inducible expression, inducible dimerization approaches can uncouple the expression of the DNA-binding domain (ZFPs [59], TALEs [36], and Cas9 [55, 79]) from that of the transcriptional regulatory domain. This allows basal accumulation of inert components that are activated, via dimerization, with precise spatiotemporal control (Fig. 3b). A comprehensive study of modular, light-activated TALE effectors utilized TALE fused to light-sensitive cryptochrome 2 (CRY2) protein and its binding partner, CIB1, fused to an epigenetic effector domain [36]. CRY2 and CIB1 fuse upon CRY2 conformational change induced by blue-light stimulation. AAV transduction of the light-inducible CRO-TALE: CIB1-VP64 system to primary cortical neurons activated a twofold induction of endogenous target genes, while CRY2-mSin3 led to repression. Gene regulation was observed within 4 h of light stimulation and persisted for 12 h, with transcript levels continuing to rise 30 min after the end of illumination, despite dissociation of CRY2-CIB1 dimers within 15 min. The authors went on to demonstrate a loss of H3K9 acetylation at the promoter targeted by CRY2-mSin3, demonstrating the utility of this approach for neuroepigenetic editing.

Additional development of light-activated dimerization includes the combination of light-sensitive dimerizing proteins, GIGANTEA (GI) and LOV, with ZFPs for controlled dimerization in human cells [59]. In this case, GI-ZFP and LOV-VP64 proteins were tested for activation of a luciferase reporter in HEK293 cells. Similarly to light-inducible TALEs, a short light pulse led to persistent induction of the target gene for 4 h, with expression saturating after 12 h [59]. Similarly, light-induced dimerization of dCas9-CIB1 and sgRNA-CRY2 activated endogenous *ASCL1* gene activity in HEK293 cells up to 3 h following light pulse, normalizing after 18 or 30 h [45, 46].

While light-induced dimerization is not a straightforward therapeutic approach, chemically induced dimerization addresses issues of spatiotemporal control and carries therapeutic potential. One study used a rational design strategy, based on the crystal structure of the Cas9/sgrNA/DNA complex, to split Cas9 into two fragments and added rapamycin-sensitive dimerization domains to either half [77] (Fig. 3c). Split dCas9-VP64 significantly activated expression of endogenous target genes following rapamycin treatment, although there was ~10% activation with rapamycin alone. To apply inducible dimerization to neuroepigenetic editing, additional studies must assay the temporal specificity and efficacy of these approaches in catalyzing chromatin and DNA modifications in neurons in vivo.

Consideration of the relative merits of various editing tools, delivery methods, and approaches to spatiotemporal control are critical to the application of epigenetic editing in

neuroscience. A small but impactful collection of studies has demonstrated the potential utility of these approaches to elucidating the causal molecular function of epigenetic modifications in brain diseases.

4 Applications to Neuropsychiatric Disorders

4.1 Neurodegenerative Disorders

Pharmacological modulation of DNA- or histone-modifying enzymes has shown promise in preclinical studies of neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases [80, 81]. However, small-molecule drugs broadly inhibit enzymatic activity, leading to global epigenetic and transcriptional changes. To address this limitation, several studies have applied gene targeting to the study of neurodegenerative disorders. One early study utilized a 6-OHDA lesion model of Parkinson's disease, which causes death of midbrain dopaminergic neurons and motor abnormalities [63]. The authors targeted *Gdnf* for epigenetic activation, since prior studies have found that overexpression of GDNF protects against dopaminergic cell death in Parkinson's disease models. Stereotaxic striatal delivery of AAV-ZFP-p65 transcriptional activators targeting *Gdnf* protected against loss of dopaminergic neurons as well as the behavioral sequelae [63].

More recently, a CRISPR/Cas9 deletion approach tested the functional relevance of a GWAS-identified risk variant (single nucleotide polymorphism, SNP) in a distal enhancer of *a-synuclein* (*SMT4*), a gene implicated in Parkinson's disease [28]. CRISPR/Cas9 gene editing in pluripotent stem cells was used to generate the two enhancer alleles distinguished by the identified SNP. This approach led to the discovery that α -synuclein is regulated differently by specific alleles, via direct recruitment of specific transcription factors [28]. Applying this approach in neurons, to directly interrogate the mechanism by which enhancer hPTMs regulate allele-specific transcription factor binding, can exploit such a finding.

Epigenetic editing approaches have also been applied to the study of Huntington's disease (HD), which is characterized by mutant *HTT* that harbors an extended CAG leading to a polyglutamine (Q) repeat in the expressed protein. An ingenious approach to perturb mutant *HTT* expression examined the notion that longer CAG repeat domains contain more ZFP target sites and should thus be bound and repressed more at any given ZFP concentration [72]. This study systematically explored the binding modes of different-length ZFPs to long repetitive DNA tracts, and their relative efficacy in gene repression mediated by ZFP fused to a Kox1 domain. Kox1 recruits the co-repressor KAP1, inducing long-range repression through the spread of heterochromatin. Remarkably, in vivo, AAV-mediated delivery of the most effective *HTT*-ZFP-Kox1 to a HD mouse model was sufficient to reduce HTT protein aggregate, protect against decline in motor coordination, and alleviate the clasping phenotype [72], establishing a proof-of-principle for the therapeutic potential of neuroepigenetic editing in this heritable neurodegenerative disease.

More recently, CRISPR/Cas9 nuclease has been applied to mesenchymal stem cells (MSCs) extracted from the bone marrow of YAC128 mice, which carry mutant *HTT* [47]. Lentiviral delivery of CRISPR/Cas9 was sufficient to nick mutant *HTT* DNA and to reduce expression of mutant *HTT* in MSCs [47]. As in the case of CRISPR/Cas9 gene editing in Parkinson's

disease [28], this study falls short of an application in neurons in vivo but holds promise for the utility of CRISPR/Cas9-mediated neuroepigenetic editing.

Further evidence of the therapeutic potential of targeted epigenetic editing in neurodegenerative disease comes from a study of Friedreich ataxia (FRDA), a neurodegenerative and cardiac disease caused by a repressive trinucleotide (GAA) repeat expansion in the first intron of the *Frataxin* gene [82]. A TALE-VP64 transcriptional activator targeting the human *Frataxin* promoter was tested for efficacy by nucleofection into a mutant FRDA cell line. TALE-VP64 expression increased primary transcript elongation past the GAA repeat and led to higher levels of mature mRNA [82]. VP64 is a viral transcriptional activator that does not interact with the epigenome [83]. Indeed, there was no effect on transcription when TALE-VP64 was combined with inhibitors of either histone deacetylase or DNA methyltransferase. This innovative approach was then tested in fibroblasts derived from a FRDA mouse model, YG8R, containing a human mutant *Frataxin* knockin gene to the mouse *Frataxin* locus. YG8R mouse fibroblasts nucleofected with *Frataxin*-TALE showed increased Frataxin mRNA and protein relative to control plasmid. To advance the therapeutic potential of this approach, it will be necessary to deliver *Frataxin*-TALE-VF64: to all neurons and cardiomyocytes, as well as to address potential immunogenicity of the TALE fusion.

4.2 Neuropsychiatric Disorders

Chronic exposure to drugs of abuse or stress regulates transcription factors, chromatin-modifying enzymes, and hPTMs in discrete brain regions. Furthermore, addiction and depression are highly heritable, yet it has been difficult to identify the specific genes involved, which suggests a possible role for epigenetic gene regulation in contributing to their heritability. We investigated the mechanism linking chromatin dynamics to reward pathology by applying ZFP-p65 or ZFP-G9a, targeting histone H3 lysine 9/14 acetylation (H3K9/14ac), a transcriptionally active mark, or H3K9me2, which is associated with transcriptional repression, respectively, to the *Fosb* gene. Considerable prior research has implicated this gene in the actions of drugs of abuse and of stress [6, 84]. We found that these ZFPs were sufficient to modify histones at the targeted region of the *Fosb* promoter in nucleus accumbens, a brain reward region, and to control drug- and stress-evoked transcriptional and behavioral responses. Intriguingly, *Fosb*-ZFP-G9a was sufficient to block cocaine-induced *Fosb* activation via interference with CREB phosphorylation [34], providing direct evidence of the hierarchy between chromatin modifiers and transcription factors in gene regulation.

We further examined the consequences of cell-type-selective induction of *Fosb* in the brain, with respect to stress susceptibility. *Fosb*-ZFPs were expressed in specific neuronal cell types using Cre-dependent HSV expression in mice transgenic for Cre recombinase in one of two types of NAc principal neuronal. We found that stress susceptibility is oppositely regulated by the specific cell type and targeted histone modification [65], as predicted from the cell-type-specific expression profile of *Fosb* following stress [85]. This work presents the first demonstration of cell- and gene-specific targeting of histone modifications, which model naturally occurring transcriptional phenomena underlying social stress behavior.

Fosb is just one of thousands of genes under epigenetic control in the context of stress and drug exposure. For example, there is growing evidence that cyclin-dependent kinase 5 (Cdk5) expression in NAc influences reward-related behaviors [86]. A recent study found that HSV transmitted ZFP-p65 and ZFP-G9a targeting the *Cdk5* locus in NAc were sufficient to bidirectionally regulate *Cdk5* gene expression via enrichment of their respective histone modifications at the *Cdk5* promoter [35]. Further, *Cdk5*-targeted H3K9/14ac increased cocaine-induced locomotor behavior, as well as resilience to social stress. Conversely, *Cdk5*-targeted H3K9me2 attenuated cocaine reward but had no effect on stress-induced behavior. These data are especially compelling given that conventional *Cdk5* overexpression or knockdown caused opposite behavioral phenotypes [86–88], demonstrating the importance of targeted epigenetic remodeling tools to invoke subtle, yet physiologically relevant changes in gene regulation.

While CRISPR/dCas9-mediated neuroepigenetic editing has not yet emerged in the literature with respect to neuropsychiatric disease, a recent study applied CRISPR/Cas9 gene editing to investigate mechanisms underlying autism spectrum disorder (ASD) [73]. CRISPR/Cas9 was used to generate a germline heterozygous mouse mutant lacking *Chd8*, which encodes chromodomain helicase DNA-binding protein 8, for which a de novo mutation has been strongly associated with ASD. CPID8 encodes an ATP-dependent chromatin remodeler and regulates many ASD risk genes involved in neurodevelopment and synaptic function. *Chd8* heterozygous mice displayed a partial ASD phenotype while ChIP- and RNA-sequencing revealed a broad role for CHD8 in genome regulation in specific brain regions [73]. To complement the global knockout approach, AAV co-expressing *Chd8* sgRNA and a GFP-KASH nuclear affinity domain [48] was injected into the adult NAc of a germline Cas9 knock-in transgenic mouse [64]. FACS of GFP-KASFI nuclei from these animals confirmed *Chd8* knockout specifically in AAV-infected cells. This innovative, brain region-specific deletion approach demonstrated that loss of *Chd8* in the ventral but not the dorsal striatum recapitulated the acquired motor learning phenotype observed in germline mutant animals [73]. A related study performed RNA-seq on induced human pluripotent stem cells nucleofected with *CHD8*-targeted CRISPR/Cas9 and differentiated to heterozygous knockout cerebral organoids [74]. Similar to results in neural progenitor cells, CHD8 was found to regulate genes involved in neurogenesis, neuronal differentiation, forebrain development, Wnt/ β -catenin signaling, and axonal guidance [74], pathways that are relevant to both basic and aberrant neurobiological function.

4.3 Glioblastoma

While locus-specific gene editing is the most current innovation for targeted cancer immunotherapy [89], treatment of glioblastoma was one of the first translational applications of ZFPs. This study applied *VEGF*-ZFP-vErbA to repress target site expression via specific deacetylation of histones H3 and H4 in a tumorigenic glioblastoma cell line to levels comparable to that in a non-angiogenic cell line [90].

5 Applications to Basic Neuroscience

A large body of literature has demonstrated that basal and induced gene expression in neurons is under complex transcriptional control and that stable epigenetic modifications are inherent to this regulation [4, 6, 7, 22, 91–94]. However, a limitation of past research is the lack of causal evidence linking induced changes in histone and DNA modifications to aberrant neural gene expression. Locus-specific epigenetic editing allows the experimental discrimination between the mere presence and functional relevance of chromatin and DNA modifications.

Several studies have utilized neuroepigenetic editing to decipher the causal role of DNA methylation in neuronal gene regulation. Interestingly, an early study utilizing ZFPs in cell lines found improved target specificity when a prokaryotic DNA methyltransferase was mutated to a less active form, reducing spurious genomewide methylation [95]. Similarly to the endogenous mechanisms of methylated DNA-mediated gene repression, ZFP-initiated DNA methylation led to enrichment of H3K9me2 and depletion of H3K4me3, which may explain the reported inheritance of the induced methylation in this case. Furthermore, the methylation status persisted beyond expression of the ZFP, indicating that targeted DNA methylation can be exploited for lasting gene repression [95]. However, one limitation of this approach was the use of an integrated, rather than endogenous, target site. In fact, another investigation, which targeted a DNA methyltransferase and the histone methyltransferase GLP to the *VEGF* gene in a human cell line, found that, upon loss of the targeted methyltransferases, the induced epigenetic marks, in this case DNA methylation and H3K9me2, returned to baseline, indicating that the methylation was not stable during cell division [96]. Differing results from additional studies [78, 97] suggest that cell line, gene target, and delivery method contribute to the stability and inheritance of induced epigenetic modifications. While one study suggests that the behavioral effects of histone methylation in vivo can persist beyond expression of the transgene [34], a systematic investigation is necessary to determine the persistence of epigenetic editing in neurons in vivo, especially considering that these cells are generally nondividing.

CRISPR/Cas9 gene editing (e.g., gene knockout via indel formation) has been applied lately to neurons in vivo, demonstrating the potential of CRISPR/dCas9 in neuroepigenetic editing. An innovative dual AAV system was used to target methyl-CpG-binding protein (*Mecp2*) mutations that underlie Rett syndrome, a neurodevelopmental disease, with Cas9 and sgRNA under control of the neuronal *Mecp2* and *Synapsin 1* promoters, respectively [48]. As referenced above, co-expression of sgRNA with KASH-GFP allows FACS of infected neurons by driving GFP to the outer nuclear membrane; in this case over 70% of primary cortical neurons showed loss of *Mecp2* expression when Cas9, but not dCas9, was co-injected [48]. To validate the potential utility of CRISPR/Cas9 gene editing in specific brain regions, AAV-sgRNA and AAV-Cas9 were injected into mouse hippocampus. *Mecp2* indels (induced mutations) were found in 70% of FACS-sorted nuclei, while *Mecp2* protein levels were reduced by 60%.

More recently CRISPR/dCas9-mediated neuroepigenetic editing has been applied to DNA methylation of the mammalian genome [51]. In this study, as noted earlier, dCas9 fused to

Tct1 or Dnmt3 was expressed with lentivirus to induce demethylation (via hydroxymethylation) or de novo methylation, respectively. Brain-derived neurotrophic factor (BDNF) expression levels were induced, and cytosine hydroxymethylation was detected in cultured mouse cortical neurons following dCas9-Tet1 + *BDNF*-sgRNA expression, as quantified in cells obtained by FACS of sgRNA-infected cells co-expressing mCherry fluorescent protein [51]. The utility of this approach was validated with lentiviral dCas9-Tet1 and *Bdnf*-sgRNA delivery to a transgenic mouse containing a methylation-sensitive GFP reporter, suggesting that the viral delivery of dCas9-Tet1 and sgRNA may be viable for CRISPR/dCas9 targeted DNA methylation in rodent brain [51].

Of particular interest in basic neurobiology is the regulation of immediate early genes, which are rapidly induced upon neuronal stimulation and important for neuronal plasticity. For example, while *c-Fos* induction is known to be regulated by several enhancers scattered around the gene, little is known about its coordinated regulation in response to stimulation. To address this, one group defined activity-dependent *c-Fos* enhancers based on activity-induced enrichment of specific hPTMs and transcription factors as well as induction of enhancer RNAs [98]. Activity-dependent *c-Fos* enhancers were then individually targeted with dCas9-KRAB, and the activity-specific induction of *c-Fos* from each enhancer was quantified by enhancer RNA and mRNA expression [98]. This study, which highlights the potential of neuroepigenetic editing to define molecular mechanisms of stimulus-induced gene expression, uncovered that BDNF-induced *c-Fos* expression is controlled by a subset of coordinated, activity-dependent *c-Fos* enhancers [98].

Finally, given the importance of chromatin-based transcriptional mechanisms in neural development, one key study investigated epigenetic programs that drive neuronal gene expression patterns during cerebellar granule cell differentiation [31]. In this case, DNase hypersensitive site (DHS) mapping and RNA-sequencing were combined to identify developmentally regulated promoter and enhancer elements, whose function was predicted based on the presence of specific hPTMs (e.g., H3K27me2) [31]. To directly test the causal relevance of DHS opening in enhancer function, dCas9-VP64 was targeted to enhancers of *Grin2c*, which encodes a developmentally regulated NMDA-type glutamate receptor subunit that mediates mature synaptic function. Epigenetic editing in cultured cerebellar granule cells with dCas9-VP64 and sgRNAs targeting either of two developmentally regulated *Grin2c* DHS sites was sufficient to specifically activate *Grin2c* expression [31]. This study, as well as that described above on *c-Fos* enhancers [98], marks a notable advance in efforts to functionally validate enhancer elements, a critically important application of epigenetic editing both in the brain and other tissues [25, 53].

6 Future Applications: Combinatorial Approaches

Gene expression profiling studies in the brain have revealed that complex regulatory networks of mRNA expression underlie normal brain function, development, and neuropsychiatric disease [2, 16, 94, 99]. Thus, attempts to control neuronal function and behavior by epigenetic editing of a single locus may be limited in their application. Recent advances in combinatorial approaches to epigenetic editing have been applied to numerous types of epigenetic modifications. For example, Cas9 can be combined with multiple

sgRNAs [46] to achieve efficient multiplexed genome editing in mammalian cells [100, 101]. In general, two main approaches to multiplexing are apparent: those involving multiple effector domains and those involving multiple gene targets.

Comprehensive analyses of both approaches in yeast and mammalian cell lines reveal potential applications in neuroscience. For example, an exhaustive quantitative study of 223 yeast chromatin regulators fused to ZFPs and targeted to a synthetic transcriptional reporter found that the precise location of ZFP binding relative to the TSS was a determining factor in its efficacy, as measured by gene expression and histone modification [102]. Reporter gene activity revealed that activation domains generally function when recruited to specific locations (e.g., promoters and enhancers), while repression is controlled throughout a gene, perhaps through heterochromatin spreading mechanisms or disruption of transcription elongation. The observed regulatory hierarchy informs future selection of loci targeted for combinatorial regulation, given that a single site may have differential effects on repression or activation based on its location relative to specific gene elements.

The combination of multiple effectors has also been applied to mimic the natural cooperative recruitment process inherent to gene activation. To test whether functional domains fused in tandem would increase transcriptional activation, one study fused dCas9 to a series of more than 20 functional domains and assessed their potency in activating a fluorescent reporter in HEK293 cells [103]. Additionally, a highly innovative design for the co-recruitment of functional domains is that of dCas9 fused to a repeating peptide array termed SunTag, a protein scaffold that recruits multiple copies of an antibody-functional domain fusion [104]. Comparison of the efficacy of lentivirus expression of dCas9-VP64 and dCas9-SunTag-VP64 in cell culture found that recruitment of multiple VP64 proteins via the SunTag potentiated VP64-mediated activation [104]. The result is extremely promising given the versatility of the anti-body-based SunTag design. Such multiplexing approaches identified tandem recruitment arrangements that could activate genes between 280 and 20,000 times relative to control [103, 104], which may be useful in certain applications (e.g., iPSC differentiation) but undermines the goal of epigenetic editing, stated earlier, to recapitulate physiologically relevant levels and modes of gene regulation.

Beyond combinatorial effector domains, simultaneous epigenetic editing of multiple target loci has great potential utility for gene network regulation in the brain [12, 16, 105]. Although multiplexed epigenetic editing has not yet been accomplished in neurons, multiplexed CRISPR/Cas9 gene knockout was used to investigate the functional redundancy of DNA methyltransferases. In this study, a cocktail of AAV-Cas9 and AAV-sgRNAs targeting the *Dnmt3a*, *Dnmt1*, and *Dnmt3b* gene loci was stereotactically injected into the hippocampus of adult mice [48]. GFP-KASH FACS and next generation DNA sequencing found that approximately 62% of all transduced neurons contained indels in both *Dnmt1* and *Dnmt3a*, compared to ~35% which contained simultaneous modification of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* [48]. Additional in vivo multiplexing studies have targeted lung epithelial tissue with a single AAV vector containing sgRNAs targeting multiple tumor suppressor genes [64], as well as combinatorial methods to drive cell differentiation [55, 79]. The results of these studies underscore the challenge of multiplexed gene targeting in vivo and remain a potential source of inspiration for approaches to combinatorial neuroepigenetic editing.

7 Conclusion

Experience and learning regulate genome-wide deposition of hPTMs and expression of the myriad enzymes that catalyze and metabolize them. Herculean efforts in next generation ChIP- and RNA-sequencing of brain regions have convinced the field that, indeed, regulation of the epigenetic landscape contributes importantly to neuronal development and function. Further, precise spatiotemporal transcriptional control is crucial to the highly specialized role of neurons in integrating external stimuli. However, major questions remain about the functional role played by many of the epigenetic modifications observed to date in the brain. That is, given the promiscuity of the enzymes involved, it is challenging to obtain direct causal evidence of the function of epigenetic remodeling that occurs at a single gene in a single cell type within a given brain region of interest in awake, behaving animals. Neuroepigenetic editing methods are poised to address this limitation, and, while this field is in its relative infancy, important advances have already been made to directly test the functional role of specific histone and DNA modifications in the mammalian brain *in vivo*. This work is central to delineate a precise understanding of epigenetic regulation in the brain as well as to eventually devise targeted epigenetic therapies for neuropsychiatric illness.

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Fig. 1.

Epigenetic editing tools. Three main approaches to epigenetic editing have been applied in neurons. ZFPs and TALEs rely on a protein DNA-binding domain tethered to an effector domain, while the CRISPR/dCas9 system utilizes an RNA-DNA interaction to guide the dCas9-effector domain fusion to the target gene. Effector domains act to modify histone tails and/or DNA at target loci. ZFPs are shown with Zn⁺ stabilizing ion (gray dot) and TALEs are shown with C- and N-terminal domains in yellow

Fig. 3.

Spatial and temporal control of neuroepigenetic editing, **(a)** Cell-type-specific expression of an epigenetic editing tool by injecting Cre-dependent virus into the brain of a transgenic animal expressing Cre recombinase in specific cell types [65]. **(b)** Light-inducible dimerization of dCas9 with an effector domain is accomplished by fusion of each to CRY2 and C1BN domains, respectively. Upon blue-light stimulation, CRY2 and C1BN heterodimerize, bringing the effector domain in proximity with the dCas9-bound locus [55]. Similar methods can be applied to ZFPs [59] and TALEs [36]. **(c)** Chemically inducible CRISPR-mediated epigenetic editing is accomplished by expression of a split-dCas9 N- and C-terminals fused to rapamycin-sensitive heterodimerization domains, FRB and FKBP,

respectively. Upon rapamycin administration, dCas9 halves are brought into proximity and translocate to the nucleus [77]

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