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Genetically Engineering the Nervous System with CRISPR-Cas

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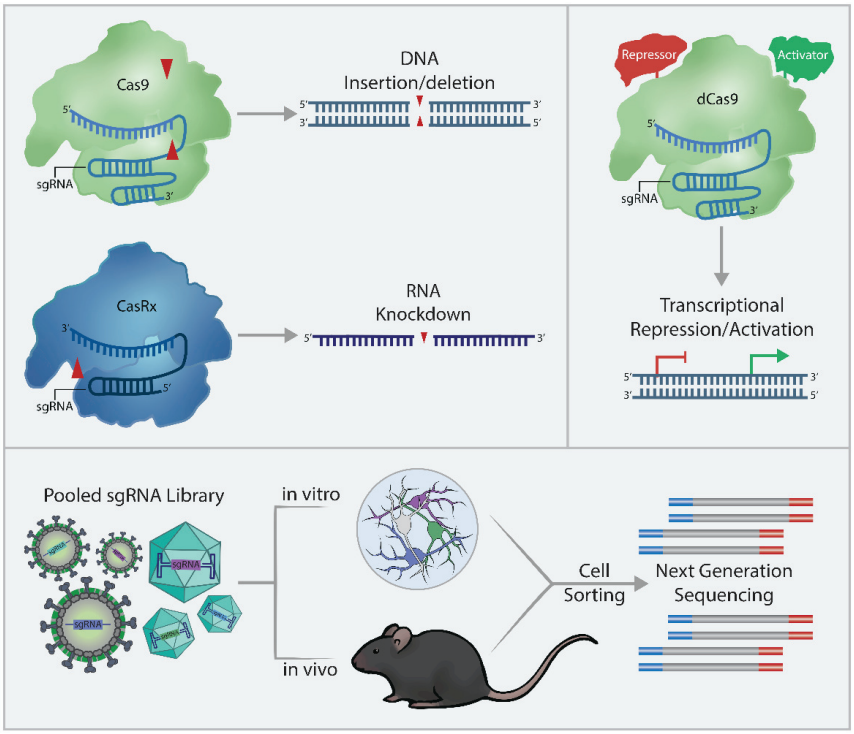


Abstract

The multitude of neuronal subtypes and extensive interconnectivity of the mammalian brain presents a substantial challenge to those seeking to decipher its functions. While the molecular mechanisms of several neuronal functions remain poorly characterized, advances in Next-Generation Sequencing (NGS) and gene-editing technology have begun to close this gap. The Clustered Regularly Interspaced Palindromic Repeats – CRISPR Associated Protein (CRISPR-Cas) system has emerged as a powerful genetic tool capable of manipulating the genome of essentially any organism and cell type, an attribute which has advanced our understanding of complex neurological diseases by enabling the rapid generation of novel, disease-relevant *in vitro* and transgenic animal models. In this review, we discuss recent developments in the rapidly accelerating field of CRISPR-mediated genome engineering. We begin with an overview of the canonical function of the CRISPR platform, followed by a functional review of its many adaptations, with an emphasis on its applications for genetic interrogation of the normal and diseased nervous system. Additionally, we discuss limitations of the CRISPR editing system and suggest how future modifications to existing platforms may advance our understanding of the brain.

Introduction

Complex behavior is driven by extensive structural and genetic interactions in the mammalian central nervous system (CNS). Historically, neuroscientists have examined these interactions with a variety of histological, electrophysiological and pharmacological techniques. While indispensable, these techniques nonetheless lack the specificity of targeted genetic approaches to dissect neuronal function. Recent advances have allowed the coupling of high-throughput Next-Generation Sequencing (NGS) technologies with the cell-type specificity of modern molecular genetics to interrogate complex network interactions and behaviors at unprecedented scale and resolution. The ability to read, write and manipulate genomes with cell-type specificity is critical, especially considering the cellular heterogeneity of various CNS structures (Chung et al., 2005). Early attempts at targeted gene editing were performed with Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), which relied on programmable DNA-binding proteins coupled to active endonucleases to cleave specific DNA sequences (Kim et al., 1996; Carroll, 2011; Joung and Sander, 2013). While suitable for a variety of applications (Gaj et al., 2013), these systems have nonetheless fallen out of favor for new genome editing systems due to relative disadvantages such as their extensive protein engineering requirements. Recent advances in gene editing technology have culminated in the discovery of CRISPR-Cas9, a bacterial immune system which has been repurposed for mammalian genome editing applications (Jinek et al., 2012). Unlike its predecessors, CRISPR nucleases target DNA in an RNA-directed manner, using a programmable single guide RNA (sgRNA) to target complementary DNA sequences for cleavage.

Since its initial adaptation, novel CRISPR-Cas variants have continued to be discovered in diverse microbial species, which differ in endonuclease size, substrate preference and target recognition requirements (Ran et al., 2015; Abudayyeh et al., 2017). Moreover, several nuclease variants have been engineered for expanded targeting capacity and improved fidelity (Kleinstiver et al., 2015; Kleinstiver et

al., 2016; Slaymaker et al., 2016; Chen et al., 2017). Perhaps most versatile are the catalytically inactive variants designed to function as DNA-binding proteins, which can regulate transcription, modify the epigenome, target RNA for destruction and facilitate base-editing through the action of their coupled enzymatic domains (Dominguez et al., 2016; Rees and Liu, 2018; Pickar-Oliver and Gersbach, 2019). The highly flexible and multifunctional character of this platform has established CRISPR-Cas as the predominant genome editing system in use today. Here we provide an overview of CRISPR-Cas technology, followed by a review of its many adaptations for genetic interrogation and modification. Throughout this article, we emphasize applications of CRISPR systems in the field of neuroscience and discuss the potential of this technology to advance our understanding of the brain.

CRISPR-Cas

Isolated from *Streptococcus Pyogenes,* the Type II CRISPR-Cas9 system (spCas9) was the first enzyme repurposed from its native role as a bacterial adaptive, immune system for genome editing applications in eukaryotic cells (Jinek et al., 2012). While spCas9 remains the most popular CRISPR nuclease, various CRISPR-Cas systems with divergent structures and properties have been discovered. These systems are broadly categorized by their nuclease composition, with those containing multi-subunit nuclease structures pertaining to Class 1 and those composed of a single protein pertaining to Class 2. Within Class 2, systems are further subdivided into types II, V, and VI, which pertain to DNA-targeting Cas9 and Cas12a and RNA-targeting Cas13, respectively (Shmakov et al., 2017). As Class 2 systems have been used in the majority of neuronal gene editing experiments, they will therefore be the focus of this review. Class 1 systems and their uses are described elsewhere (Cameron et al., 2019; Pickar-Oliver et al., 2019). The prototypical CRISPR nuclease, spCas9, is an RNA-guided DNA endonuclease that relies on an RNA duplex comprised of a CRISPR RNA (crRNA) and a transactivating

crRNA (tracrRNA) for its activity (Fig. 1A). CRISPR RNAs direct Cas9 enzymes to their intended genomic targets, whereas tracrRNAs are responsible for stimulating Cas9's endonuclease activity and mediating pre-crRNA processing and maturation. Although discovered as two distinct RNAs in nature, it was experimentally determined that the essential elements of the tracrRNA-crRNA duplex could be combined into a chimeric single guide RNA (sgRNA). Therefore, genome editing utilizing this system only requires the Cas9 protein and the sgRNA. Cas9-DNA targeting occurs when the Cas9-bound sgRNA hybridizes to its target-DNA proximally to a short sequence known as the protospacer adjacent motif (PAM), which is used for target recognition. Once Cas9 binds to the genomic target site, it will create a double strand break (DSB) ~3 bases upstream of a PAM-containing locus with sufficient crRNA complementarity. DSB formation initiates the nonhomologous end joining (NHEJ) DNA repair mechanism, which due to the error prone nature of this repair pathway, creates insertion and deletion (Indels) mutations at the DSB break/repair site (Fig. 1A). If the DSB occurs within the protein coding region of a gene, a loss of protein function can occur from the deletion of relevant codons or from a shift in the reading frame, often creating a truncated protein – collectively leading to a null allele/gene knockout (KO) (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). Alternatively, if a donor DNA template is provided, Homology Directed Repair (HDR) can occur instead of NHEJ. This phenomenon can be harnessed to specifically modify the genome at precise loci (Fig. 1B) (Cong et al., 2013; Mali et al., 2013; Wang et al., 2013). However, HDR mediated DNA repair via existing technology remains very inefficient and therefore, its use in non-dividing cells (i.e., neurons) *in vivo* has limited utility (Chu et al., 2015; Maruyama et al., 2015).

The Type V nuclease Cas12a, (previously known as Cpf1 - CRISPR from *Prevotella* and *Franciscella 1)*, is a related DNA targeting enzyme that departs mechanistically from Cas9 in ways that may be advantageous. For example, unlike Cas9, Cas12a processes its own CRISPR array (crRNA precursors) into mature crRNAs, independent of any ancillary enzymes and a tracrRNA. Cas12a also

recognizes a different PAM sequence (Cas12a – TTTV; Cas9 – NGG), generates staggered cuts (discussed additionally below) and requires a much shorter guide RNA than its Cas9 counterpart (~40 nt – Cas12a; ~100 nt – Cas9). Cas12a's compact guide RNA architecture and self-crRNA processing ability make it well suited for multiplexed gene-targeting, particularly through the use of custom crRNA arrays encoding multiple crRNAs. Recently, these properties were optimized and harnessed for large scale gene-editing, with Campa and authors reporting the ability to deliver and express 20 crRNAs and Cas12a from a single vector, simultaneously (Campa et al., 2019). The continued discovery and development of new CRISPR-Cas systems with advantageous properties is highly encouraging for the future of biomedical research and therapeutic development.

Gene Disruption in the Mammalian Brain via CRISPR-Cas and NHEJ

Targeted gene disruption is a popular approach for dissecting the functional role of many synaptic and neuronal proteins *in vivo* (Gray et al., 2011; Uezu et al., 2016), although historically, this has required conventional mutant germline engineering, which is experimentally time-consuming and can generate deleterious phenotypes, and is generally prohibitive for multigene perturbation. Gene disruption with CRISPR-Cas has been demonstrated as a promising alternative to existing gene KO strategies. Several groups have begun to apply CRISPR-Cas to disrupt genes in mature neurons in vitro and in vivo by taking advantage of targeting Cas9 to specific loci and relying on NEHJ repair pathways to create indels which lead to a high rate of gene disruption (Incontro et al., 2014; Swiech et al., 2015). The earliest studies that implemented CRISPR-Cas for neuronal gene editing *in vivo* established the lack of toxicity of prolonged Cas9 expression in neurons while also creating the first transgenic and viral platforms for their expression and delivery (Platt et al., 2014; Swiech et al., 2015). Using these transgenic mice, Platt and authors also demonstrated the high knockout frequencies (84% biallelic, 9% monoallelic;

159 NeuN) achievable in neurons transduced with AAV-sgRNAs. Swiech and authors sought to expand the
160 applicability of CRISPR for broad *in vivo* use by adapting Cas9 for packaging into popular viral vectors for
161 gene delivery into the brain (Swiech et al., 2015). The Adeno-Associated Virus (AAV) DNA packaging limit
162 (~ 5 kb) is a major limitation for viral delivery *in vivo*, therefore packaging the Cas9 transgene (~ 4 kb),
163 sgRNA cassette and other necessary expression components into a single vector is infeasible. To
164 circumvent this, Swiech and authors developed an AAV-CRISPR system that expresses spCas9 and its
165 respective sgRNA from separate AAV vectors. Applying AAV-CRISPR to target various genes *in vitro* and
166 *in vivo* recapitulated the substantial editing frequencies observed in transgenic Cas9 mice. For example,
167 targeting MeCP2 in cultured neurons produced morphological defects concurrent with MeCP2 loss of
168 function. Furthermore, multiplexed targeting of several DNA methyltransferase genes within the
169 dentate gyrus was capable of producing context-specific freezing deficits in mice that received
170 contextual fear conditioning, while sparing behavioral performance in other tasks (open field test, novel
171 object recognition, elevated plus maze.)

172 Traditional gene editing strategies have relied heavily on engineered viral vectors for *in vivo*
173 construct delivery (Yin et al., 2017). Although AAV and Lentiviral (LV) vectors are widely used for their
174 ability to stably express transgenes for extended periods, the potential drawbacks of viral delivery and
175 prolonged Cas9 expression for therapeutic gene editing have received increased attention. For example,
176 higher cellular concentrations of Cas9 have been shown to decrease specificity, presumably because off-
177 target cleavage is the only possibility after all target sites have been destroyed (Davis et al., 2015). This
178 observation has raised concerns for therapeutic development that rely on viral gene transfer, which in
179 the case of AAV-mediated gene expression, persists for several years after delivery (Nathwani et al.,
180 2011; Wojno et al., 2013; Colella et al., 2018; Guilbaud et al., 2019). Engineered ribonucleoprotein
181 complexes (RNP; Cas9 protein bound to a guide RNA) and Cas9-encapsulating nanoparticles have been
182 developed as non-viral alternatives for local, transient CRISPR expression in the brain.

Staahl and authors introduced a cell permeable Cas9-RNP capable of transient and titratable gene disruption (Staahl et al., 2017). Cas9-RNPs were designed with repeating SV40 nuclear localization sequences (NLS), which have been previously reported to enhance cell-penetrance (Liu et al., 2015). Pre-assembled Cas9-RNPs were injected into the S1 primary somatosensory cortex, the V1 primary visual cortex, the dorsal striatum and the hippocampus of Ai9-tdTomato mice. Reporter activation increased in a dose-dependent manner with larger administered doses of Cas9-RNP. Furthermore, RNP injection into the dorsal striatum did not induce a significant immune response, which has been a point of concern after a report of an anti-Cas9 immune responses (Chew et al., 2016).

Recently the nanoparticle-based CRISPR-Gold system was used to target mGluR5, a metabotropic NMDA receptor involved in ASD (Autism Spectrum Disorder) - related hyperexcitation (Lee et al., 2018). CRISPR-Gold RNPs containing mGluR5-targeting guides were infused into the striatum of FMR1 KO mice, which significantly reduced exaggerated stereotypies (excessive digging and jumping). Analysis revealed 14.6% of striatal mGluR5 genes contained LOF mutations, while mGluR5 mRNA and protein levels decreased by roughly 50%. Despite modest editing efficiency, these results indicate the potential of nanoparticle-based systems to deliver CRISPR and therapeutically edit genes in the brain. While CRISPR-Gold administration was sufficient to reverse the behavioral phenotype, additional optimization of nanoparticle entry into neurons will likely expand the use of non-viral, nanoparticle-based methods for genome editing in neuroscience.

Another group engineered membrane-permeable nanocomplexes to deliver Cas9 RNPs into the brain (Park et al., 2019). CRISPR nanocomplexes were generated by fusing an amphiphilic R7L10 peptide to Cas9 RNPs to permit cellular entry. R7L10-Cas9-RNPs exhibited remarkable *in vivo* stability and longevity, sustaining high levels of expression for over week, which declined below detection thresholds after three weeks. Unlike virally delivered CRISPR transgenes that remain stably expressed for extended

periods, nanocomplex-delivered RNPs possess limited opportunity to perform their gene targeting functions. Remarkably, *in vivo* targeting of beta-secretase 1 (*Bace1)* in the hippocampal CA3 region of 5XFAD transgenic mice produced an editing efficiency of 45% which significantly reduced Aβ plaques and Aβ42 secretion. Surprisingly , a single hippocampal injection of *Bace1*-targeting nanocomplexes elicited persistent improvements in contextual and associative memory 3 months after treatment (Park et al., 2019). While the decay rates of injected RNPs and their potential off-targeting effects remain to be determined, additional research could accelerate the development of injectable RNP therapies for focal neurologic disease.

Genetically modified animals have been instrumental in understanding genetic contributions to neuronal development, function and disease. Conventionally, establishing transgenic animal strains has been a time- and labor-intensive process that requires several months for completion and specialized facilities capable of single-cell zygote microinjection and embryonic stem cell (ESc) manipulation (Capecchi, 2005). In recent years, many of these constraints have been overcome by CRISPR-Cas9 genome editing. This includes the ability to rapidly produce transgenic animals containing multiple mutations with relative ease, which is a significant improvement over traditional transgenic production approaches. For a more detailed discussion on generating transgenic/knock-in mice with CRISPR-Cas, we direct the reader to the following articles (Yang et al., 2014; Henao-Mejia et al., 2016; Williams et al., 2016).

While the broad availability of genetically modified mice has contributed to their widespread use in biomedical science, rats remain the preferred animal model in behavioral neuroscience research. The paucity of available transgenic rat models has left an unmet demand for additional transgenic rat lines (Ellenbroek and Youn, 2016). Germline genome editing with CRISPR-Cas9 has emerged as a highly efficient method for producing transgenic strains, as such CRISPR-Cas9 was used to generate transgenic

Cre--dependent Cas9 wild-type rats and Cre dependent Cas9-Nickase [Cas9(D10A)] rats and an improved Cre recombinase (iCre) rat line under the control of the dopamine transporter promoter (DAT-iCre) on the Long-Evans background (Back et al., 2019). To show that gene targeting was Cre-dependent, Back and authors infused AAVs encoding iCre and TH-targeting sgRNAs into the midbrain. Four weeks after infusion, a 45% and 60% decrease in TH immunoreactivity was observed in the substantia nigra and striatum, respectively. To determine the targeting efficiency achievable with the double-transgenic rat (DAT-iCre +/ Cas9 +), AAVs encoding *Manf* sgRNAs were infused into the midbrain. After 4 weeks, only 3% of dopaminergic neurons demonstrated *Manf* immunoreactivity; additionally, nearly 90% of non-dopaminergic neurons remained *Manf* $^{+}$, thereby illustrating the potential of these lines to facilitate highly specific genome editing with extremely high editing efficiencies. With the availability of neuron-specific Cre-driver lines (GABAergic, D1, D2, Parvalbumin), these Cre-dependent Cas9 knock-in rat lines present a significant advancement for future gene studies in behavioral neuroscience.

Gene Modification in the Mammalian Brain via CRISPR-Cas and HDR

Currently the factors governing DNA repair pathway choice remain unclear. In general, the NHEJ mediated pathway appears to be far more efficient and active compared to the HDR mediated pathway (Cox et al., 2015). It has been generally believed that HDR is largely restricted to the S/G_2 phases of the cell cycle, which may restrict harnessing HDR's full potential in post-mitotic cells such as neurons (Saleh-Gohari and Helleday, 2004). The restriction of HDR activity to the S/G2 phases may be due to the presence of conditions favorable to recombination such as the presence of proximal sister chromatids or the increased expression of requisite repair machinery, both are conditions which may preclude robust HDR activity in terminally differentiated neurons; this, however, remains to be determined.

Cas9's canonical function is to cleave DNA, but this function can be harnessed to introduce foreign transgenes and introduce new sequences utilizing the HDR pathway (Fig. 1B). Low neuronal HDR

activity has largely discouraged gene-editing attempts in the brain. However, recently evidence has surfaced demonstrating the successful modification of neuronal genes in the mouse brain.

Mikuni and authors developed single-cell labeling of endogenous proteins by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-mediated homology-directed repair (SLENDR) (Mikuni et al., 2016). The authors specifically intended to target neural progenitors at embryonic days 12 and 15 (E12, E15), when these cells should still possess HDR activity. They subjected embryonic brains to *in utero* electroporation (IUE), transfecting the cells with sgRNAs, Cas9 coding plasmids, and a hyperactive Piggyback transposase system to allow the stable integration of these transgenes and donor templates consisting of single stranded oligonucleotides (ssODNs). This approach enabled the modification of targeted genes so they would possess N- or C- terminal epitope tags. Essentially the system is designed to allow one to target relatively few neurons *in vivo* and allow epitope tags, even tags as big as the GFP coding region to be added to protein coding regions of endogenous neuronal genes, to allow for sparse labeling of neurons and facilitation of protein localization studies. The authors reported modification efficacies as high as 7.5% of targeted neurons when the targeting was performed at E12, and slightly lower levels when the targeting was performed at E15. It's important to point out that NHEJ indels will occur at a much higher efficiency compared to HDR using this system. However, for protein localization studies, this is acceptable and additionally the authors specifically targeted the beginning and end of the protein coding regions, to reduce the chance that indel formation would have a consequence on protein structure and function.

Nishiyama and authors created a similar system as Mikuni and authors, but utilized a viral approach. Their system, referred to as vSLENDR (AAV/CRISPR-based Viral-mediated Single-cell Labeling of ENdogenous proteins via HDR system), was shown to allow HDR mediated gene modification of neurons in the mouse adult brain (Fig. 1B). They observed gene modification efficiencies *in vivo*

(Nishiyama et al., 2017) as high as ~30% of targeted neurons, which provides proof-of-principle for HDR-mediated editing in mature neurons. While encouraging, the mechanism of HDR-mediated editing will require additional characterization and subsequent optimization before it can be broadly applied for *in vivo* studies.

Additional Gene Modification Strategies

While broadly considered an inherently error-prone process, various NHEJ-dependent DNA-editing tools have been developed that demonstrate the remarkably high editing frequency and precision of NHEJ repair (Fig. 1C). These tools, designated Homology Independent Targeted Insertion (HITI), Homology-mediated end-joining (HMEJ and Homology-independent universal genome engineering (HiUGE) have been shown to effectively integrate exogenous DNA sequences at similar frequencies (20% to over 50%). The first of these, HMEJ, exploits homology-dependent processes by coupling donor templates harboring sgRNA recognition sites with targeted, Cas9-mediated DNA cleavage. HMEJ-DNA donors contain 5' and 3' distal sgRNA sites that, upon cleavage, release a long donor cassette which encourages integration into the cleaved genomic site. When applied to adult mouse neurons *in vivo* HMEJ produced knock-in frequencies of ~50%. Although homology-dependent (HD) strategies ensure locus specificity through extensive donor template homology, unique template production is generally restrictive for high-throughput experimentation. Therefore, unrestricted by locus homology, homology-independent (HI) systems have gained more traction. The HITI and more recently developed HiUGE systems also exploit NHEJ repair to introduce DNA payloads. Both HITI and HiUGE incorporate similar components and mechanisms to achieve targeted transgene integration, for example the use of a non-homologous donor vector with sgRNA recognition sequences is ubiquitous among NHEJ-mediated systems. However, HITI and HiUGE depart as HiUGE donors contain self-targeting sgRNAs, whereas HITI donors require sgRNA recognition sequences to be manually matched between

the target and donor; the addition of a self-targeting guide RNA to HiUGE vectors permits the development of "all-in-one" donor libraries that may function complimentarily with large-scale CRISPR genetic screens.

Regulable Gene Editing with inducible CRISPR-Cas Systems

Germline editing with CRISPR-Cas9 has proven remarkably useful for genetically modifying animals (Li et al., 2013; Chapman et al., 2015; Remy et al., 2017). However, germline modifications can produce undesirable developmental phenotypes providing little benefit for studies interrogating gene function in adult animals. Furthermore, temporally precise manipulations may be required for studying gene function in dynamically regulated processes. In such situations it may be beneficial to deploy temporally regulable systems capable of gene editing within tightly restricted windows. Towards this aim, CRISPR-Cas9 has been combined with several other technologies to develop systems that can be regulated genetically, optically, or with small molecules (Dow et al., 2015; Zetsche et al., 2015).

Some of the first inducible CRISPR systems were regulated by components of the popular tetracycline-dependent promoter (Tet) system (Dow et al., 2015; de Solis et al., 2016), which can be regulated in Tet-on (rtTA) and Tet-off (tTA) configurations (Fig. 2) (Gossen and Bujard, 1992; Gossen et al., 1995). de Solis and authors developed the first doxycycline (dox)-inducible Cas9-based editing system that saw use in the brain. First Cas9 was placed under the control of the Dox-dependent TRE3G promoter to attempt to temporally regulate Cas9 expression and subsequent genome editing (de Solis et al., 2016). However, TRE3G-driven Cas9 exhibited leaky expression *in vitro,* prompting the development of regulable sgRNA expression vectors, which successfully regulated gene-editing events in a dox-dependent manner. To determine if this dox-regulable CRISPR-Cas9 system was suitable for *in vivo* applications, AAV vectors encoding Cas9 and Dox inducible sgRNAs were infused into the basolateral amygdala (BLA). *In vivo* genome-editing analysis revealed that only animals receiving doxycycline

contained indels at the target locus. Additionally, dox-inducible and constitutively expressed systems exhibited near identical levels of gene editing, demonstrating that spatiotemporally precise editing is achievable in the brain without significant loss of efficiency. Additional Cre and Dox inducible CRISPR systems have been developed based on the smaller SaCas9 endonuclease. For further discussion of the saCas9 orthologue and these inducible tools, we direct the reader to (Kumar et al., 2018; Zhou et al., 2018b).

While the conditional Tet- and Cre- based systems are frequently used to restrict gene expression temporally and spatially, their specificity and regulation is largely transcriptionally mediated. In cases where swift gene-editing is desirable, it is beneficial to reduce the response rate of the system. Post-translationally regulated processes circumvent the *de novo* transcription and translation involved in transcriptionally mediated responses, permitting a more rapid response to dynamic cellular environments. Additionally, reducing the permissible window for gene-targeting events could significantly reduce the off-target modifications reported with constitutively active Cas9. Several inducible Cas9 enzymes have been developed whose activities are post-translationally regulated with small molecules (Fig. 2) (Davis et al., 2015; Liu et al., 2016a). These small molecule-responsive systems utilize the human Estrogen Receptor ligand-binding domain (ERT) fused to Cas9 to trigger gene editing events in the presence of the ERT ligand 4-hydroxytamoxifen (4-OHT). Davis and authors introduced a 4-OHT-inducible Cas9 nuclease whose enzymatic activity was inhibited by a strategically placed, self-splicing intein (Intein-Cas9) (Davis et al., 2015). Intein-Cas9 was engineered such that its enzyme activity would only be restored after administration of 4-OHT, which activates intein-protein self-splicing and permits Cas9's adoption of a catalytically active form. A related 4-OHT inducible Cas9 enzyme was introduced in 2016, dubbed "iCas". However, this system departs from its predecessor by employing ERT2 as a subcellular carrier versus a covalent inhibitor. As the ERT2 ligand binding domain permits translocation into the nucleus when bound by 4-OHT, fusing multiple copies of the ERT2 domain to Cas9

enables bidirectionally regulable genome editing in human cells. Both of these systems demonstrated improved editing specificities over wild type Cas9, although iCas9 exhibited lower background activity and higher on-target editing when benchmarked against intein-Cas9. While, intein-Cas9 and iCas9 show promise for studying dynamic processes in the brain, to our knowledge, they have yet to see use in such experiments.

Advances in photoinducible protein biology have culminated in the development of systems that can control gene-editing and transcription with blue-light irradiation (Fig. 2) (Nihongaki et al., 2015; Polstein and Gersbach, 2015). Nihonkagi and authors achieved photoinducible gene editing by conjugating fragments of a Cas9 nuclease to protein elements of a dimerizing, light responsive system dubbed 'Magnets' (Kawano et al., 2015). The fungal-derived Magnet system consists of two photoinducible protein elements termed "positive Magnet" (pMag) and "negative Magnet" (nMag), which are named on the basis of their electrostatic properties (Kawano et al., 2015). This system demonstrated that gene editing could be bidirectionally regulated by light irradiation, albeit with modest indel frequencies and a relatively slow response time (maximal editing ~ 48 hours). As these limitations may limit paCas9's usefulness *in vivo*, additional engineering and optimization are likely required before this technology can be robustly applied in animal studies. While light inducible and optogenetic technologies are widely used in neuroscience research, photoactivatable gene-editors have yet to be applied to the nervous system.

Genomic Regulation with Nuclease Deficient Cas9

Cas9's capabilities have expanded beyond conventional genome editing by adapting the system into a programmable DNA-binding module suited for targeting diverse protein domains to specific DNA sequences (Fig. 3). To achieve this, Cas9's catalytic activity was abolished by introducing point mutations

into the RuvC1 (D10A) and HNH (H840A) domains to generate nuclease deficient or dCas9. Catalytically-inactive Cas9 retains DNA-binding capability with no apparent loss of targeting or binding specificity (Qi et al., 2013). As discussed later, dCas9-effector fusions provide seemingly endless applications for non-mutagenic genome modification, including transcriptional regulation, epigenome editing, cellular imaging and RNA interference.

Transcriptional regulation with dCas9

CRISPR-based transcriptional regulators provide researchers with the ability to assess the functional relevance of specific genes in a variety of neuronal contexts. By manipulating endogenous loci, CRISPR-based overexpression and gene silencing strategies also circumvent the shortcomings of cDNA overexpression and RNAi-mediated silencing such as potential protein mislocalization or widespread off-targeting. The first systems endowing activator and repressor capabilities to the CRISPR platform utilized fusions of tetrameric Herpes Simplex Viral Protein 16 (VP64), the NF-κB trans-activating subunit p65, (p65) or the Kruppel Associated Box domain of Kox1 (KRAB) to dCas9 (Gilbert et al., 2013) (Fig. 3E). When directed to promoter or enhancer sequences, dCas9-VP64 and dCas9-KRAB were capable of inducing or suppressing gene-specific transcription, respectively. These capabilities encouraged their ready adoption for mapping putative cis-regulatory elements in neurodevelopment and neurodegeneration studies (Frank et al., 2015; Heman-Ackah et al., 2016; Huang et al., 2017). Although this first generation of transcriptional regulators could modestly alter transcription, several reports demonstrated that gene expression could be amplified with the provision of multiple sgRNAs per targeted promoter (Gilbert et al., 2013; Maeder et al., 2013; Konermann et al., 2015; Savell et al., 2019). This observation suggested that the overall copy number and enzyme cooperativity of the recruited effectors was responsible for differences in gene expression. Capitalizing on this observation, other

groups developed additional CRISPR activator (CRISPRa) and CRISPR interference (CRISPRi) systems with enhanced transcriptional regulatory capabilities (Tanenbaum et al., 2014; Chavez et al., 2015; Konermann et al., 2015). These second-generation systems employ diverse scaffold architectures to recruit transcriptional regulators and maximize effector potency and recruitment.

Early second-generation systems employed an epitope-based scaffolding strategy to increase activator recruitment known as SUperNova (SunTag) (Fig. 3C) (Tanenbaum et al., 2014). The SunTag scaffold is a peptide array composed of tandem repeating GCN4 epitopes. Transcriptional regulators conjugated to short-chain variable fragments (scFv) with high affinity for the GCN4 epitope can effectively bind the SunTag scaffold, facilitating the formation of multimeric regulatory structures at targeted DNA sequences. Essentially the system is designed to recruit many VP64 transcriptional activation domains to the promoter to enhance transcriptional activation. Indeed, expressing dCas9-SunTag with scFv-bound VP64 activators dramatically increased targeted gene expression compared to dCas9-VP64.

Another study (Konermann et al., 2015) examined the regulatory potential of sgRNAs designed to recruit transcriptional activators using RNA aptamers (Fig. 3B). Analysis of sgRNA secondary structures identified regions that were non-interacting with the Cas9 endonuclease and found that mutating distal base pairs in these regions had no influence on DNA binding or cleavage. By substituting sgRNA stem loops with MS2 aptamers that could recruit MS2 Coat Proteins (MCP) fused to p65 and heat shock factor 1 (HSF1), it was determined that dCas9-VP64 could upregulate transcription at significantly higher levels when co-expressed with RNA aptamer-containing sgRNAs versus standard sgRNAs.

A separate group screened putative activator domains for gene activation potency, identifying VP64, p65 and the Epstein-Barr Virus R Transactivator (Rta) as the most potent transcriptional activators. However, dCas9-p65 and dCas9-Rta both exhibited lower transcription rates than the original

dCas9-VP64 chimera. To overcome this, combinations of activators were fused with the aim of cooperatively inducing higher gene expression (Chavez et al., 2015). Using dCas9-VP64 as a starting framework, a tripartite fusion of VP64-p65-Rta (VPR) (Fig. 3A) was tethered to dCas9 and subsequently assayed for induction capacity, which revealed that gene expression was upregulated between 22 and 320-fold when compared to dCas9-VP64.

Second-generation activators were screened for maximal induction of *ASCL1* and *NEUROD1* genes in HEK293T cells, revealing SAM (Konermann et al., 2015), SunTag (Tanenbaum et al., 2014) and VPR (Chavez et al., 2015) as the most potent gene activators. Subsequent assays revealed SAM as the most consistent in activating high levels of gene expression. Notably, the increased transcription of several tested genes reached orders of magnitude above that induced by dCas9-VP64 (Chavez et al., 2016). While newly developed CRISPR activators undergo validation in several common cell types, few have seen any use in neuronal contexts. Savell and authors have recently introduced lentiviral vectors capable of robust neuronal VPR expression *in vitro* and *in vivo (in vivo* discussion continued below) (Savell et al., 2019). Gene overexpression assays in primary cultured neurons demonstrated VPRs ability to robustly overexpress single or multiple genes with high specificity. Notably, multiplexed gene activation with VPR recapitulated earlier reports of sgRNA-dose responsiveness, demonstrating effective activation with the use of single sgRNAs which also increased significantly with the use of additional sgRNAs targeting the same gene.

In contrast to transcriptional activators, few dCas9 repressors capable of enhanced transcriptional downregulation have been developed. Recognizing this deficit, Yeo and authors proceeded to perform a similar screen to identify dCas9-repressors capable of robustly inhibiting gene expression (Yeo et al., 2018). Of the screened transcriptional repressors, the bipartite dCas9-KRAB-MeCP2 fusion emerged as the most potent (Fig. 3F).

Regulating Transcription *in vivo* with dCas9

Ectopic gene overexpression mediated through viral vector delivery is a popular strategy to investigate neuronal gene regulation (Lentz et al., 2012; Haggerty et al., 2020). As previously described, numerous CRISPR activator systems have been developed enabling the potent activation of multiple genes in various tissues types. However, until recently, these technologies have been limited to *in vitro* applications because of the difficulty of efficiently delivering the large and numerous transgenes required to cells *in vivo* simultaneously. Recently, elements of the SAM and VP64-SunTag system were combined to develop a new dCas9-based transcriptional activator, dCas9-SunTag-p65-HSF1 (SPH) (Fig. 3D), for *in vivo* gene regulation (Zhou et al., 2018a). To develop the SPH platform, the VP64 tetramers in the SunTag system were replaced with the p65-HSF1 effector domains from the SAM system. When combined, these components potently induced gene expression, surpassing the SunTag, VPR, and SAM activators. In order to circumvent the difficulties associated with viral delivery of large multi-component systems to the nervous system, the authors generated transgenic mouse line harboring a Cre-dependent SPH system. The potency of gene induction observed when benchmarked against similar 2nd generation activators suggests that the SPH activator may present an advancement in technologies enabling genome wide GOF screens. Considering that cell-type and circuit-specific multiplex strategies will likely be required to successfully interrogate gene networks *in* vivo, Zhou and authors performed feasibility experiments on SPH's multiplex gene activation capabilities. Using a combination of AAV vectors encoding neuron specific Cre (hSyn-Cre and CamKIIα-Cre) and long sgRNA arrays targeting multiple genes (eight coding genes and two long noncoding RNAs), Zhou and authors were able to induce robust overexpression of several targeted genes simultaneously. When coupled to currently available genome wide CRISPRa sgRNA libraries, these SPH mice provide a critical tool for endogenous gene overexpression and clear a path for *in vivo* genome wide screening in the brain.

Although CRISPR-Cas has recently been applied to advancing transgenic rat production, genetic technologies are overwhelmingly limited to laboratory mice. To overcome this shortcoming, Savell and colleagues sought to optimize the previously developed dCas9-VPR activator for behavioral neuroscience by developing neuron-optimized viral vectors capable of potent, multiplexed gene expression *in vivo*. By examining VPR expression under the control of several promoters, they were able to identify and produce a lentiviral system that permitted robust VPR expression *in vitro* and *in vivo* under the control of the neuron specific Synapsin promoter. This neuron-optimized lentiviral VPR system was applied in various neuronal contexts and notably, was capable of potent, isoform-specific induction of various BDNF transcripts *in vivo* (Savell et al., 2019).

Until recently, RNA interference (RNAi) and conditional Cre-loxP systems have been the predominate methods used for gene knockdown and knockout respectively. However, evidence documenting the significant off-target effects of short hairpin (shRNA) and small interfering RNAs (siRNA) has accumulated (Castanotto and Rossi, 2009; Jackson and Linsley, 2010; de Solis et al., 2015). Alternative methods for gene knockdown such as CRISPR-based repressors have been proposed, due to their ability to potently silence gene expression within various contexts, however applying CRISPRi technology for neuronal editing *in vivo* has seen limited use.

Recently, a lentiviral-based CRISPRi system was developed for use in the mammalian brain (Fig. 3E). Using the dCas9-KRAB repressor, synaptotagmin I (*Syt1),* vesicle associated membrane protein I (*Vamp1)*, syntaxin 1A (*Stx1a)* and synaptosome associated protein 25 (*Snap25),* genes responsible for vesicular neurotransmitter release, were targeted in cultured hippocampal neurons. To compare the efficiency of CRISPRi and RNAi-mediated knockdown, sgRNAs and shRNAs were tested for each target gene. CRISPRi produced ~90% reduction in mRNA and protein levels of all genes targeted, compared to a modest reduction produced by RNAi. Additionally, whole-cell patch-clamp recordings of CRISPRi-

targeted hippocampal neurons revealed significant reductions in excitatory postsynaptic potentials (EPSCs), as expected from disruption of the neurotransmitter release pathway (Zheng et al., 2018).

Numerous studies have reported the potential for Cas9 endonucleases to bind off-target sites (Kuscu et al., 2014; Wu et al., 2014). This, coupled with the observed potency of the dCas9-KRAB repressor, raises concerns for severe off-target silencing. The authors used a "pseudo-target fishing strategy" to determine the frequency of off-targets by expressing dCas9-KRAB with sgRNAs containing unique mismatches with the *Syt1* locus. This strategy revealed that *Syt1* expression levels remain largely unchanged, indicating that mismatched sgRNAs were incapable of efficiently directing dCas9-KRAB to the *Syt1* locus (Zheng et al., 2018).

As cell-type specificity is essential for the interrogation of gene and cell function in the brain, the dCas9-KRAB repression system was modified to restrict targeting to glutamatergic (αCaMKII-dCas9-KRAB) or GABAergic (VGAT-dCas9-KRAB) neurons. Lentiviral infusion into the dentate gyrus revealed a roughly 20% transduction rate of neurons confined to the granule layer. Analysis of dCas9-KRAB^{+} DG neurons revealed that *Syt1* expression was completely abolished in a cell-type specific manner. Likewise, whole-cell patch-clamp revealed that EPSCs within αCaMKII-expressing neurons were almost completely abolished, with a similar reduction in GABAergic neuron IPSCs (Zheng et al., 2018).

Targeting *Syt1* within glutamatergic and GABAergic neurons enables altering of the inhibitory – excitatory (I-E) ratio within the hippocampus. As the hippocampus is implicated in various forms of learning and memory (LaBar and Cabeza, 2006), the authors subjected mice to multiple spatial and associative learning tasks after CRISPRi mediated I-E shifting. Animals receiving αCaMKII-dCas9-KRAB (shift towards inhibition) exhibited significant performance reductions in spatial memory related tasks (Morris water maze, Barnes Maze, T maze) compared to animals receiving VGAT-dCas9-KRAB (shift towards excitation). In tests of associative memory (fear conditioning), CaMKIIα driving animals

demonstrated reducing freezing levels in response to a cued stimulus (tone) in contrast to VGAT driving animals which exhibited slightly enhanced freezing, illustrating that alterations of the I-E ratio within the hippocampus could bidirectionally regulate spatial and contextual fear memory (Zheng et al., 2018).

CRISPR based Epigenome Editors

DNA methylation is vitally involved in neurodevelopment and in dynamic gene regulation across various networks in the central nervous system (Smith and Meissner, 2013). Cytosine methylation within promoter regions permits the controlled regulation of various processes ranging from basic gene transcription to higher-order functions such as learning, memory and cognition. Historically, epigenetic studies have been incapable of determining the functional relevance of specific methylation events due to the limitations of the methylation-inhibiting small molecules 5-azacytidine and 5-aza-2'-deoxycytidine (Heerboth et al., 2014). Although these compounds could be locally injected to induce regional CpG hypomethylation, these shortcomings are largely prohibitive for the precise investigation of disorders such as Angelman's, Fragile-X, Rett syndrome, and Prader-Willi Syndrome, all which exhibit significant neurological phenotypes and aberrant CpG methylation (Butler, 2009). Recent advances in epigenome engineering technology have produced CRISPR-based epigenome editors that couple the programmable targeting of CRISPR with enzymes involved in the DNA methylation pathway (Fig. 3G-J) (Liu et al., 2016b; Lei et al., 2017; Liu et al., 2018a).

As dynamic DNA methylation has been proposed to regulate activity-dependent gene expression, Liu and authors sought to determine whether their lentiviral dCas9-TET1 system could induce Brain-derived Neurotrophic Factor (BDNF) expression by targeting the BDNF IV promoter for demethylation in cultured primary neurons (Fig. 3H) (Liu et al., 2016b). Neuronal dCas9-Tet1 expression successfully increased BDNF expression 6-fold, however 'no sgRNA' controls also produced a nearly 2-fold increase in BDNF expression, demonstrating this system's potential for non-specific gene induction.

529 CRISPR-epigenome editors have also been used preclinically for therapeutic studies for example, dCas9-
530 TET1 was used to demethylate the CGG trinucleotide expansion in the 5' UTR of the Fragile X Mental
531 Retardation 1 (FMR1) gene in models of Fragile X Syndrome (FXS) (Persico and Napolioni, 2013; Liu et
532 al., 2018a). dCas9-TET1 targeting to the FMR1 5' UTR in *in vitro* derived FXS neurons significantly
533 reduced CGG trinucleotide hypermethylation and the associated hyper-excitable phenotype.
534 Remarkably, dCas9-TET1 treated iPSc-induced FXS neurons retained high-levels of FMRP expression
535 months after their engraftment into live mouse brains.

536 Beyond the transcriptional regulation mediated by dynamic DNA methylation, histone
537 modifications gatekeep gene expression by altering chromatin conformation and the accessibility of cis-
538 regulatory elements to DNA binding proteins (Yarrington et al., 2018). CRISPR-based epigenome editors
539 have been used to uncover the functional importance of discrete regulatory elements (Hilton et al.,
540 2015; Chen et al., 2019). Using dCas9-p300 and dCas9-HDAC8 (Fig. 3I-J), the histone modifications at the
541 2nd enhancer (Enh2) of the neuronal immediate early gene (IEG) Fos were shown to fine tune the degree
542 of activity-induced transcription. In other words, the type of histone modification installed by
543 p300/HDAC8 could slightly increase or decrease activity-dependent Fos transcription. However, inducing
544 a heterochromatic state with HDAC8 could not completely silence Fos activity and inducing a
545 euchromatic or "pro-transcriptional" state was insufficient to induce Fos transcription without neuronal
546 activity. This observation contrasts the constitutive gene activation mediated by other CRISPRa systems,
547 which if targeted to Enh2, would presumably induce Fos without neuronal activity. The effectiveness of
548 CRISPR-based epigenome editors highlights the potential for these new tools to elucidate the functional
549 relevance of non-coding and epigenetically regulated elements to animal behavior, neuronal function
550 and disease.

551

Engineering the Neuronal Transcriptome with RNA-Targeting CRISPR Effectors

Programmable DNA-targeting Cas9 nucleases have been used for *in vivo* gene studies, however; tools enabling the study of RNA function are severely lacking. Recently, the diverse group of Class II CRISPR-Cas systems has been expanded to include the Type VI, RNA-targeting Cas13 family of effectors (Abudayyeh et al., 2016; Abudayyeh et al., 2017). Despite their recency, RNA-targeting CRISPR systems have been engineered for targetable RNA visualization, knockdown, base-editing, and most recently, *in vivo* isoform manipulation (Figs. 4 and 5) (Abudayyeh et al., 2017; Cox et al., 2017; Konermann et al., 2018). The Cas13 family of endonucleases are characterized by a single-effector protein containing two Higher Eukaryotes and Prokaryotes Nucleotide-Binding (HEPN) ribonuclease (RNAse) domains (Abudayyeh et al., 2016). Unlike their DNA-targeting counterparts, Cas13 effectors do not require tracrRNAs for pre-crRNA processing, nor do they require PAM sequences for nucleic acid targeting and non-self-recognition. Instead, sequences that are enriched proximally to protospacer targeting sites are referred to as protospacer flanking sequences (PFSs). Notably, several Cas13 variants have been shown to not require PFSs for RNA cleavage (Cox et al., 2017). Multiple studies have reported a large amount of divergence amongst the Type VI family, often reporting little sequence conservation among Cas13 nucleases other than the characteristic HEPN RNase domains; for a more complete discussion of their individual properties we suggest reviewing (Shmakov et al., 2017).

Numerous studies have compared the knockdown ability of multiple Cas13 subtypes and orthologues to RNAi, which have overwhelmingly demonstrated that Cas13's RNA knockdown capabilities are superior to those of shRNAs (Abudayyeh et al., 2017; Cox et al., 2017; Konermann et al., 2018). Additionally, the recently discovered Cas13d effector – *Ruminococcus flavefaciens*-Cas13d (CasRx) (Fig. 4A) – has been shown to more effectively silence gene expression than other well-established methods such as CRISPRi (Konermann et al., 2018). When targeted to the endogenous *B4GALNT1,*

575 *ANXA4* and *HOTTIP* genes in HEK293FT cells, CasRx demonstrated a remarkable median knockdown
576 efficiency of 96% compared to 53% knockdown produced with sequence-matched shRNAs.
577 Furthermore, CasRx did not generate any detectable off-target transcriptional changes, which starkly
578 contrasts shRNA-induced silencing of an excess of 500-900 off-target genes (Konermann et al., 2018).
579 CasRx also outperformed CRISPRi (dCas9-KRAB) mediated repression, which produced a median 53%
580 knockdown rate when targeted to the same genes. Other recently described Cas13 subtypes have been
581 shown to robustly knockdown RNA in mammalian cells, compared to Cas13a (LwaCas13a-msfGFP-NLS)
582 (Abudayyeh et al., 2017) and Cas13b (PspCas13b-NES) (Cox et al., 2017), CasRx demonstrated greater
583 transcript knockdown ability (median knockdown rates; Cas13a - 80%; Cas13b – 66%; CasRx; 97%).
584 Remarkably, of 14 sgRNAs targeted to both coding and non-coding RNA, CasRx yielded at least ~80%
585 transcript knockdown, suggesting that CasRx could be used to regulate any RNA in the cell.

586 Several degenerative diseases have been linked to mutations within individual pre-mRNA
587 elements. For instance, mutations within exons 45-55 or exon 23 of the Dystrophin gene produce the
588 muscle degeneration associated with Duchenne's Muscular Dystrophy (DMD) (Ousterout et al., 2015;
589 Long et al., 2016) . Likewise, neurodegenerative tauopathies such as Frontotemporal dementia with
590 parkinsonism linked to chromosome 17 (FTDP-17) is associated with point mutations in exon 10 of the
591 *MAPT* gene, which determines which Tau protein isoform is expressed in neurons (Boeve and Hutton,
592 2008). As previous studies have reported success in models of DMD using exon-skipping strategies
593 (Nelson et al., 2019), Konermann and authors, tested whether dCasRx could efficiently drive isoform
594 selection by developing a dCasRx-RNA splice effector fusion (Fig. 4B).

595 Pre-mRNA splicing is mediated by interactions between cis-acting elements (splice
596 acceptor/donor sites, intronic branchpoint nucleotides, etc.) and the trans-acting spliceosome (Matera
597 and Wang, 2014). Within the cohort of pre-mRNA interacting molecules are the heterogeneous nuclear

598 ribonucleoproteins (hnRNPs), a ubiquitously expressed group of splice factors that facilitate alternative
599 splicing by inhibiting exon exclusion (Wang et al., 2015). The hnRNPa1-CTD was fused to dCasRx and
600 targeted to several putative splicing elements, which successfully induced exon-skipping in a
601 fluorescence splicing reporter. In order to determine whether skipping Exon 10 of the *MAPT* gene could
602 decrease the accumulation of pathogenic (isoform 4R) tau, cortical neurons differentiated from patient-
603 derived iPSCs were transduced with AAV encoding dCasRx-hnRNPa1 and three Exon 10 targeting
604 sgRNAs; dCasRx-hnRNPa1 mediated exon-skipping was shown to reduce 4R/3R ratios by 50%, a level
605 similar to unaffected controls (Konermann et al., 2018).

606 These results demonstrate the ability of type VI, RNA-targeting Cas13 effectors for enhanced
607 RNA interference and manipulation. In the past, applications of dCas13 effector fusions have been
608 limited by their large size. Therefore, CasRx's short coding sequence (~ 2.9 kb) makes it highly suited for
609 use in AAV vectors. As described above, the CasRx fusion and three sgRNAs fell below AAV packaging
610 limitations, a characteristic that may inspire the future development of CasRx-based effectors that are
611 capable of elucidating RNA function in the brain.

612

613 **Base and Prime Editing**

614 Existing CRISPR technologies equip researchers with a powerful, multifunctional platform to
615 investigate a staggering number of biological questions, however these tools are not without drawbacks.
616 DSBs created by Cas9 nucleases often result in haphazard DNA repair and indel formation, which can
617 frequently produce extensive sequence heterogeneity and yield several unwanted or deleterious DNA
618 products. Technologies have been developed that circumvent problematic DSBs and imprecise cellular
619 DNA repair processes through the use of enzymes (Fig. 5) that can alter RNA and DNA nucleotides *in*
620 *situ*, or more recently, prime editors that can faithfully install edits through reverse-transcription of an

RNA template (Fig. 6). These technologies, termed base editors, rely on dCas9 fusions to nucleobase deaminases to directly install point mutations without the need for DSBs. Existing base editors are collectively able to catalyze all possible transition mutations (C to T and A to G - point mutations) in DNA, with recent developments in RNA base editing allowing the conversion of A to I, and C to U bases as well. (DNA and RNA base editors are extensively discussed in (Rees and Liu, 2018). As of yet, no studies have reported the use of base editors in any neuronal context. However, the growing number of single nucleotide polymorphisms (SNPs) implicated in psychiatric and neurological diseases and the finding that the mRNAs of various neuronal ion channels and synaptic receptors undergo RNA editing may prompt the future use of these tools in neuroscience laboratories (Behm and Ohman, 2016).

Prime editors present the latest advance in precision gene editing. Anzalone and authors introduced a Cas9 nickase (Cas9n)-based system that couples the DSB-free editing strategy pioneered with base-editors to an sgRNA-based RNA donor template (Fig. 6) (Anzalone et al., 2019), a strategy similar to one recently introduced in yeast (Sharon et al., 2018). Prime editors are multi-component systems comprised of a chimeric Cas9n-reverse transcriptase and a Prime Editing guide RNA (pegRNA). Both the target locus and the desired DNA edit are encoded on the pegRNA, which harbors the standard Cas9 sgRNA components and a 3' extended RNA template. Cas9n cleavage of the PAM-containing strand allows donor-template invasion and hybridization, which permits RNA-template reverse transcription and effective installation of the desired edit. This prime editing strategy was shown to successfully introduce broad classes of edits with lower indel frequencies than Cas9-mediated HDR in multiple cell types *in vitro*, including a modest editing frequency (6-8%) in primary neuronal cultures. Although a promising development, the frequency of genome-wide off targets and unintended reverse transcription products remain unknown. This, in concert with the modest editing frequency achieved with the latest prime editor, may preclude its immediate use *in vivo*. Nonetheless, this technology

644 presents an exciting new development towards achieving high-fidelity, corrective gene editing with
645 CRISPR.

646 **CRISPR Screens**

647 The recent exponential advances in next-generation sequencing technologies and the easy
648 design and production of large numbers of unique sgRNAs has facilitated the high-throughput
649 investigation of various psychiatric and neurodegenerative disorders, cancer, and essential gene
650 functions through large-scale CRISPR screens (Fig. 7). CRISPR-mediated-screens combine high-
651 throughput, single-cell sequencing technologies with genome-wide sgRNA targeting libraries optimized
652 for gene knockout (Sanjana et al., 2014; Doench et al., 2016; Morgens et al., 2017; Wang et al., 2018; Liu
653 et al., 2019), activation (Horlbeck et al., 2016; Joung et al., 2017; Chong et al., 2018; Liu et al., 2018b;
654 Sanson et al., 2018) and silencing (Horlbeck et al., 2016; Liu et al., 2017; Sanson et al., 2018)
655 applications. Recent applications of CRISPR-screening have produced new experimental pipelines that
656 permit the unambiguous contribution of risk-associated genes to disease phenotypes (Thyme et al.,
657 2019) and the determination of cellular-lineage and heredity in developmental studies (McKenna et al.,
658 2016; Raj et al., 2018). For example, CRISPR-Cas9 was recently used to perform a mutant-phenotyping
659 screen on schizophrenia-associated genes identified in human genome-wide association studies (GWAS)
660 (Thyme et al., 2019). Cas9 was used to mutagenize several risk-associated genes in developing zebrafish.
661 These mutants were then subjected to behavioral and structural analysis which allowed Thyme and
662 authors to successfully uncover phenotypes for multiple understudied genes. A separate zebrafish study
663 deployed a large-scale CRISPR-Cas9 technique (GESTALT, see McKenna et al., 2016 for additional
664 background) combined with single-cell RNA-seq (scRNA-Seq) to determine cellular fate and lineage
665 characteristics in developing brains. Paired with an inducible Cas9, DNA barcodes harboring specific
666 target sequences were used to indicate whether DNA editing occurred in a specific cell; because

667 genomic barcode expression results in cellular progeny with identical barcode sequences, this allowed
668 Raj and authors to determine the lineage histories for a plethora of cell types in the developing zebrafish
669 brain.

670 While most screens are performed *in vitro* or *ex vivo,* two CRISPR-Cas9 mediated *in vivo* screens
671 have recently been reported in the brain (Chow et al., 2017; Jin et al., 2019). A recent *in vivo* screen
672 (Perturb-Seq, first described by Dixit et al., 2016) aimed at systematically uncovering the phenotypes of
673 a large panel of autism-spectrum disorder (ASD) related genes, was performed by coupling cell-type
674 specific transcriptomics and a lentivirus-mediated sgRNA library targeting 35 putative ASD-risk genes.
675 Ventricularly injecting lentiviral-sgRNA libraries to developing embryos *in utero* permitted postnatal,
676 single-cell transcriptional profiling and identification of multiple gene clusters from cortical and striatal
677 tissue. CRISPR perturbation coupled with a scRNA-seq readout readily enabled differential gene
678 identification, subsequent perturbation and phenotyping for a number of ASD-risk genes involved in
679 distinct molecular pathways across variant cell types.

680 The earliest reported *in vivo* screen was directed at investigating the functional and tumorigenic
681 consequences of significantly mutated genes (SMGs) that were previously identified in tumor samples
682 taken from human glioblastoma multiforme (GBM) patients (Chow et al., 2017). A pooled AAV-sgRNA
683 library (mouse Homolog Tumor Suppressor library – mSTG) targeting various risk-genes was
684 hippocampally or ventricularly infused into mice, which produced GBM-characteristic tumor growth at 4
685 months post-injection. Histological, transcriptomic and genetic characterization of AAV-CRISPR
686 mediated GBM tumors, *in vivo* and *ex vivo,* permitted the successful identification and correlation of
687 single and co-occurring tumor drivers to GBM mutations identified in human patients.

688 Although CRISPR-based screens are heavily used in oncology research (Hart et al., 2015; Tzelepis
689 et al., 2016; Chow et al., 2017) these tools have garnered significantly less attention for large-scale

genetic studies in disease-relevant cell types such as differentiated neurons. Tian and authors recently performed several CRISPRi screens to elucidate functional contributions of various genes to cell survival, differentiation, transcriptional regulation and morphology in human inducible pluripotent stem-cell (hiPSC) derived neurons (Tian et al., 2019). In an initial survival screen, dCas9-BFP-KRAB and the lentiviral H1 sgRNA library were used to target ~2300 genes comprising the "druggable genome". CRISPRi-mediated gene knockdown uncovered a strong neuronal dependence on sterol/cholesterol metabolism genes and enhanced neuron survival when members of the integrated stress response (DLK, JNK, PERK) were knocked down. Tian and authors also performed screens that identified common regulators of variant transcriptional programs in iPSCs and neurons, as well as several genes that contributed to neuronal longevity and morphology.

Existing Challenges for CRISPR Gene Editing

Despite the explosive progress of CRISPR-mediated genome engineering in the last decade, significant challenges for clinical and preclinical applications remain. For example, concerns regarding CRISPR's immunogenicity, targeting efficiency, fidelity and optimal delivery will need to be addressed before CRISPR can fulfil its full clinical and research potential.

Delivering CRISPR *in vivo* can be mediated via viruses, RNPs/nanoparticles, or a combination of viruses and transgenic animals (e.g., Cas9 mouse). For preclinical studies utilizing small animals, these delivery methods are sufficient, since experiments can be conducted where useful data can often be generated by targeting/manipulating a small body region of approximately a few cubic millimeters. Virus and RNPs can adequately deliver their necessary cargo to regions of this size reasonably well. However, improvements could be made to increase the ease of delivery and the area of tissue that could be effectively targeted with CRISPR systems. For example, conventional AAVs and LVs need to be

stereotaxically injected intracranially to gain access to the brain and usually will not transduce more than a few cubic millimeters of tissue. More recently, AAVs with modified capsids have been developed that can cross the blood brain barrier, so they can be administered systemically and transduce brain cells (Chan et al., 2017). However, while these developments are encouraging, they still need more development for clinical utility. Notably, non-human primate research and clinical human studies will generally benefit from less invasive routes of delivery that can target far larger regions of the brain than just a few cubic millimeters. This will be an important hurdle to overcome if CRISPR is to ever realize its full potential at treating CNS diseases.

For any gene modification technique, its specificity and accuracy are paramount, especially for clinical applications. While high-fidelity spCas9 variants have been developed (Kleinstiver et al., 2016; Slaymaker et al., 2016; Chen et al., 2017; Casini et al., 2018; Chatterjee et al., 2018; Hu et al., 2018), the off-targeting frequencies and loci for therapeutic sgRNAs will need to be thoroughly characterized *in vitro* before use in human therapies. For gene knockout in preclinical applications, SpCas9's fidelity is likely sufficient, especially since researchers can perform independent experiments with differing sgRNAs designed to knockout out their intended gene. Given that differing sgRNAs would likely not exhibit the same off targets, if the same phenotype is obtained with both sgRNAs, then their result would likely be due to the knockout of the intended target. While CRISPRi and CasRx have been demonstrated to be highly accurate methods to target specific genes for transcriptional inhibition and knockdown (Gilbert et al., 2013; Konermann et al., 2018; Yeo et al., 2018) , the latest generation of CRISPRa (Suntag, VPR and SPH systems) still requires whole genome sequencing to determine their targeting specificity. This is especially important, given how effective these newer systems are at inducing gene expression.

735 Preclinical studies using CRISPR-Cas have generated significant enthusiasm for the future of
736 personalized gene therapies. However, as CRISPR becomes implemented clinically, aspects of its safety
737 for use in human therapies have received extensive scrutiny. Recently, various pre-clinical studies have
738 described the immunogenicity of CRISPR nucleases following systemic (IV) administration to laboratory
739 mice (Chew et al., 2016; Nelson et al., 2019). Host anti-vector and transgene responses are discussed
740 elsewhere (Sun et al., 2003; Rabinowitz et al., 2019; Wang et al., 2019). Additionally, pre-existing
741 adaptive immunity against *Streptococcus pyogenes* and *Staphylococcus aureus* Cas9 have also been
742 reported in humans (Charlesworth et al., 2019). However, these findings are unsurprising given the high
743 frequency at which these bacteria infect humans (Lowy, 1998; Roberts et al., 2012). While SpCas9 and
744 SaCas9 remain two of the most broadly used CRISPR enzymes, new orthologues derived from non-
745 pathogenic bacterial species may be required for human therapies where pre-existing immunity is a
746 concern. Alternatives such as orthologue specific-epitope engineering or short term suppression (Chew
747 et al., 2016) may theoretically ameliorate immune responses in the short-term. However, the long-term
748 expression of AAV-mediated therapies and their potential for genome-insertion at DSB sites (Miller et
749 al., 2004; Hanlon et al., 2019), may limit the feasibility of immunosuppressive approaches.

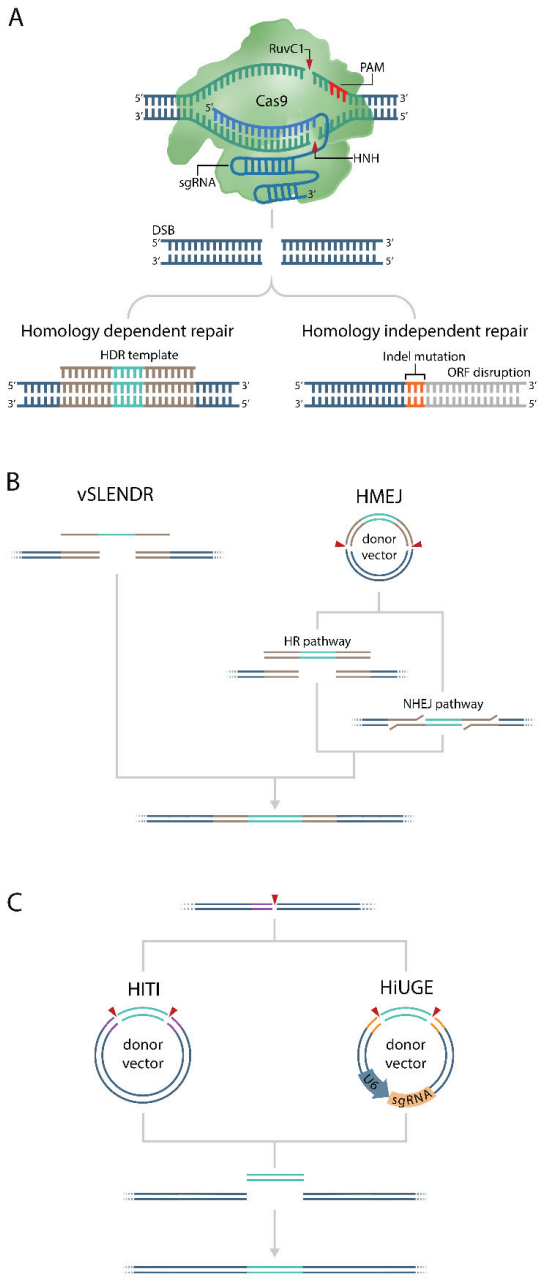
750 The low efficiency of precise editing (corrective editing via HDR, Prime Editing etc), in neurons is
751 another significant hurdle for the use of CRISPR for neuroscience research and human therapy. The
752 available data indicate that precise editing occurs at relatively low levels in neurons, limiting the utility
753 of these methods and currently making them unlikely to have any benefit clinically. Although precise
754 editing occurs relatively infrequently in most cell types compared to NHEJ-mediated indel formation,
755 disorders that afflict mitotically active cell populations may be more amenable to precise editing. For
756 example, hemopoietic progenitor cells can be genetically modified ex vivo, clonally selected for the
757 precise modification, expanded and then re-implanted, essentially bypassing the issue of inefficient HDR
758 mediated precision editing. This has also been demonstrated in a mouse model of hereditary

tyrosinemia type I (HTI), where AAV-CRISPR was delivered via tail injection (Yin et al., 2014). Although only a small percentage of liver cells (<1%) harbored the gene correction, the treated hepatocytes were able preferentially repopulate the liver, as the introduced gene correction provided a fitness advantage over unedited cells. Unfortunately, given that neurons are post-mitotic, CRISPR mediated precision editing has limited utility for the foreseeable future until methods are developed to increase the efficiency of precision

Conclusions and Future Directions

The CRISPR-Cas system has emerged as a highly adaptable platform with extensive utility in multiple areas of biomedical and basic science. Given its ability to target nearly any gene or RNA transcript, alter gene expression and modify epigenetic states with high specificity, CRISPR-Cas represents an invaluable tool helping drive the rapid pace of discovery in biological sciences. While early studies only demonstrated its use in peripheral tissues, recent efforts have produced CRISPR-Cas systems amenable for use in the central nervous system. Additionally, the development of CRISPR-expressing animals, as well as the discovery of AAV-compatible orthologues, have provided substantial tools for probing neuronal function at multiple levels of analysis. While newly developed CRISPR-transgenics may be crossed with existing Cre-driving lines, newly developed and CNS-optimized tools will likely require viral vector encoding and delivery. Challenges associated with viral vectors such as packaging constraints, low virus infectivity and low gene editing efficiencies remain limiting factors for using CRISPR in the brain. In order to maximize the therapeutic and research potential of available systems, existing delivery methods must be optimized and new, more effective ways of introducing these systems must be developed. Undoubtedly, future improvements and applications of CRISPR-Cas technology will surface. Despite these challenges, recent advances in CRISPR-Cas technology have provided researchers with powerful new tools for engineering the neuronal genome.

Figures:



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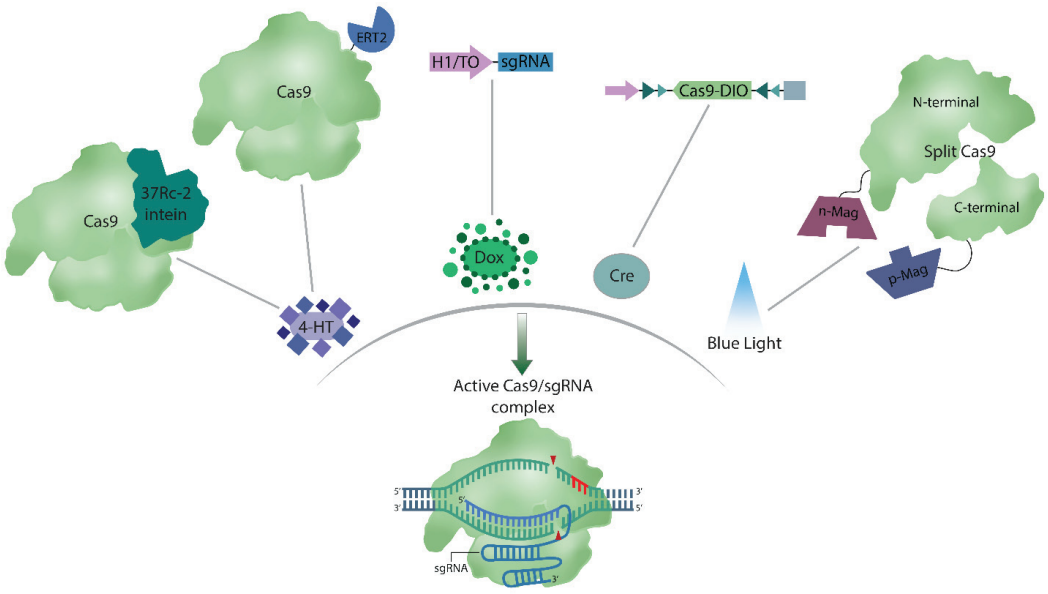
788 **Figure 1. CRISPR-Cas9 mediated genome editing. A)** Cas9 target recognition occurs through sequence
789 complementarity between a Cas9 associated single guide RNA (sgRNA) and a genomic target sequence.
790 Target recognition requires the presence of a proximal 3' protospacer adjacent motif (PAM), which
791 facilitates Cas9 binding and endonucleolytic cleavage. Cas9's dual catalytic domains, HNH and RuvC1,
792 mediate complementary and non-complementary strand cleavage, respectively. Double stranded breaks
793 (DSBs) repaired by non-homologous end joining (NHEJ) can introduce short insertion/deletion (indel)
794 mutations that cause frameshifts capable of disrupting protein coding sequences, causing loss of gene
795 function. Alternatively, Homology-Directed Repair (HDR) can be used for site-specific, sequence
796 alteration by supplying DNA templates encoding user-specified modifications. **B)** The Viral-mediated
797 Single-cell Labeling of ENdogenous proteins via HDR system (vSLENDR) and Homology-mediated end
798 joining (HMEJ) knockin strategies exploit homology dependent repair pathways to introduce foreign
799 sequences. vSLENDR and HMEJ both require long homology arms flanking the DSB site for efficient gene
800 insertion. However, HMEJ utilizes a hybrid NHEJ/HDR strategy which departs from the HDR-based
801 vSLENDR strategy by also requiring DSBs to release the donor DNA template (2B – Red arrows). **C)**
802 Homology Independent (NHEJ) knockin strategies mediate sequence insertion by forming DSBs at
803 desired target sites and donor templates simultaneously. Homology Independent Targeted Integration
804 (HITI) utilizes a donor template that is flanked by sgRNA recognition sites that match the genomic target.
805 Simultaneous donor/target cleavage and repair stimulate donor template insertion. Homology-

806 Independent Universal Genome Engineering (HiUGE) also requires simultaneous donor and target
807 cleavage, however, HiUGE donor vectors encode both a donor template and a self-targeting sgRNA.

808



809

810 **Figure 2. Inducible CRISPR-Cas systems.** CRISPR-Cas9 genome editing can be spatially and temporally
811 regulated with a variety of genetic, small molecule, and optical techniques. Intein-Cas9 and iCas can be
812 regulated with the small molecule 4-hydroxytamoxinfen (4-HT). Whereas 4-HT induced intein splicing
813 renders Intein-Cas9 constitutively active, iCas is bidirectionally regulable. Gene targeting sgRNAs can be
814 transcriptionally regulated with the doxycycline response H1/TO promoter. Additionally, both sgRNA
815 and Cas9 expression cassettes can be rendered Cre-dependent with the insertion of flanking loxP sites.
816 Split architecture Cas9 systems have also been rendered photoinducible through fusions to light
817 responsive, heterodimizering molecules.

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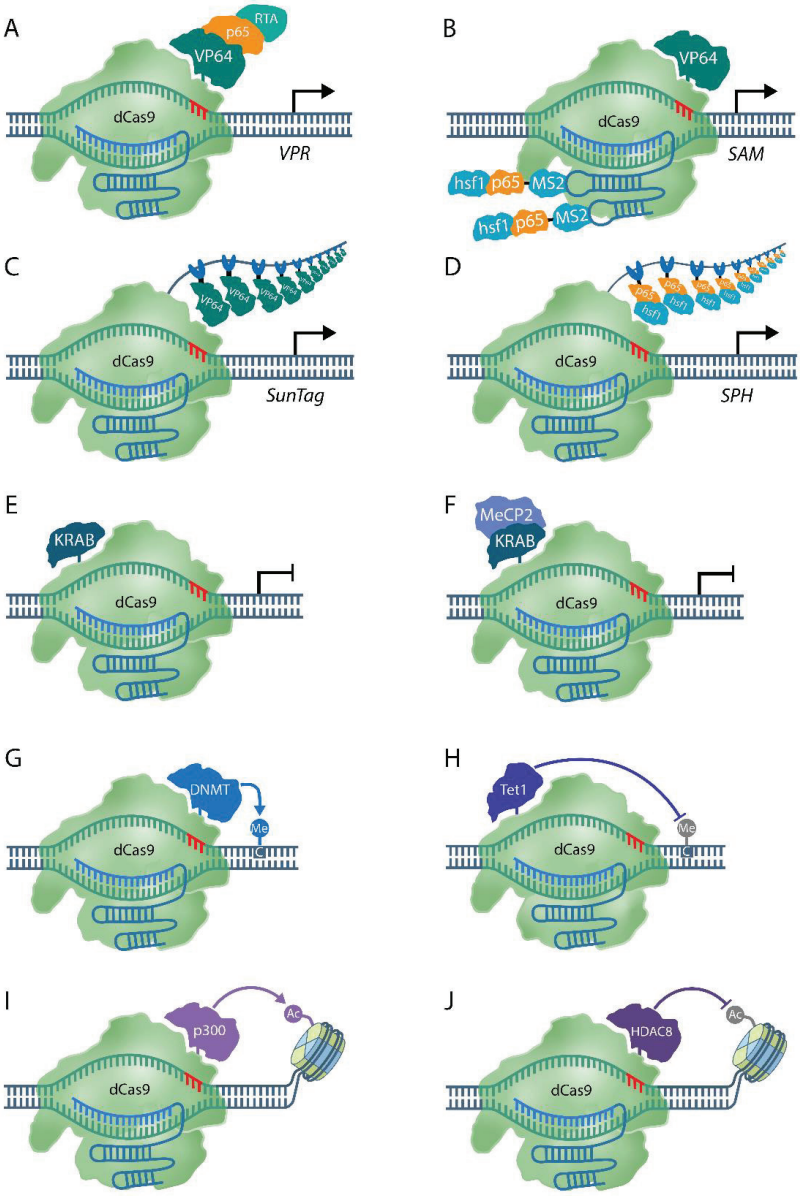

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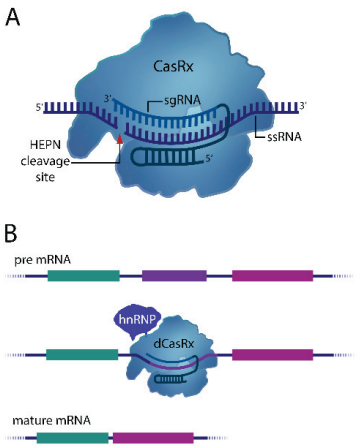
Figure 3. Transcriptional and epigenetic regulation with CRISPR-Cas Transcriptional control can be achieved by fusing various transcription regulating enzymes to catalytically inactive Cas9 (dCas9). The CRISPR-based activators Suntag, VPR, SAM and SPH employ various architectures to recruit transcription activating molecules. **A)** VPR deploys traditional peptide linkers to fuse the tripartite VP64, p65 and Rta effector to dCas9. **B)** The Synergistic Activator Mediation (SAM) uses the MS2 RNA aptamer to recruit MS2 Coat Proteins (MCP) fused to a p65-HSF1 domain to induce transcription. **C)**The Suntag system utilizes the a GCN4-epitope array to localize VP64 activators to transcription start sites (TSS). **D)** Relatedly, the Suntag-p65-HSF1 (SPH) system uses the Suntag scaffolding array to recruit p65-HSF1 dimers in lieu of VP64. **E)** The dCas9-KRAB (Krüppel-Associated Box) and **F)** the improved dCas9-KRAB-MeCP2 (Methyl CpG binding Protein 2) transcriptional repressors use similar strategies inhibit transcription. **G)** dCas9 fused to the DNMT3A (DNA Methyltransferase 3A) enzymatic domain can de-novo methylate CpG dinucleotides in a programmable manner. **H)** dCas9 fused to Ten-eleven Translocation's (TET1) catalytic domain facilitates successive cytosine oxidation and demethylation at methylated CpG sites. dCas9-DNMT3A/TET1 can effectively regulate gene transcription by targeting CpG containing promoter regions for epigenetic modification. **I)** dCas9 C-terminally fused to the catalytic core of the human p300 acetyltransferase ($p300^{core}$) or **J)** Histone Deacetylase 8 (HDAC8) can regulate the acetylation status of Histone 3 Lysine 27 (H3K27) residues to regulate transcription from promoters and both distal and proximal enhancers.



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841

842 **Figure 4. RNA targeting with CasRx**. CasRx can efficiently target and cleave RNA via its dual Higher
843 Eukaryotes and Prokaryotes Nucleotide-Binding (HEPN) nuclease domains. Unlike DNA targeting Cas9
844 endonucleases, several Cas13 orthologues do not exhibit protospacer flanking sequence (PFS; PAM site
845 analogue) requirements. Mutating HEPN catalytic residues (R295A, H300A, R849A, H854A) preserves
846 CasRx's RNA binding ability, allowing CasRx to be adapted for fusion constructs. **Splice Isoform**
847 **Engineering |** Decatalyzed CasRx (dCasRx or dCas13d) fused to the splicing factor hnRNP1 can be
848 targeted to various splice elements (splice acceptors, splice donors, intronic branch points etc) to induce
849 exon skipping and isoform selection.

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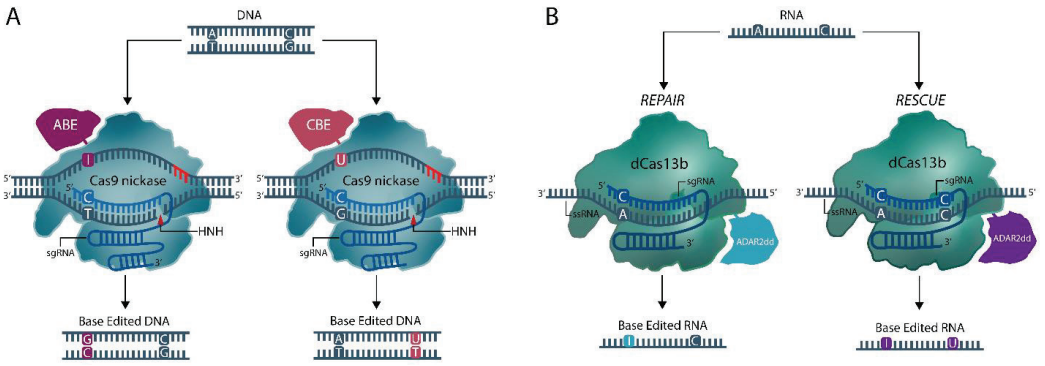

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852

853 **Figure 5. Base Editors. A)** Adenine (ABE) and Cytosine base editors (CBE) catalyze the deamination and
854 alteration of DNA nucleobases via chimeric Cas9n-DNA deaminase fusions. Nicking (single strand DNA
855 cleavage) of the non-edited strand increases base-editing efficiency by inducing cells to repair the
856 cleaved strand using the edited strand as a template. **B)** The Cas13-based RNA base editor RNA-Editing
857 for Programmable A to I Replacement (REPAIR) mediates the conversion of Adenosine to Inosine, while
858 RNA Editing for Specific C to U Exchange (RESCUE) mediates the conversion of Cytosine to Uracil.

859

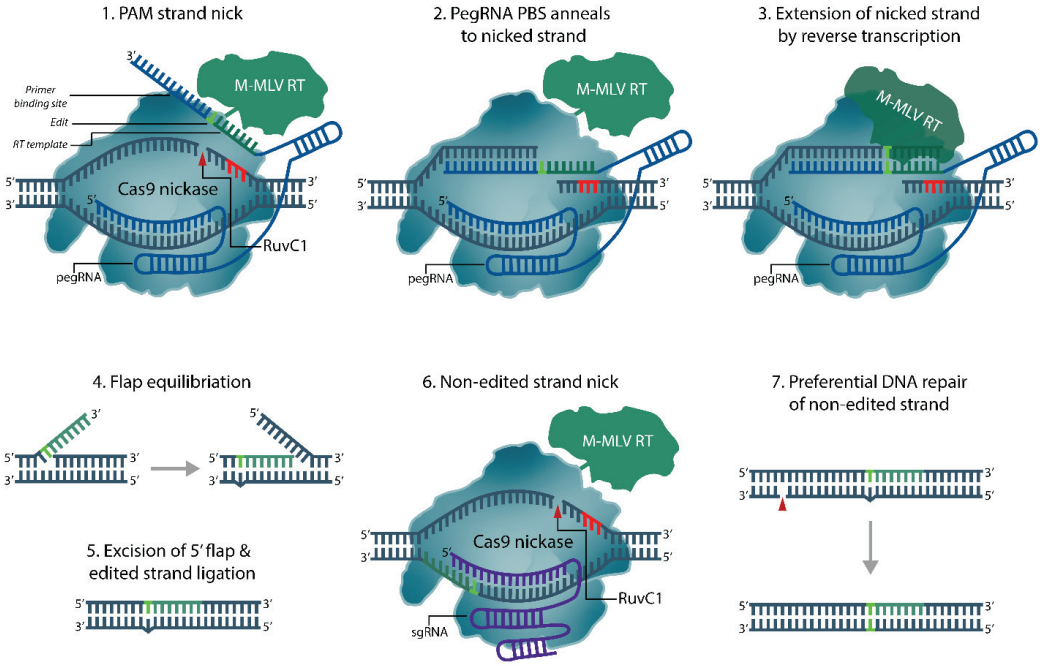

Figure 6. Prime Editing. Prime Editors (PE) utilize a partially decatalyzed Cas9(H840A) nickase, a Prime-Editing RNA (pegRNA) and an engineered reverse transcriptase (RT) to precisely introduce DNA edits; pegRNAs contain a primer binding site (PBS) which anneals to the nicked target strand, allowing sequence extension through reverse transcription and production of the edited strand. pegRNA-PBS reverse transcription produces an edit-containing 3' flap and an unedited 5' flap which undergoes preferential degradation by endogenous 5'-3' exonucleases. The remaining edited 3' flap anneals and is ligated, resulting in a mismatched heteroduplex which can be resolved by cellular DNA repair pathways. Targeting the unedited strand with a separate sgRNA increases editing efficiency and stimulates preferential DNA repair to permanently install edited DNA.

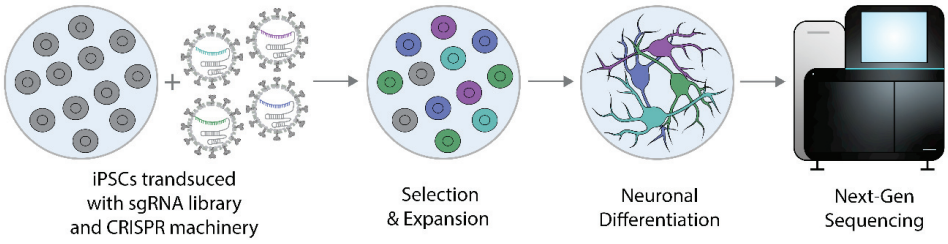

Figure 7. High Throughput Genetic Screening with CRISPR. Large scale genetic screens can be performed in inducible pluripotent stem cell-derived neurons (iPSCs) expressing CRISPR machinery. Transduction of iPSCs with pooled lentiviral sgRNA libraries permits the selection and expansion of construct-positive cells before *in vitro* neuronal differentiation. CRISPR-KO, CRISPRi and CRISPRa can be coupled with single cell -omics and next generation sequencing technologies for genome-wide or targeted gain- and loss-of function screens.

884

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