Unraveling of Central Nervous System Disease Mechanisms Using CRISPR Genome Manipulation

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ABSTRACT: The complex structure and highly variable gene expression profile of the brain makes it among the most challenging fields to study in both basic and translational biological research. Most of the brain diseases are multifactorial and despite the rapidly increasing genomic data, molecular pathways and causal links between genes and central nervous system (CNS) diseases are largely unknown. The advent of an easy and flexible CRISPR-Cas genome editing technology has rapidly revolutionized the field of functional genomics and opened unprecedented possibilities to dissect the mechanisms of CNS disease. CRISPR-Cas allows a plenitude of applications for both gene-focused and genome-wide approaches, ranging from original "gene scissors" making permanent modifications in the genome to the regulation of gene expression and epigenetics. CRISPR technology provides a unique opportunity to establish new cellular and animal models of CNS diseases and holds potential for breakthroughs in the CNS research and drug development.

KEYWORDS: Central nervous system, CRISPR-Cas, CRISPR, brain, CNS disease, genome editing

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Introduction

Central nervous system (CNS) disorders represent a global burden for the society in terms of disability, human suffering, and economic losses. However, effective therapeutic intervention, let alone the cure, is still lacking for most CNS disorders, mainly due to the lack of thorough understanding of disease-relevant cellular and circuit mechanisms. The completion of the Human Genome Project in 2003 followed by rapid development of gene sequencing technologies has substantially advanced our understanding of the genetic architecture of neurological diseases. The finding of large effect-size rare familial mutations has provided invaluable insight into biological pathways and mechanisms underlying many common and devastating neurological diseases including amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), or schizophrenia (SZ). However, most CNS disorders are a result of complex interaction of multiple low effect risk and protective alleles, which work in concert with environmental factors to determine individual's genetic risk to a disease. Although large-scale genomic investigations have uncovered the involvement of hundreds of coding and noncoding sequences in polygenic neurological diseases, their functional roles in the nervous system are largely unknown.[1–3]

During the past 10 years, understanding of the brain has advanced with tremendous leaps, fueled by the development of cutting-edge technologies such as optogenetics and high-resolution imaging tools.[4,5] New technologies have made it possible to manipulate and visualize neural activity in specific cell types in real time, which has truly transformed our ability to understand the function of the nervous system at the systems level. At the same time, the development of precise genome engineering methods has led to a revolution in the functional genomic research across biomedical fields. In particular, the advent of RNA-guided genome editing tool CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas (CRISPR-associated protein) has opened new avenues for both basic and translational research.[6,7] The impressive power of CRISPR, method based on bacterial adaptive immune system components, relies on its unprecedented easy and adaptable design which works across species.[8] In addition to its principal application for generating site-specific genomic modifications, quickly increasing number of novel innovative CRISPR applications have been developed for regulating epigenome, controlling gene expression, and labeling genomic sequences.[9] CRISPR requires only 2 key components to function: a nuclease (eg, Cas9) and sgRNA (single-guide RNA) which directs nuclease to specific genomic sites. By simply exchanging the recognition sequence of the expressed sgRNA, CRISPR can be targeted to new genomic positions. By expressing several sgRNAs, the system also enables multiplex genome editing at high efficiencies, making it an attractive tool for studying multigenic disorders and gene interactions.

The advent of CRISPR offers tremendous potential to dissect the complexity of the CNS and to pursue CNS disease-causing factors.[10] CRISPR facilitates the generation of traditional animal models, such as mice, by reducing the time required for the production of new transgenic lines and allowing simultaneous engineering of multiple loci in one generation. The universal design of CRISPR allows its utilization in

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any species or existing model systems independent of their genetic background, opening up the avenue for the development of novel, more accurate animal models. In addition to generating new transgenic animal lines, CRISPR can be used for spatially and temporally controlled in vivo gene manipulation in the CNS.

Beyond classical reverse genetic approaches, the efficiency and multiplexing capabilities of the CRISPR enable a high-throughput forward screening of "genotype to phenotype" functions in various model systems. In combination with recent breakthroughs in stem cell and 3-dimensional (3D) culture technologies, CRISPR facilitates the studying of mechanisms underlying CNS diseases in human-derived in vitro model systems.

The goal of this review is to provide basic understanding of the rapidly developing CRISPR technique and its potential in studying the mechanisms of CNS diseases. The review highlights recent technical advancements along with the benefits and challenges of diverse CRISPR applications for the neuroscience research.

Gene Editing Methods

Genome editing methods refer to a variety of technologies used to add, remove, or modify specific DNA sequences in the specific site of an organism's genome. Genome editing tools based on DNA-cutting nucleases provide a general method for inducing site-specific sequence modifications in the genome of broad range of organisms and cell types.[11,12] The first group of genome editing tools, including meganucleases, zinc finger nucleases (ZFNs), and transcription activator–like effector nucleases (TALENs) achieve sequence-specific DNA-binding via protein-DNA interaction.[13–17] These programmable nucleases have enabled important advances in genome editing in a variety of different cell types and organisms, but their use is limited by laborious design and engineering of new specific protein modules for each new target site. More recently, the discovery and development of RNA-guided genome editing tool CRISPR-Cas has led to a giant leap in the field of genome engineering.[6,7,18] The simplicity and high efficiency of the CRISPR system allow affordable genome editing now in a wide variety of organisms.

CRISPR-Cas—RNA-guided genome editing tool

CRISPR-Cas is an RNA-mediated adaptive immune system mechanism, which bacteria and archaea use to protect themselves from foreign nucleic acids such as viruses and plasmids.[19,20] Microbial CRISPR-Cas systems are divided into Class 1, with multisubunit effector complexes, and Class 2, with single protein effectors.[21] First identified in *Streptococcus pyogenes*, the native type class II type II CRISPR-Cas9, is so far the best characterized of bacterial CRISPR systems. A major breakthrough in the development of CRISPR system as a genome editing tool was the description of single synthetic guide RNA (sgRNA), which can be easily engineered to target Cas endonuclease into a genomic locus of interest (Figure 1A). When bound to the genome, Cas endonuclease induces a double-strand DNA break (DSB) at the target location. This chromosomal DSB is detected in cells as potentially lethal damage, which activates 1 of 2 highly conserved natural DNA repair machinery pathways to repair breaks in eukaryotic cells (Figure 1B). The highly error-prone nonhomologous end joining (NHEJ) pathway leads to unpredictable introduction of insertions and deletions (INDELs) of various lengths, which can disrupt the translational reading frame of coding sequence and consequently results in gene knockout. Homology-directed repair (HDR)-mediated repair induces a precise recombination event between damaged target locus and native or engineered DNA donor template. Thus, HDR can be used for introducing specific point mutations or inserting and deleting desired sequences into the genome.

Since the initial description of CRISPR-Cas9 as a programmable tool to cut DNA in vitro,[6,7] numbers of papers have been published to show that the method can be used to cut and edit DNA in a variety of cells and organisms encompassing different types of human cells and model organisms important for biomedical research, embryonic stem cells (ESCs), as well as crop plants and livestock.[6,9,18] The advantages of CRISPR over previous gene editing methods rely on its simple and adaptable design and affordable costs. Double-strand break in the target site can be induced by introducing only 2 components, sgRNA and Cas nuclease, into cells or an organism. The only required engineering is a 20-nt (nucleotide) target-complementary sequence in sgRNA. A unique advantage of CRISPR-based systems over previous programmable nucleases, such as ZFNs and TALENs, is an ease of multiplexing. By introducing several sgRNAs in parallel, single nuclease can simultaneously induce mutations in multiple genes.[6,22,23]

Along with the most commonly used CRISPR-Cas9 system from *S pyogenes*, several other natural and synthetic CRISPR nucleases have recently been described for improved specificity, targeting scope, and spatiotemporal resolution.[24–31] In addition to its most popular role as a tool to make permanent site-specific modifications in the genomic DNA, a diverse set of CRISPR-based applications have been developed for, ie, modulating gene expression, regulating epigenome and labeling of specific genomic regions,[8,28] and more recently to track and modify RNA[32-34] (Figure 2).

Gene Editing in the CNS

The mammalian brain is composed of a complex network of functionally and morphologically differentiated neuronal types with highly specialized functions. Recent development of sensitive next-generation sequencing methods has further highlighted the diversity of neuronal cells in terms of highly variable transcriptional profile.[35,36] Although all neuronal types share a single genomic blueprint, the expression and function of specific genes is strictly dependent on the brain area, microenvironment, and neuronal connections, which

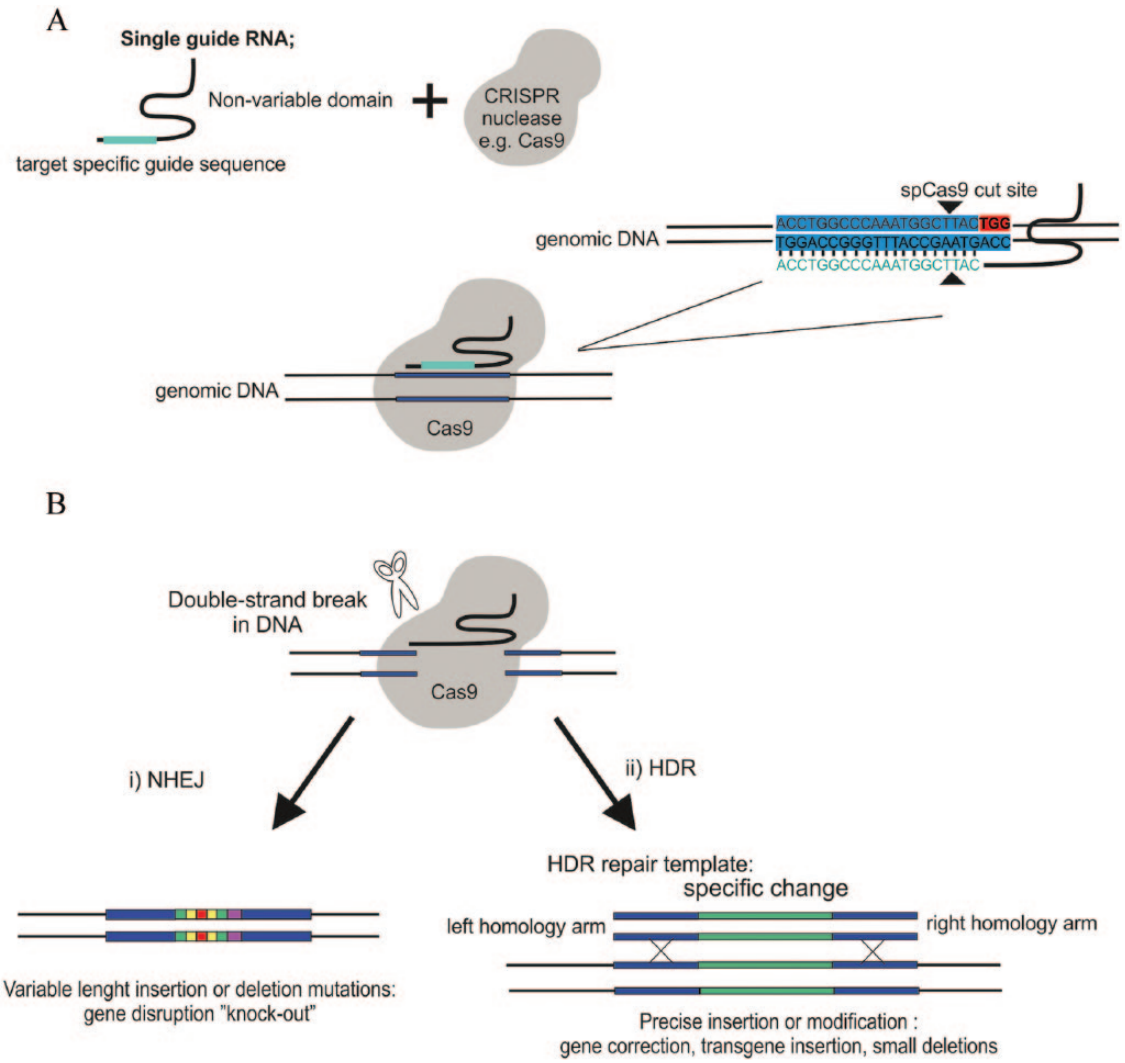

Figure 1. Principle of CRISPR gene editing. (A) Single guide RNA (sgRNA) consists of 20-base sequence (guide sequence) specific to the target DNA 5´ of a nonvariable scaffold sequence. sgRNA directs Cas nuclease (here Cas9) to its genomic locus via Watson-Crick base-pairing and targeting of Cas9 can be easily changed by altering only the 20-nt guide sequence within the sgRNA. The only absolute requirement for the Cas nuclease-mediated cleavage is the location of the proto-spacer adjacent motif (PAM) sequence at the 3´ end of the DNA target sequence. Cas nucleases differ in their PAM requirement (5´-NGG-3´ for spCas9) and cleavage pattern, expanding the genomic loci which can be reached and allowing more flexibility for the design of target sites. (B) Repair pathways. After Cas nuclease has incorporated a sequence-specific double-strand break (DSB) in the genomic DNA, the cell repairs cut DNA strand using natural repair pathways. (i) In the absence of a donor template, the cell will repair DSB mainly by error-prone nonhomologous end joining (NHEJ) pathway, which joins the ends of damaged DNA together. This results typically in random insertions and deletions (INDELs) at the site of editing and gene knockout. (ii) Alternatively, HDR pathway can be activated in dividing cells by providing a separate DNA donor template containing sequences homologous to the regions flanking the DSB. HDR requires a recombination event between the damaged target and intact donor strands of DNA and is thus the more accurate mechanism for DSB repair. HDR can be used for generating specific insertions, point mutations, or deletions. DSB, double-strand break; HDR, homology-directed repair; INDEL, insertion-deletion; NHEJ, nonhomologous end joining.

poses a huge challenge for attempts to study genotype-phenotype relationship.

Deciphering of normal and pathological gene functions in the human CNS is restricted mainly on postmortem samples and more recently on human stem cell–derived in vitro cultures. Thus, cellular and animal models play a significant role in both basic and translational neuroscience research. CRISPR has turned out to be exceptionally agile and affordable method for generating new mouse models as well as enabled the development of novel models of human disease in species not previously accessible for genetic manipulation.[37,38] In addition to promoting the generation of new transgenic animal models, CRISPR-based strategies have been applied to in vitro–cultured neural cells, ex vivo brain slices, and in vivo embryonic and adult mouse brain.[10]

Because most of the studies on the functions of programmable nucleases have been conducted in dividing cell lines,[6,7,18,39] the outcomes of targeted genome editing tools may vary considerably in the highly differentiated neural tissue. In postmitotic neurons, HDR is infrequent and NHEJ is considered to be the

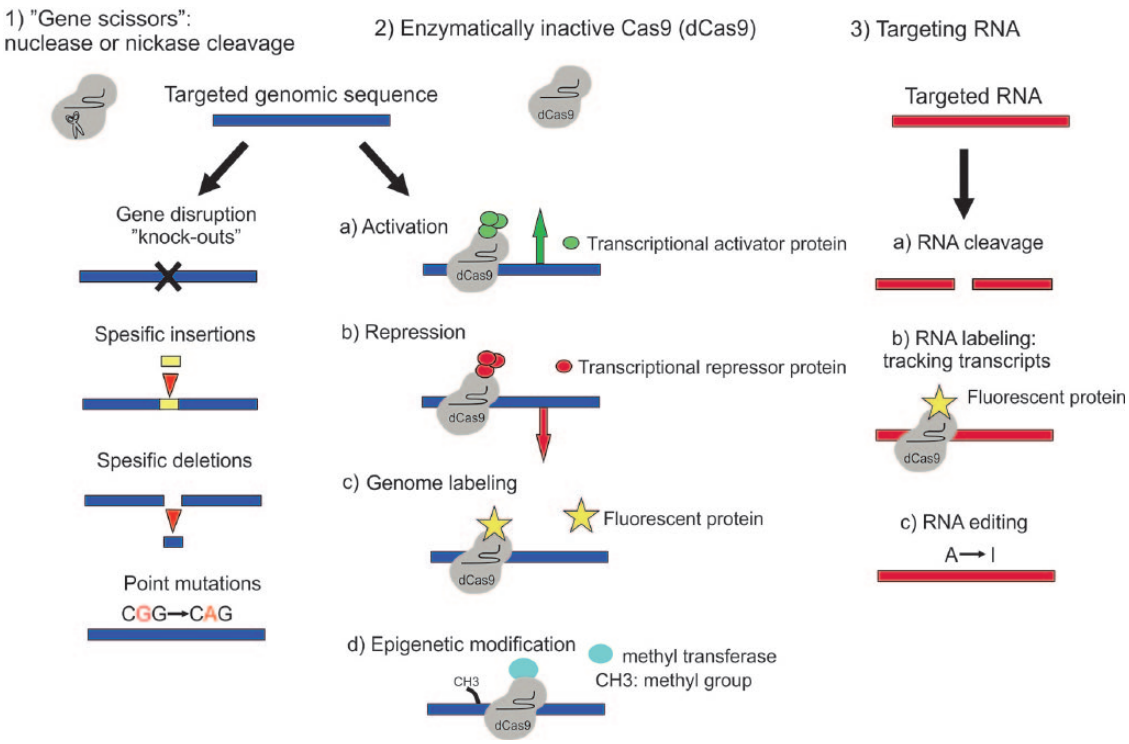

Figure 2. Emerging applications of CRISPR technologies. (1) Beyond the original "gene scissors" making targeted modifications in the genomic DNA, (2) CRISPR can be used as a general RNA-guided platform to direct effectors into the specific sites at the genome. Two mutations convert Cas9 into catalytically inactive, nuclease dead Cas9 (dCas9). dCas9 does not induce double-strand breaks but can be fused to transcriptional activators (2a) or repressors (2b) to regulate gene expression, fluorescent marker proteins to label genomic sequences (2c) In the picture please change 2e) with 2c) epigenetic modifiers such as methyltranferases or demethylases to alter packaging of DNA into chromatin (2d). Instead of targeting chromosomal DNA, CRISPR-based methods can be used for regulating levels of small noncoding RNAs (microRNAs) and messenger RNAs (mRNAs) (3a) and tracking (3b) and editing of full-length transcripts (3c).

major pathway for DNA repair,[40,41] limiting the use of CRISPR applications relying on HDR repair in the mature nervous system. During the development, proliferative neural tissues use mainly HDR and failure to faithfully repair the genome leads to apoptosis.[42,43] Recently, however, novel strategies have been developed to facilitate targeted gene editing in the nervous system.[44,45]

Modeling CNS Disease In Vivo

Accelerated generation of rodent models

For past decades, genetically modified mouse has been a primary experimental model for studying gene function and human disease. Conventional gene targeting methods rely on introducing mutations through homologous recombination (HR) in mouse ESCs,[46] which is slow, laborious, and limited to strains for which germline-competent ESCs exist. The discovery that CRISPR components can be injected into ESCs or directly into 1-cell embryo to generate transgenic founders (Figure 3A)[22] has revolutionized the field of mouse translational genetics.

With its unprecedented efficiency and ease of use, CRISPR provides several advancements for modeling neurological disorders in mouse models.[47,48] The time required to generate a transgenic mouse is now months, not years, and the costs a fraction of conventional techniques. It has been estimated that generation of a transgenic mouse line carrying a short insertion or deletion costs up to 80% less when using CRISPR technique in zygote in comparison with traditional gene targeting in mouse ESCs.[47] For the first time, it is feasible to create mouse model panels in which each model carries a mouse allele that has been modified to carry a different mutation, covering a complete allelic series of patient-specific mutations.[47] The accuracy of modeling complex human traits is improved by accessibility of any mouse strain with CRISPR genome editing. The mouse humanization project aims at exchanging of mouse genes with their human homologues, which is expected to increase the translational relevancy of mice studies.[47]

The full power of CRISPR in mouse genetics relies on the possibility to simultaneously engineer multiple loci to study the combinational effects of several genes. It has been shown that using CRISPR, 3 to 5 mutations can be generated in one generation.[22] As tens to hundreds of different mutations of individual genes and of tens to hundreds of genes comprise the causative genetic landscape of most of the CNS disorders, assessing combinatorial gene effects is imperative to understand their cause. Conditional CRISPR approaches have been developed for inducible and tissue-specific CRISPR editing, allowing the spatiotemporal assessment of gene functions in transgenic mice.[30,49]

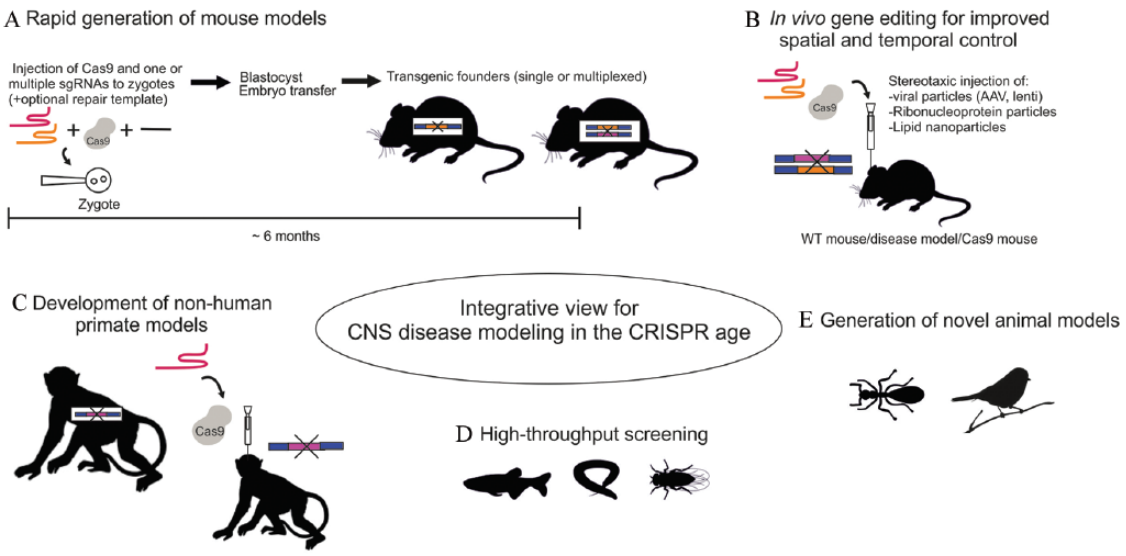

Figure 3. CRISPR facilitates in vivo modeling of CNS disorders by speeding up the generation of rodent models and increasing the diversity and accuracy of model systems. (A) CRISPR enables rapid generation of mouse models regardless of genetic background. CRISPR components can be injected directly into 1-cell embryo to generate transgenic founders, significantly reducing the time required to generate a transgenic mouse. By introducing several sgRNAs at the same time, multiple alleles can be knocked out in one generation, circumventing the need for time-consuming cross-breedings and accelerating the study of synergistic gene effects behind polygenic CNS disorders. (B) Instead of creating germline animal models, CRISPR components can be delivered directly to the brain via stereotaxic injection of viruses or preassembled nucleoprotein/lipid nanoparticles. Alternatively, somatic genome engineering can be achieved by sgRNA delivery into mouse lines expressing Cas9 either constitutively or in a conditional manner. In vivo gene editing offers improved spatial and temporal control and can be applied to any mouse line (eg, existing disease models) and to other mammalian systems including nonhuman primates. (C) CRISPR provides the first efficient means to generate transgenic nonhuman primate models for CNS diseases. Primate models provide a unique model to study higher cognitive functions and age-related neuronal diseases which are difficult to assess in rodent models. Primate models may also help in filling the translational gap between rodent models and human disease facilitating the development of new therapeutics. (D) CRISPR is a powerful tool for high-throughput in vivo screening of genotype to phenotype relationships in simple model organisms with well-characterized nervous system structure. (E) CRISPR technique works across species and can be used to generate novel animal models in species not previously accessible for genetic modification (such as ants and songbirds). A broader range of animal models allows assigning the model system for the scientific question and facilitates a comparative approach between standard species and novel model organisms.

Although CRISPR inarguably facilitates the disease modeling in mouse in many ways, the technology still has its limitations. Introduction of specific INDELs is less efficient than the generation of knockout animals, and the larger the fragment to be modified, the lower the recombination efficiency.[50] Despite the high homology of mouse and the human genome, mouse models differ in several fundamental biological features from humans and the translational value of mouse models is often questioned especially when studying complex neurological diseases and higher brain functions. The prefrontal cortex, which guides higher cognitive functions and whose dysfunction is observed in many neurological disorders, is poorly developed in mice.[51] In addition, the amount and organization of the white matter is different in mouse and human brains.[52] Many CNS diseases associated with aging, such as PD and Alzheimer disease (AD), have different cellular phenotypes in mice and humans.[53,54] For example, an overt neurodegeneration, which is the most important pathological feature in patient brains, is absent in genetic rodent models of AD and PD. Modeling of human neuropsychiatric disorders in mice is extremely challenging given that many of the symptoms cannot be assessed in mice.[55]

Novel animal models—leveraging diversity

CRISPR technique has worked in all species tested so far, ranging from worms to primates, which has expanded biological research beyond traditional, genetically tractable model organisms.[8] Focus is increasingly directed to larger, long-lived mammals, which are more closely related to humans than rodent models.[51,56] First studies from transgenic pigs and primates generated using ZFN, TALEN, and viral methods supported the idea that larger mammals can recapitulate pathophysiological events and clinical symptoms of neurological disease better than rodent models.[57–61] CRISPR technique has been successfully used to engineer transgenic pig, sheep, cow, and primate models.[62–64] In a pig model, 3 genes associated with familial early-onset PD were successfully mutated simultaneously.[65] The adoption of CRISPR method in primate research will evidently foster the generation of primate models of human neurodegenerative and neuropsychiatric diseases (Figure 3C) such as autism, PD, and AD.[51,66]

Although nonhuman primates provide physiologically the most relevant research model for many CNS disorders, especially those related to higher cognitive functions,[51] technical challenges and fundamental bioethical considerations limit

their usage. Genetic mosaicism interferes with the interpretation of the results, and although successful knockout studies have been published,[62,67] the gene replacement via the CRISPR system remains elusive in primates. Long breeding times, small litter sizes, and expensive maintenance costs limit the broader use of primate models of disease. Lack of public support has significantly reduced primate research in Europe and the United States. Advocates say that primate models are indispensable for bridging the translational gap between rodent models and human disease.[55,56] At the moment, more than 80% of drugs passing preclinical testing will fail in human trials,[68] and the proportion of failures is particularly high with CNS drugs.[69] As primate gene engineering is now within reach, the possible use of nonhuman primates in research must be weighed in terms of the indispensable value of the research for understanding and curing devastating human diseases.

In addition to mammalian models, CRISPR has proven as a powerful and efficient tool for both reverse and forward genetic studies in simpler model organisms, such as zebra fish, flies, and worms (Figure 3D).[70–72] Due to their well-characterized genome and nervous system structure, short generation cycles, and reasonable maintenance costs, these model organisms allow a high-throughput in vivo screening of genotype to phenotype relationships and of potential therapeutic agents. As an example, multiplexed guide RNAs were used to make knockout at 48 loci in zebra fish, leading to the identification of 2 novel genes involved in electrical synapse formation.[72]

The emergence of CRISPR has boosted the generation of novel animal models, which offer important advances for comparative neuroscience research (Figure 3E).[73] Recently, CRISPR method was used to modify the genome of songbirds, which are valuable models for vocal learning, social interactions, and brain development.[74,75] Tree shrews, the world's tiniest mammals, have a close phylogenetic relationship to primates and provide a feasible model to study basic neuronal functions in real time.[76,77] In their pioneering study of CRISPR-engineered ants, Yan et al showed that knocking-out odorant-receptor function causes aberrant social behavior and defective neural development.[78]

Local manipulation of the CNS networks

Although transgenic animal models provide an invaluable tool to study gene functions, they have certain limitations. In transgenic animals, first, the modified gene is typically in every cell which complicates the tracking of cell type–specific gene functions. Second, developmental compensation for deleted genes may obscure phenotypical effects.[79] Although conditional transgenic strategies can be used to improve the spatial and temporal interrogation of gene functions,[30,49] the technique is costly and time-consuming and suitable models are not always available. CRISPR-mediated in vivo gene editing in the brain provides fast and area-specific interrogation of gene functions, which can be applied to any animal species or to existing disease models at any developmental age (Figure 3B).[10,80,81]

Several studies have reported successful gene knockouts in both dividing neuronal precursors as well as postmitotic neurons in the brain.[81–84] In the first proof-of-principle demonstration of CRISPR-mediated knockout in neurons in vivo, Straub et al disrupted the NMDA-receptor function in a sparse population of pyramidal neurons using in utero electroporation of Cas9 and sgRNA directed to knock out *Grin1* gene.[82] In their pioneering study of acute knockout in the brain, Swiech et al used dual adeno-associated viral (AAV) system to perform single and multiplex knockout in the hippocampus of adult mice. The targeted disruption of one gene (*mecp2*) led to 70% of deduction of MECP2-positive cells in the dentate gyrus, and contextual fear-conditioning paradigm behavioral tests of these mice revealed impaired contextual learning similar to *mecp2*-mutant mice.[81] In the same study, multiplex disruption of a family of demethyltransferases (*Dnmt1, Dnmt3a*, and *Dnmt3b*) in the dentate gyrus of adult mice showed lower editing efficiencies but was still able to recapitulate some of the findings from *Dnmt3a* and *Dnmt1* knockout mice.[81]

Similar dual AAV-mediated strategy was successfully used to knock out mutant huntingtin (*HTT*) gene in the brain of Huntington disease (HD) mouse model in vivo.[83,84] Non–allele-specific knockout of mutant *HTT* in adult striatal neuronal cells was shown to efficiently and permanently eliminate polyQ_ expansion–mediated neuronal toxicity and alleviate motor deficits and neurological symptoms associated with HD.[84] More recently, Monteys et al showed that an allele-specific CRISPR knockout strategy based on single-nucleotide polymorphism (SNP) heterozygosity in the promoter region of mutant and wild-type *HTT* gene is sufficient to reduce human mutant HTT expression in HD mouse model.[83]

Challenges of in vivo editing in the brain

Although these samples illustrate the efficiency of CRISPR in creating targeted gene deletions in the brain,[80–82] there are only few examples of specific gene replacements in the nervous system (Table 1).[44,45,92] This reflects the challenge of using HDR pathway in postmitotic tissues, as HDR enzymes are predominantly active during S and G2 phases of the cell cycle.[93,94] In utero electroporation allows the introduction of CRISPR machinery into mitotic neuron precursors which still undergo HR.[44,82,92] SLENDR (single-cell labeling of endogenous proteins by CRISPR-Cas9–mediated HDR) technique uses in utero electroporation to insert a sequence encoding an epitope tag or a fluorescent protein to a gene of interest by CRISPR-Cas9–mediated HDR.[95] SLENDR enables multiplex labeling of several neuronal proteins simultaneously and provides a method for localizing endogenous proteins and live monitoring of protein dynamics in the mammalian brain with micrometer to nanometer resolution.[95] Recently, the same group who developed SLENDR showed that precise genome editing via

Table 1. Examples of CRISPR-mediated in vivo gene manipulation in the rodent brain.

REFERENCES	GENE	NUCLEASE	TYPE OF MODIFICATION	DELIVERY	VALIDATION
Dimos et al[85]	*Grin1*	spCas9	KO	In utero electroporation	Functional (electrophysiology)
Yagi et al[86]	*Mecp2*	spCas9	KO	Dual injection of AAV-Cas9 and AAV-sgRNA into hippocampus and primary visual cortex of adult mice	Sequencing, immunostaining, electrophysiology, Western blot, behavioral analysis
Yagi et al[86]	*Dnmt1, Dnmt3a, Dnmt3b*	spCas9	KO (multiplexed)	Dual injection of AAV-Cas9 and AAV-sgRNAs into hippocampus of adult mice	Sequencing, Western blot, behavioral analysis
Pires et al[87]	*HTT*	spCas9	KO	Dual injection of AAV-Cas9 and AAV-sgRNAs into hippocampus of adult BacHD[a] mice	Genomic DNA PCR, qRT-PCR
Bhinge et al[88]	*HTT*	spCas9	KO	Dual injection of AAV-sgRNA and AAV-Cas9 into one side of the striatum in homozygous HD140Q-KI[b] mice	Western blot, immunostaining, sequencing
Xu et al[89]	*Mertk*	spCas9	KI	Subretinal injection of AAV-Cas9 and AAV-rMerkt-HITI into RCS rat[c]	Immunostaining, qPCR, sequencing
Kampmann[90]	*Mecp2, Nlgn3, Drd1*	AsCpf1	KO (multiplexed)	Dual injection of AAV-sgRNA and AAV-asCpf1 into hippocampus of adult mice	Sequencing, immunostaining
Dickinson and Goldstein[70]	β-*Actin, CaMKIIα*	spCas9	KI of HA and EGFP tags	Dual injection of AAV-Cas9 and AAV-HDR (repair template + sgRNA) into different brain areas of WT and J20 mice.[d] Single injection of AAV-HDR into different brain areas of Cas9 expressing mice	Immunostaining
Shalem et al[91]	*tdTomato*	spCas9	KO	Injection of Cas9 ribonucleoprotein particles into multiple brain regions of Ai9 tdTomato mouse[e]	Genomic DNA PCR, immunostaining, sequencing

Abbreviations: AAV, adeno-associated virus; AsCpf1, *Acidaminococcus* sp. Cpf1; KI, knockin; KO, knockout; sgRNA, single-guide RNA; PCR, polymerase chain reaction; qRT-PCR, reverse transcription-PCR; spCas9, *Streptococcus pyogenes* Cas9.
[a]BacHD mice are transgenic for a full-length human mutant huntingtin (*mHTT*).
[b]In HD140Q-knockin mice, exon 1 of endogenous mouse *Htt* is replaced with exon 1 of human *HTT* with 140 CAG.
[c]Royal College of Surgeons (RCS) rat, a model for retinitis pigmentosa.
[d]J20 mice, Alzheimer disease model expressing a mutant form of the human amyloid precursor.
[e]Transgenic mice which harbor loxP-flanked STOP cassette preventing transcription of a CAG promoter–driven red fluorescent protein variant (tdTomato).

HDR is possible in mature postmitotic neurons in the mouse brain via vSLENDR (virus-mediated single-cell labeling of endogenous proteins via HDR) method combining CRISPR-Cas9–mediated DNA cleavage and the efficient delivery of donor template with AAV.[44]

To circumvent challenges in harnessing HDR in postmitotic neurons, Suzuki et al described a novel NHEJ-mediated targeted integration strategy "HiTi" (homology-independent targeted integration).[45] HiTi allows for robust DNA knockin in both dividing and nondividing cells in vitro and in vivo in the mouse brain. The therapeutic potential of this technique was demonstrated by in vivo correction of *Mertk* gene in a retinal degeneration rat model, which was able to partially restore retinal function.[45]

The main barrier for in vivo applications of CRISPR technology is a large transgene size of commonly used nucleases, such as spCas9 (4.2 kb).[10,49] Widely used AAV delivery systems have limited packaging capacity,[96] which renders it difficult for incorporation of nuclease along with sgRNA expression cassettes and necessary genetic elements. Lentiviral vectors have bigger packaging capacity than AAVs and can be used to deliver Cas9, reporter gene, and several sgRNAs using a single vector.[97,98] However, lentiviral vectors integrate into the host genome and permanent expression of CRISPR components may facilitate undesirable off-target effects, limiting the utility of lentiviral strategy for in vivo applications requiring high level of precision.

Diverse strategies have been developed to overcome the delivery challenges associated with Cas9. Several studies have shown efficient in vivo gene modification by delivering Cas9 and sgRNA in separate AAVs.[44,45,81,84] Smaller Cas9 orthologues, such as *Streptococcus aureus* Cas9 (saCas9), have been successfully packed with sgRNA and fluorescent reporter molecule into a single AAV and used for in vivo gene editing.[99]

CpfI family of endonucleases requires only single transcript array to drive several guide RNAs, providing a simplified system for multiplex editing in vivo.[100,101]

Several nonviral platforms have been developed and employed for transient expression of CRISPR components in vivo. Staahl et al used Cas9-sgRNA ribonucleoprotein (RNP) particles to express CRISPR components in diverse neuronal subtypes in the different brain areas.[102] The penetration of RNP complex into the cells was facilitated by an insertion of an optimized pattern of Simian vacuolating virus 40 (SV40) nuclear localization sequences to Cas9 sequence. Recently, gold nanoparticles were successfully used for delivery of Cas9 along with sgRNA and donor DNA template to facilitate HDR in various cell types in vivo.[103]

Constitutive and conditional Cas9 knockin mice provide an alternative strategy for expressing of Cas9.[49] Knockout can be easily achieved by viral expression of sgRNA only and these systems can be combined with doxycycline-dependent sgRNA expression for temporal control.[104] Improved spatiotemporal control can be achieved by crossing Cre-dependent Cas9 mice with one of the hundreds of different Cre-or tamoxifen-inducible CreER driver lines available.[105]

Modeling of Neuronal Function in Human-Derived Stem Cells Systems in the CRISPR Age

Although animal models provide valuable information into pathophysiology and mechanisms of neurological disorders, the relevance of animal studies to human disease is often questioned due to inherent species-specific differences. Around 20% of genes in humans lack an identifiable one-to-one orthologue in mouse and even apparently orthologue genes can have divergent roles in different species.[106] A major limitation with animal models is that most of the neurological disorders are a result of complex interaction of multiple risk and protective alleles along with nongenetic factors, whose accumulation along lifetime determines individual's genetic risk to disease.

Until this decade, studying of the mechanisms of human CNS disease has been limited to postmortem samples from patients having neurological disease. The opportunity to produce neurons from human ESCs (hESCs) and, in particular, human-induced pluripotent stem cells (hiPSCs) has revolutionized the field of in vitro modeling of human disease.[107,108] In combination with recent development of 3D organoid cultures and expanding genome editing and regulation toolkit,[109] it is now possible to dissect the pathophysiology of human neuronal diseases in a relevant genetic and cellular context.

Using CRISPR in cellular models of CNS diseases

The iPSC-based disease models have been generated for several neurodevelopmental and neurodegenerative disorders (reviewed by Imaizumi and Okano[110]), including SZ,[110,111] Parkinson,[112] AD,[86] and ALS.[85] The primary concern surrounding the studies conducted in hiPSCs derived from cases and controls is genetic heterogeneity. With CRISPR genome engineering, it is possible to target individual genetic variants in controlled genetic background in constant biological circumstances (Figure 4), bypassing the intrinsic variability of iPSC lines derived from different individuals.

By repairing the genome of hiPSCs or neurons derived from a known disease-affected donor, it is possible to test the effects and sufficiency of particular gene variant on phenotypic presentation (Figure 4A). Such CRISPR-mediated gene-corrected isogenic control cell lines have been generated for several rare inherited neurological diseases, which has given a valuable insight into the cellular pathophysiology underlying these diseases.[87,88,89,114] Many of these studies have shown that gene correction is sufficient to reverse phenotypic abnormalities associated with the disease. For example, CRISPR-mediated deletion of a CGG repeat expansion in the fragile X mental retardation 1 (*FMR1*) gene was sufficient to reverse the transcriptionally repressed chromatin state of *FMR1* and restore the normal expression levels of FMR1 in FXS patient-derived cells.[113] Similarly, correction of a CAG repeat expansion in *HTT* gene coding Huntington protein reversed phenotypic abnormalities observed in HD patient-derived stem cells.[89] This study also clearly illustrated the importance of isogenic controls for disease modeling using hiPSCs. Although the correction of *HD* gene rescued most of the phenotypic abnormalities observed in HD neurons, a number of apparent gene expression differences detected between HD and nonrelated healthy control lines were absent between HD and corrected isogenic control lines.[89]

Another approach for dissecting genotype-phenotype relationships is to introduce a mutation in the genome of a healthy donor with random background (Figure 4A). Paquet et al used a novel CRISPR-Cas9–based genome editing framework "CORRECT" to introduce specific homozygous and heterozygous knockin mutations of amyloid precursor protein (APP) and presenilin 1 (PSEN1) genes.[115] Cortical neurons derived from these edited hiPSCs displayed genotype-dependent disease-associated phenotypes similar to neurons derived from patients with AD.[115] In their recent paper, Fink et al showed that knocking out *UBE3A*, a gene deleted in Angelman syndrome, in an isogenic CRISPR-Cas9 gene–edited cell line led to disrupted neuronal maturation and replicated the cellular phenotype observed in hiPSCs-derived neurons from patients with Angelman syndrome.[116]

Tackling polygenity

Although most initial hiPSC studies have focused on monogenetic forms of CNS disorders, hiPSCs, having the same complex genetics as the affected individuals, are anticipated to mean a real breakthrough for dissecting the pathophysiology of polygenic diseases. Genetic risk factors associated with complex neurological diseases are challenging to tackle as risk variants are present not only in patients but also in unaffected

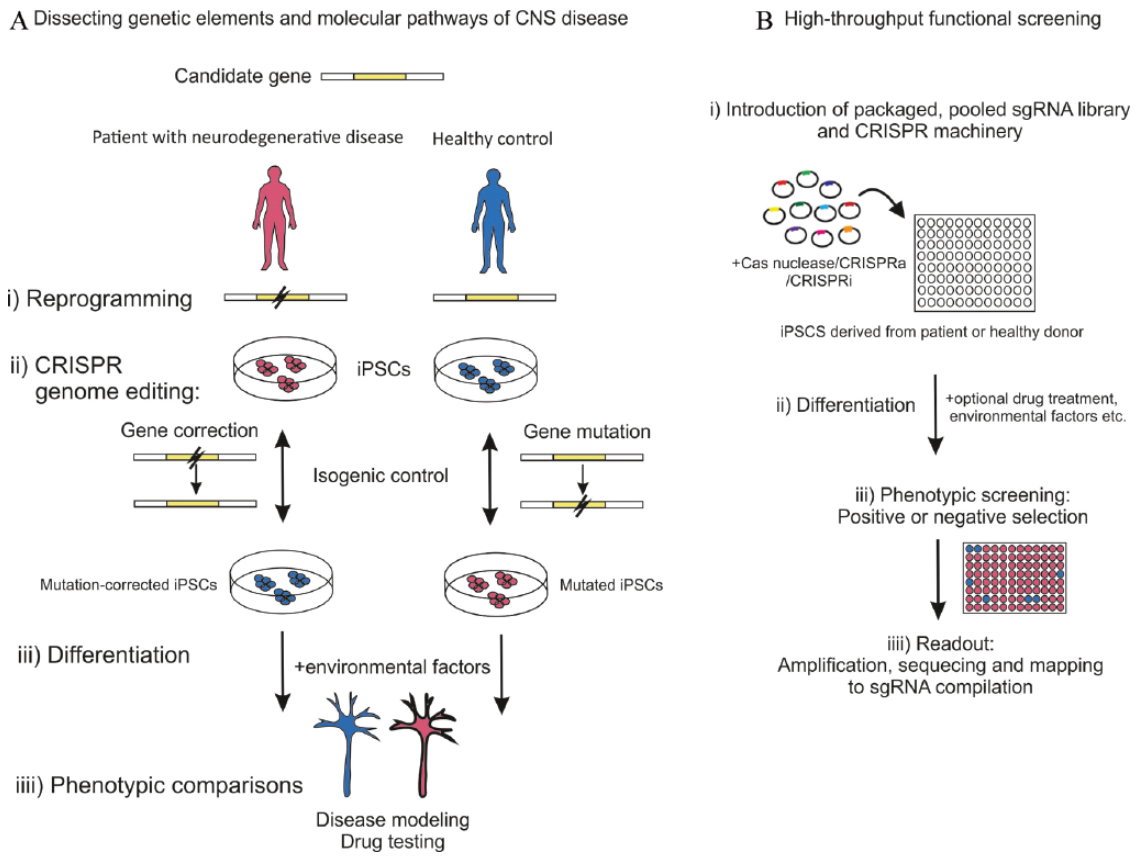

Figure 4. The combination of human iPSC technology with CRISPR gene editing in CNS disease modeling and drug development. (A) Gene-edited iPSCs enable dissecting of genetic components and molecular pathways of CNS disease in precisely controlled human model. Somatic cells (eg, skin fibroblasts) from a patient with neurogenerative disease and healthy control can be reprogrammed into iPSCs. Isogenic cell lines (cells with identical genetic background) are generated using CRISPR gene editing either by correcting pathogenic mutation in patient-derived iPSCs or inducing mutation into cells derived from healthy control. Edited iPSCs are differentiated into specific types of cells, such as neurons, and the phenotypes of isogenic lines are compared. Isogenic cell lines can also be used to study synergistic gene effects and the interplay between genetics, epigenetics and environmental factors. (B) CRISPR can be used for high-throughput functional screening of genetic elements underlying CNS disease. In a pooled CRISPR screen, targeted cells are treated with a pooled sgRNA library containing typically 103-105 of different sgRNAs and mutated cells are then screened for a selected phenotype. Genomic DNA of isolated cells is then subjected to next-generation sequencing (NGS) to identify sgRNA representation in the selected subpopulation and to draw a causal link between genetic perturbation and observed phenotype. In addition to characterization of basic biological mechanisms and discovery of novel genetic elements, CRISPR-based platforms can be used to screen new therapeutic agents and genes involved in resistance to drugs.

individuals and as protective alleles might obscure phenotypes relevant to disease biology. Intriguingly, many of risk variants localize to regulatory DNA elements, not in exons, and are thought to be involved in the regulation of gene expression.[117] For example, SNP variants in *SNCA*, the gene that encodes α-synuclein, increase the lifetime risk of Parkinson disease by around 30%.[118] By introducing specific mutations in the α-*synuclein* gene using CRISPR-Cas9 in isogenic hiPCs derived from unaffected individuals, Soldner et al demonstrated that 2 known risk-associated SNPs in noncoding region of *SNCA* affect the binding of transcription factors and transcriptional regulation of the *α-synuclein* gene.[119] This study supported the idea that elevated levels of α-synuclein are not only correlated with the risk of PD but also highlighted the power of gene editing techniques in unraveling the molecular consequences of common risk variants which presumably cause subtle phenotypic effects difficult to monitor heterogeneous cell lines.[119]

Rapid screening of phenotype-to-genotype relationships

In addition to allowing easy and fast modification of known genetic risk factors, CRISPR enables high-throughput forward genetic screens for the unbiased discovery of novel genetic elements and characterization of basic biological mechanisms (Figure 4B).[90,120] CRISPR screens can be used in multiple approaches, such as knockout (loss-of-function), knockdown (inhibition), and activation screens, and can target both coding and noncoding regions in the genome of various cellular and animal models.[91,121,122] CRISPR-based screening platforms have been developed for identifying cellular factors controlling vulnerability and cellular processes underlying neurodegenerative disease.[90,123]

A particularly exciting approach uses Cas9 knockin mice for in vivo high-throughput genetic screens.[49,124] In their recent study, Chow et al developed an AAV-mediated CRISPR screen

to identify functional suppressors in glioblastoma.[124] Using stereotaxic delivery of 2 AAVs carrying astrocyte-specific Cre recombinase and a sgRNA library targeting genes commonly mutated in human cancers, they were able identify multiple drivers and co-occurring drivers for glioblastoma in the native microenvironment of the mouse brain.[124]

Off-target Effects—Concern About the Specificity

The specificity of CRISPR nucleases remain the major concern for the use of technology, in particular for clinical applications.[125] Off-target mutations generated at sites other than the intended on-target site may cause genomic instability and disrupt the functionality of normal genes. A number of studies have assessed the specificity of CRISPR and a large body of work has been dedicated to minimize off-target mutations.[126–131] Improvement of the specificity of CRISPR technique has been reported with novel CRISPR nucleases and engineered Cas9 variants,[130,131] truncated sgRNAs,[132] and paired Cas9 nickases.[31] Delivery of CRISPR nuclease as messenger RNA or protein instead of plasmid will limit the duration nuclease is active in cells, reducing off-target effects.[125,133]

Conclusions and Future Perspectives

The CRISPR system provides a powerful approach for precisely modifying genomic sequences, allowing the interrogation of gene function in unprecedented specificity and efficiency. With easy design and possibility of multiplexing of guide RNAs, the advent of CRISPR has greatly simplified genetic manipulation across species. Novel CRISPR-based strategies are developed at a furious pace and new natural CRISPR systems are harnessed for gene manipulation studies. CRISPR technology has substantially accelerated the understanding of functional organization of the genome at the systems level and facilitated the establishment of causal links between genetic variations and biological phenotypes.

However, until today, the exploitation of CRISPR in neuroscience has lagged behind many other fields of biomedical research. Precise genome editing via HDR has been considered to be largely restricted to dividing cells and the delivery of CRISPR genome editing machinery in the brain in vivo has been challenging. Validation of Cas nuclease efficiency and specificity is particularly complicated in the complex and diverse architecture of the mammalian brain. Recently, novel HDR-independent strategies have been developed to facilitate targeted genome editing in postmitotic neurons.[44,45] Together with the progress in gene delivery methods, such as new nanoparticle-based delivery strategies and the discovery of smaller CRISPR nucleases fitting into viral vectors,[134] these advancements can be expected to boost the utilization of CRISPR in basic and translational neuroscience in the near future.

Throughout the history, neuroscientists have taken advantage of the diversity animal models and comparative approach has served as an important tool in neuroscience research.[135] With the CRISPR technique in hand, scientists have now for the first time identified a capability to implement functional gene studies in a wide range of species independently of genetic background. Different animal models can offer distinct advantages for studying specific aspects of complex CNS disorders. Assigning the model system for the scientific question and a comparative approach between standard species and new animal models can greatly promote our understanding of the brain as a whole. This is imperative for understanding of the cause and pathogenesis of the CNS disease and for the development of new effective therapeutics.

Despite the vast investments of time and money, most of the preclinical animal studies do not translate into successful treatment in humans, which is particularly evident in the case of major neuropsychiatric and neurodegenerative diseases.[55] One of the major obstacles in developing new effective treatments for CNS diseases is the lack of understanding how human neural function correlates with the findings from model organisms. By combining CRISPR gene editing with hiPSC technology, it is now possible to study and test gene functions in human "disease-in-a-dish" in vitro model systems. Genetically controlled hiPSC cultures and 3D organoids open new possibilities for drug discovery and screening and can in part help to bridge the translational gap between animal models and human clinical trials.

Although the main benefit of CRISPR has to date been seen in basic research, CRISPR holds a great potential to treat genetic diseases, especially for diseases produced by single-gene mutations.[136] Recently, proof-of-principle studies have demonstrated CRISPR-mediated inactivation of trinucleotide repeat expansions leading to fragile X syndrome and HD.[113,137] However, several challenges remain to be addressed before these and other initial studies can be translated to viable therapeutics. Most of the CNS disorders are either developmental or progressive by nature, and before the disease cause is understood better, the correct timing of the treatment is elusive. In addition, many practical issues related to the efficiency, safety, and delivery of CRISPR therapeutics need to be thoroughly interrogated before this novel technology can be accepted in medical practice.

Author Contributions

AV performed all the work associated with preparing, writing, and submitting the manuscript.

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