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CRISPR/Cas9 System: an Important Tool for Brain and Cognitive Science*

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Brain function is associated with the morphologies, organizations, synaptic connections, and circuit activities of neurons. As the building blocks of the brain, neurons display remarkable morphological diversity and form neural circuits with synaptic connections. Understanding the "wiring" connectomes among synapses and their roles in neural networks may provide insights into how the brain processes perception, cognition, memory, and relevant behavior. Meanwhile, the emergence of molecular and genetic technologies has greatly contributed to the study of neuroanatomy, synaptic connectomes, and activities of the neural circuitry. For instance, virus-based visualization tools, such as engineered PRV (pseudorabies virus) and RV (rabies virus), are generally applied to show the connections among synapses and trace the neural circuit [1-6]. These engineered viruses, without their toxic activities and replication abilities, preserve the property of retrograde synaptic transfer, thereby enabling the polysynaptic (PRV) or monosynaptic(RV) tracing of neural circuits[7]. A "reverse screen" method of synaptic labeling, termed as GRASP (GFP reconstitution across synaptic partners), can be used to test whether two neurons are connected [8]. Various genetically encoded indicator (GEI) techniques to monitor the neural activities, which include genetically encoded pH indicators (GEPIs), genetically encoded transmitter indicators (GETIs), genetically encoded voltage indicators (GEVIs) and genetically encoded calcium indicators (GECIs)[9-10]. Notably, optical technologies, as powerful cutting-edge methods in neurosciences, have also been introduced for studying brain circuitry, through these technologies, neural activities can be monitored and manipulated[11-14].

Despite the rapid progress in various technologies for studying the molecular and neural basis of cognition and memory, explicitly monitoring the genomic mechanisms of neural circuits remains difficult. Due to the specificity, ease of use and highly modular programmable nature, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated), which is the most remarkable genome-editing tool to date [15-17], may be a promising system for determining the molecular mechanisms of neural circuits involved in perception, cognition, memory, and behavior and exploring therapeutic strategies for neurological and neuropsychiatric disorders[18]. In this review, we discuss the implications of CRISPR/Cas technique in neuroscience and its potential applications in the exploration of the

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molecular and neural circuit basis of cognition and memory.

1 CRISPR/Cas9 and its applications in neuroscience

The CRISPR/Cas system is an adaptive immune system in bacteria and archaea [15]. It recognizes invading DNA or RNA through CRISPR RNAs (crRNAs) and cleaves the targets through the cooperation of the Cas proteins[19–21]. The CRISPR/Cas system undergoes three stages during an immune response to foreign DNA or RNA [22]. In the first acquisition stage, foreign DNA or RNA is inserted into the host CRISPR loci as spacers. In the second stage, CRISPR repeats with inserted spacers are transcribed into pre-crRNAs, which are further cleaved into mature crRNAs. In the third stage, the mature crRNAs guide the Cas proteins to the invaded genome loci and mediate the cleavage of the targets[23–24]. The specificity of the Cas protein-mediated cleavage depends on the PAM (protospacer-adjacent motif) in the invaded genome loci, which is discriminative from the host genome sequence[23, 25].

Cas9, an RNA-guided endonuclease in the class 2 type Ⅱ CRISPR system, cleaves foreign DNA guided by the crRNA and tracrRNA (*trans*-activating CRISPR RNA)[15, 25]. The tracrRNA functions as the mediator by binding with Cas9 protein and hybridizing with crRNA to form the crRNA-tracrRNA-Cas9 complex. By engineering the crRNA-tracrRNA duplex to a chimeric single guide RNA (sgRNA), CRISPR/Cas9 system has been widely used as genome editing tool [26–27]. In this paper, we summarize the applications and limitations of CRISPR/Cas9 system in neural gene manipulation.

1.1 CRISPR/Cas9 for neural gene editing

CRISPR/Cas9 system-mediated genome editing, like ZFN (zinc finger nuclease) and TALEN (transcription activator-like effector nuclease) editing tools, is sequence specific and requires the following steps: DNA cleavage and DNA repair. During DNA cleavage, Cas9 is guided by the sgRNA and creates a DNA double-stranded break (DSB) in a sequence-specific genome locus. During DNA repair, a targeted DSB triggers the endogenous DNA repair system, including homology-directed repair (HDR) and nonhomologous end joining (NHEJ) [28–31]. HDR can generate sequence-specific deletion, insertion, mutagenesis, and correction by homologous recombination, which is mediated by a donor template[28, 32–34]. NHEJ can create random deletions and insertions (*i.e.*, indels) in specific genome locus to cause a shift or mutation in the open reading frames of target genes [28]. Thus, combined with the HDR and NHEJ repair system, CRISPR/Cas9 system has been used as a powerful tool for the generation of gene knockout, knockin, site-specific mutagenesis, and correction.

Given that postmitotic neurons in adult brains do not undergo cell division, the neuronal genome is unlikely manipulated by HDR. Meanwhile, NHEJ, an error-prone repair system, is the dominant mechanism in DNA DSB repair in postmitotic neurons [18, 34–36]. Several researchers successfully elicited gene mutations in postmitotic neurons by using the CRISPR/Cas9 system. For example, *Grin1* (NMDA receptor subunit 1) was mutated in a mouse hippocampus through electroporation with Cas9 and sgRNA-containing vectors[37]. The loss of *Grin1* caused abnormal electrophysiological patterns and behavioral defects[37]. In another study, *Gria2* (AMPA subunit 2), in a rat hippocampal slice culture was mutated through the biolistic transfection of designed Cas9 and sgRNA expression vectors[38]. The mutation efficiency of Cas9 positive neurons were nearly 100% and approximately 90% mutations were indels with frame shifts[38]. These findings indicated that NHEJ is a major repair mechanism in postmitotic neurons and the efficiency of mutagenesis in postmitotic neurons is comparable to that observed in dividing cells or even higher[18]. Other genes, such as DNA methyltransferases (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*), were mutated in cultured neurons or mouse hippocampal gyrus by delivering the Cas9 and sgRNA through adeno-associated viral vectors [39]. The indel efficiencies in *Dnmt1*, *Dnmt3a*, and *Dnmt3b* are ～75%, ～75%, and ～50%, respectively. Furthermore, some researchers were able to promote the mutation of *EMX1* in the cochlea by delivering the Cas9 and sgRNA vectors through a cationic lipid, and they were able to obtain an indel efficiency of approximately 20% without performing electroporation or using virus vectors as delivering methods in nervous systems[39–40]. All these gene editing studies provided evidence that CRISPR/Cas9 system is a feasible genome editing platform for functional and circuitry study of neuronal genes.

1.2 CRISPR/Cas9 for gene regulation

The CRISPR/Cas9 system can also be used as a feasible platform to activate gene expression (namely CRISPRa) or inhibit gene expression (namely

CRISPRi) by fusing transcriptional activators or repressors with nuclease dead Cas9 (dCas9)[41]. dCas9 that lacks nuclease activity can only bind sequence-specific genomic sequences and transports transcriptional effectors to the targeted genes to control their expression[28].

dCas9 with or without repression domains, such as KRAB (Kruppel-associated box), targets the specific genome locus by sgRNA and tends to block the elongation or the binding of RNA polymerase, thereby inhibiting transcription processes[42–43]. When fused with an activating domain, such as VP64, dCas9-VP64 increases the expression level of a GFP reporter gene[42, 44–46]. For instance, one neuronal gene *NTF3* was reported to be transcriptionally activated to three folds by dCas9-VP64[45–46]. However, the activation efficiency elicited by dCas9-VP64 is much higher in other cell lines and models[45]. sgRNA can be engineered by adding a second structure motif (such as MS2, PP2, and Com) to recruit RNA binding proteins in order to increase activating efficiency[47–48]. When these RNA binding proteins are fused to an activation domain (such as VP64) or repression domain (KRAB), they either activate or repress the targeted gene expression[28]. Thus, the CRISPRa/i system should be widely used in genome-wide screening for identifying genes involved in neural circuit-based cognition and memory.

Apart from transcriptional activators or repressors, dCas9 can also be combined with epigenetic modifiers to modulate the epigenetic status[28]. One study has shown that when dCas9 was fused with histone demethylase LSD1 and guided to the enhancers of interested genes, the epigenetic mark, H3K4me2, near the enhancers was reduced and the gene expression was suppressed[49]. When fused with the catalytic domain of histone acetyltransferase p300, the dCas9-p300 increases H3K27 acetylation levels at the regions of targeted enhancers and activates the targeted gene expression[50]. As a powerful epigenome editing platform, CRISPR/dCas9 system is a promising tool for the exploration of epigenome, particularly its role in the regulation of neural circuit-based cognition and memory.

1.3 CRISPR/Cas9 for imaging

Visualization of genomic dynamics in living cells facilitates the elucidation of the relationships among three-dimensional architecture, gene expression, and cell behavior. However, it has remained a challenging field because of insufficient ideal imaging tools.

When fused dCas9 with enhanced GFP, the dCas9-EGFP can be used to locate any interested genomic locus in living human cells[51]. Endogenous centromeres and telomeres can be labelled by dCas9 and targeted sgRNA[52]. Furthermore, dCas9-based imaging can be combined with SunTag peptide array to amplify the fluorescent signals[53]. Different Cas9 proteins (Nm dCas9 and St1 dCas9) can be fused with various colored fluorescent proteins for the simultaneous tracking of changes in multiple genomic loci by multiple sgRNAs[54]. CASFISH, a new FISH (fluorescence *in situ* hybridization) method, labels the genomic loci through DNA sequence-specific hybridization and dCas9-mediated enzyme reactions[55]. CASFISH has fast staining protocols and exhibits better performance in preserving cellular and genomic architectures compared with traditional FISH methods[55]. All these sequence-guided dCas9 labeling tools can potential record the dynamics of genomic loci in living cells. However, the functions of genomic structure with respect to neuronal activity and the corresponding cognition and memory remain largely unknown. The relationships between the dynamics of genomic architecture and neuronal plasticity should be investigated further using a feasible CRISPR/dCas9-based system.

1.4 Limitations of CRISPR/Cas9 system

Before the CRISPR/Cas9 system can be widely used in neuroscience, its efficiency must be improved and off-target issues must be addressed[18, 56].

Strategies to change the HDR to NHEJ pathway have been used in *Drosophila* and mammals to improve the efficiency for gene editing[57–58]. Other strategies include the optimization of the timing and concentration of Cas9 and sgRNA vectors and improvement of vector delivering methods[59–60].

Off-target effects in CRISPR/Cas9 system are usually caused by mismatches of sgRNA to nonspecific genome loci or nonspecific nuclease activities[61]. The off-target effects of CRISPR/Cas9 system may lead to severe consequences in an adult nervous system, which more affected than other cells, as the error-prone NHEJ is the predominant repair mechanism in postmitotic neurons. Thus, the sequence specificity of sgRNA must be optimized from 20 bp to 17-19 bp to reduce the nonspecific targeting and improve the specificity of CRISPR/Cas9 system[62]. sgRNAs with unpaired G at the 5′ end reduce

mismatching events[63]. Strategies for improving dCas9 specificity involve the engineering of Cas9 variants, including the paired nickase Cas9 (nCas9), dCas9-Fok1 nuclease, and mutated Cas9 (*e.g.*, eCas9), which exhibit reduced off-target effects [64-65]. The efficient and precise control of gene editing, gene expression regulation, and *in vivo* imaging render the functional studies of vulnerable neurons feasible.

2 Possible further applications of CRISPR/Cas9 in neuroscience

CRISPR/Cas9 system offers a revolutionary platform for genomic editing, gene regulation, and genomic architecture imaging. However, despite its feasibility and versatility, this technique has not been widely used in neuroscience, except for neural gene knockout and knockin to study the gene function. In this paper we propose some potential applications and future directions of the CRISPR/Cas9 system in neuroscience, including neuronal subtype identification, neuronal RNA granule tracking, and neural activity recording and control. Developing molecular genetic tools with CRISPR/Cas9 system can be useful in the elucidation of neural circuit functions underlying cognition and memory processes.

2.1 Identification of diverse neuronal subtypes by enhancer mapping

The human brain consists of approximately 10 billion neurons and a thousand folds of synaptic connections. The astronomical number of neuronal subtypes and synaptic networks hinders our pace in exploring brain functions. Characterization of the neural subtypes is a challenge because of insufficient cell type-specific markers for monitoring diverse neurons. Given that the expression of cell type-specific markers is controlled by enhancers, genetic screening for neuronal specific enhancers can facilitate the identification of genetic markers that can label different neurons.

The CRISPR/Cas9 system shows feasible RNA-guided gene targeting and efficient induction of indels. These advantages have prompted several laboratories to use this system for high-throughput screening of active enhancers[66]. The fundamental strategy for the identification of functional enhancers is based on the fact that nucleotide alterations in the regulatory region of a gene may alter gene expression. For instance, through this tiling screening, a composite enhancer of gene *BCL11A* was identified in both human and mouse[67]. A distal enhancer of p53 downstream gene *CDKN1A* was also identified through the use of a CRISPR/Cas9 tiling screen[68]. In a human ES model, the enhancer of gene *POU5F1* can be characterized with the high-throughput CRISPR/Cas9 system [69]. Furthermore, on the basis of the pooled sgRNA library, another high-throughput mapping method, called MERA(multiplexed editing regulatory assay) has been developed to screen for the functional enhancers[70].

These high-throughput technologies for active enhancer screening can be used to identify neuron-specific enhancers, which are already defined. With random sgRNA library targeting to all the putative enhancers and reporter system with fluorescent proteins in the nervous system, CRISPR/Cas9 may characterize all the functional enhancers in a given neuron subtype in the high-throughput manner.

2.2 Tracking of neuronal RNA granules

Neuronal RNA granules, such as ribonucleoprotein particles (RNPs), stress granules, and processing bodies, display compositional diversity and play critical roles in synapse plasticity, thereby affecting learning and memory [71]. The localized mRNA in the RNPs controls the expression of genes involved in axon guidance and neural regeneration[72]. However, our understanding of neuronal RNA granules is limited because genetic tools for tracing and visualizing RNA granules remain insufficient.

Most of the current studies about CRISPR/Cas9 technique focus on RNA-guided DNA modification; some findings also reported that certain Cas9 variants (*e.g.*, Fn Cas9) can cleave mRNA [73-74]. This finding may open up a new area for RNA editing and modifications. The engineered Fn Cas9 can target any interested RNA by synthetically designed rgRNA (RNA-guided RNA). The mostly used Cas9 protein (Sp Cas9) has been reported to cleave single-stranded RNA *in vitro* [75]. In contrast to Fn Cas9, Sp Cas9 requires the PAM sequence for targeting the RNA[75]. Thus, the CRISPR/Cas9 system can be used to track RNA or RNA-related complexes or granules. When combined with the translational activators or repressors, this system can also regulate the localization, stability, and dynamics of RNA granules. Furthermore, the CRISPR/Cas9 system can be used to establish the molecular basis underlying neuronal plasticity and elemental unit of memory for the tracking and control of neuronal RNA granules.

2.3 Spatiotemporal control and recording of neural circuitry activity

The GEIs and optogenetic systems are the two widely used techniques for recording and manipulating neural activity [9]. However, these methods are incapable of simultaneously performing these processes at the circuitry level. Recently, a light-inducible system has been introduced to the CRISPR/Cas9 platform to spatiotemporally control gene editing and expression regulation[76]. dCas9 fused with blue light-inducible hetero-dimerizing proteins CIB1 and CRY2 can recruit transcriptional effects to the target sites when blue light appears [77]. This approach enables the recording of neural activity in circuitry level by guiding diverse sgRNA in different neurons in the circuitry through the use of the light-inducible control of the CRISPR/Cas9 system.

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