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***In vivo* cell type-specific CRISPR gene editing for sleep research**

Hiroshi Yamaguchi¹ and Luis de Lecea¹

¹Department of Psychiatry and Behavioral Sciences, Stanford University, 1201 Welch Road, Stanford, CA 94305, USA

Abstract

Sleep is an innate behavior conserved in all animals and, in vertebrates, is regulated by neuronal circuits in the brain. The conventional techniques of forward and reverse genetics have enabled researchers to investigate the molecular mechanisms that regulate sleep and arousal. However, functional interrogation of genes in specific cell subtypes in the brain remains a challenge. Here, we review the background of newly developed gene-editing technologies using engineered CRISPR/Cas9 system and describe the application to interrogate gene functions within genetically-defined brain cell populations in sleep research.

Keywords

CRISPR/Cas9; sleep

Introduction

We spend about one third of life asleep. Sleep is a conserved behavior among all animals and its mechanism is one of the major unanswered questions in biology. Scientists have been attempting to unravel the neuronal and molecular mechanism of sleep for many decades. Optogenetic¹ and chemogenetic² tools have rapidly expanded our understanding of sleep at the neuronal circuit level. Channelrhodopsin-2 (ChR2), a light-gated cation channel originally discovered in *Chlamydomonas reinhardtii*, is a key tool of optogenetics^{3,4,5}. Neurons expressing ChR2 can be excited by blue light delivered through an fiber-optic implanted into the brain. Chemogenetics utilize genetically engineered receptors and biologically inert ligands to modulate the activity of neurons. Designer receptor exclusively activated by designer drugs (DREADDs) are modified muscarinic G-protein coupled receptors which are activated by Clozapine-N-oxide (CNO) and the most common chemogenetic tool used in neurosciences⁶. Controlling the activity of neuronal circuits by cell type-specific expression of optogenetic and/or chemogenetic tools enabled researchers to interrogate the causal relationship between sleep and the activity of specific neurons in

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Competing interests statement

The authors declare no competing interests

hypothalamus^{7,8,9,10,11}, brain stem^{12,13,14,15,16,17}, ventral tegmental area¹⁸, basal forebrain^{19,20}.

On the other hand, forward and reverse genetics approaches have expanded our knowledge of the molecular mechanisms of sleep regulation in non-mammalian^{21,22,23,24,25,26} and mammalian organisms^{27,28}. For example, hypocretin/orexin is a neuropeptide expressed in ~10,000 neurons in the lateral hypothalamus^{29,30,31}. Hypocretin-deficient animals exhibit symptoms of narcolepsy, revealing an indispensable role of hypocretin/orexin in the maintenance of arousal^{32,33}. However, the role of other gene transcripts within genetically defined neuronal populations remains a significant challenge. Here, we summarize the background of recently developed gene-editing technologies using engineered CRISPR/Cas9 system and describe the application of these technologies to investigate the gene functions in the specific cell population.

Engineered CRISPR/Cas9 system

CRISPR/Cas system forms an adaptive immunity in bacteria and archaea to memorize the viral nucleic acids of previous infections and cleave invading nucleic acids during re-infection^{34,35}. There are three types of CRISPR/Cas systems. The type II system is composed of trans-activating RNA (tracrRNA)³⁶, CRISPR (Clustered regularly interspaced short palindromic repeats)³⁷ and cas (CRISPR-associated) genes³⁸. CRISPR loci are made of “repeat-spacer” array and adjacent to small clusters of cas genes including Cas9 endonuclease. The CRISPR array is transcribed as a single pre-CRISPR RNA (crRNA) and processed into small pieces of matured crRNAs which has one spacer and one repeat³⁹. While the spacer of matured crRNA is a fragment of the invading DNA integrated into the host genome during the previous infections³⁴, the repeat is complementary to tracrRNA³⁶. The duplex of crRNA and tracrRNA brings Cas9 endonuclease to the target sequence immediately adjacent to a Protospacer Adjacent Motif (PAM) by complementary base pairing with the spacer and then Cas9 cleaves the target DNA⁴⁰. The PAM sequence is a 2–6 base pair DNA sequence essential for Cas9 binding to the target loci and it varies depending on the bacterial species of the Cas9 gene⁴¹. For example, the most popular Cas9 derived from *S. pyogenes* requires 5'-NGG-3' as PAM.

Recently, engineered CRISPR/Cas9 system has been utilized as a gene editing tool⁴². Engineered CRISPR/Cas9 system contains two components: Cas9 and single guide RNA (sgRNA), a chimeric RNA composed of crRNA (spacer and repeat) and tracrRNA^{40–43}. Cas9, when combined with the sgRNA, can introduce double-strand breaks at target locations in the genome specified by the sgRNA. Researchers can specify the target sequence of Cas9 by simply changing a ~20 nucleotide spacer sequence in the sgRNA^{43,44,45}. Double strand breaks, induced by Cas9, are repaired by either non-homologous end joining (NHEJ) pathway or homology directed repair (HDR) pathway. Although the NHEJ is the most active repair mechanism, it is an error-prone pathway which can introduce small insertions or deletions (indels) at the target loci. These small indels result in amino acid deletions, insertions, or frameshift mutations leading to premature stop codons within the open reading frame of the target gene. Given the NHEJ pathway is active in both proliferating and non-proliferating cells, engineered CRISPR/Cas9 system can be

applicable to disrupt genes in post-mitotic neurons *in vivo*^{46,47,48}. Also, engineered CRISPR/cas9 system accelerates the production of knockout animals by co-injection of the Cas9 mRNA and sgRNA into the embryos⁴⁹. Funato et al used engineered CRISPR/Cas9 system to show a missense mutation in the sodium leak channel NALCN reduced REM sleep. Also, Sunagawa *et al* injected Cas9 mRNA and triple sgRNAs targeting the same gene into the embryo to efficiently generate whole-body biallelic knockout mice in a single generation. By using this method, they screened all of the *N*-methyl- *D*-aspartate (NMDA) receptor family members and found *Nr3a* as a short-sleeper gene⁵⁰. Same group also showed that the amount of REM sleep was dramatically reduced in the mice deficient in *chrm1/ chrm3* genes⁵¹. While NHEJ-mediated DSB can disrupt the gene expression by introducing premature termination codons, HDR can be used to incorporate exogenous DNA into endogenous loci. HDR is a process of homologous recombination relies on a DNA repair template with sufficient homology to the regions flanking the DSB site. Thus, HDR-mediated gene editing requires a DNA repair template, which has the desired sequence flanked by two homology arms, with the sgRNA and Cas9^{52,45}.

Cell type-specific gene editing to investigate the mechanism of sleep.

Transitions between wakefulness and sleep are regulated by the brain, which is composed of tremendously diverse cell types. Study of gene function during the last three decades has relied on the generation of systemic and conditional gene knockouts to disrupt gene expression. However, it has been challenging to knockout a gene in specific cell types in the brain. One of the most widely used methods is the Cre/loxP binary system, in which the Cre recombinase disrupt a gene through recombination of target sequences flanked by two loxP sites⁵³. The expression of Cre recombinase driven by unique gene promoters allows researchers to generate cell type-specific gene knockouts. However, recombinase-dependent conditional gene knockouts require time consuming breeding of at least two mouse lines. Even though stereotaxic injections of recombinant Adeno Associated Virus (AAV) expressing a Cre recombinase can mediate spatiotemporal gene knockouts without time consuming breeding⁵⁴, only a few cell type-specific promoters are compatible with the packaging capability of AAV. *in vivo* RNAi-based methods can reduce expression levels of multiple genes at a time, but they lack cellular specificity⁵⁵. Alternatively, a research group led by Feng Zhang showed engineered CRISPR/Cas9 system can disrupt multiple genes in the adult mouse brain by AAV-mediated delivery of Cas9 and sgRNA⁴⁸. The group also generated Cre-dependent Cas9 knockin mice⁵⁶. Since hundreds of gene-specific promoter-driven Cre mouse lines are available, researchers can specifically express Cas9 in a wide variety of cell types in the brain by crossing Cre mouse lines with the Cas9 knockin mice and can target a gene by the stereotaxic injection of AAV encoding sgRNA (Fig. 1a–b). By mating *Agrp* (agouti-related peptide)-IRES-cre mice and Cre-inducible Cas9 knockin mice, Xu et al showed that CRISPR/Cas9-mediated ablation of *leptin receptor* in the adult hypothalamic *Agrp* neurons caused severe obesity and diabetes⁵⁷. Tso et al targeted astrocytes in the suprachiasmatic nucleus (SCN) using *Aldh1L1*-cre mice crossed with Cas9 knockin mice. The stereotaxic injection of AAV carrying sgRNA against *bmal1* ablated the gene in SCN astrocytes and the mice showed significantly prolonged the circadian period of clock gene expression in the SCN and abnormal locomotor behavior⁵⁸. Our group recently

employed engineered CRISPR/Cas9 system to interrogate the role of norepinephrine (NE) secreted from the LC in the regulation of sleep-wake cycles (Yamaguchi et al Nature Communications in revision). *Tyrosine Hydroxylase* (*Th*)-Cre mice were crossed with Cas9 knockin mice to express Cas9 in the locus coeruleus NE neurons of their N1 offspring (Th/Cas9) (Fig 1a). We then packaged AAV carrying dual sgRNA targeting the gene of *dopamine beta-hydroxylase* (*dbh*), a key enzyme to produce norepinephrine from dopamine, and injected the virus into the LC of Th/Cas9 mice (Fig 1b). Mice showed reduced wakefulness during the dark phase even in the presence of salient stimuli, indicating that NE from the LC is involved in the maintenance of arousal. Taken together, cell type-specific Cas9 expression combined with AAV-mediated delivery of sgRNA enabled the interrogation of gene functions in the specific cell subtypes in the brain.

Despite the benefits of engineered CRISPR/Cas9 system to disrupt genes in the specific neuronal subtypes, there are some limitations (Table 1). First, since CRISPR/Cas9-mediated gene disruption depends on the random small indels by error-prone NHEJ, the target neuronal population could become mosaic composed of cells have non frame-shift monoallelic and/or biallelic mutations. However, multiple sgRNA strategy might circumvent this problem. Sunagawa et al simulated the efficiency of gene disruption using multiple sgRNA targeting a same gene and exploited triple CRISPR strategy to knockout a gene⁵⁰. We also adopted the dual sgRNA strategy to improve the efficiency of gene disruption in the LC NE neurons (Fig 1b) (Yamaguchi et al Nature Communications in press). We collected genomic DNA from the tissue punch of dual sgRNA-infused LC, and then sequenced *dbh* loci and revealed the percentages of frameshift mutations in all mutated *dbh* loci were 82.6% and 85.4%, respectively (Yamaguchi et al Nature Communications in press). We then found the 85% reduction in *dbh* immunoreactivity in sgRNA-infected LC cells (Fig 1c). Another limitation of using engineered CRISPR/Cas9 system is its off-target effects^{59,60,61,62}. Since Cas9 tolerate mismatches of sgRNA and DNA complex, it is possible that Cas9 cleaves the open reading frame of other genes which has homology with the target sequence. Akcakaya et al actually showed that engineered CRISPR/Cas9 system with promiscuous sgRNAs induces substantial off-target mutations besides on-target mutations *in vivo*⁶³. On the other hand, they also showed on-target indel mutations and no off-target mutations using sgRNAs which have relatively few closely matched sites. Therefore, it is essential to design sgRNAs with the lowest number of closely matched sites using *in silico* tools^{64,65}.

Future direction and conclusion

The CRISPR/Cas9 toolbox is rapidly expanding since the first application to gene editing. For example, epigenome-editing tools using a nuclease deficient Cas9 (dCas9) fused to the catalytic domain of DNA methyltransferases^{66,67,68} or histone modification enzymes^{69,70} has been recently developed. It allows researchers to control epigenetic modifications in target loci. Sleep deprivation impairs cognitive functions such as learning and memory and its impact on the epigenome has been studied⁷¹. Thus, it will be interesting to investigate if modulations of epigenetic modification using dCas9-based tools in specific neuronal population mimics or dampen the detrimental effect of sleep deprivation on cognitive functions. Also, engineered CRISPR/Cas9 system enabled genetic modification in animals other than rodents. Therefore, researchers may investigate the gene functions in sleep

regulation using animals (e.g marmosets) which have similar sleep patterns and characteristics to humans.

In summary, engineered CRISPR/Cas9 system may shed light on our understanding of gene functions within the genetically-defined brain cell types in sleep research. Also, we anticipate the future application of expanding CRISPR/Cas9 toolbox will open a new avenue of sleep research.

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Highlights

- Sleep is regulated by the brain composed of tremendously diverse cell types.
- Gene-editing technologies using engineered CRISPR/Cas9 system was recently developed
- Engineered CRISPR technologies enabled the interrogation of gene functions within genetically-defined brain cell populations in sleep research.

Figure 1.

Cre-dependent gene editing of *dbh* in the locus coeruleus using engineered CRISPR/Cas9 system. (a) Design for expressing Cas9 in LC-NE (b) Schematic of the AAV vector encoding dual sgRNA targeting *dbh* and experimental design. (c) Representative immunofluorescence images of the LC from Th/Cas9 mice injected with AAV encoding control sgRNA (left) or sgRNA targeting *dbh* (right). Scale bar, 100 μ m.

Table 1,

Comparison of methods for cell-type specific gene disruption in the brain

	Method			
	Cre/loxp conditional KO	AAV-Cre/loxp conditional KO	RNAi	CRISPR/Cas9
Cell-type specificity	High	High	Low	High
limitations	Time-consuming breeding Leaky expression of Cre in non-target cells	Cell type-specific promoters are not always available	Off-target effects	Off-target effects Mosaicism