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INSIGHT

Conditional Genome Editing in the Mammalian Brain Using CRISPR-Cas9

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Neuronal ensembles with distinct morphological, biochemical, and functional identities are organized into complex circuits in the mammalian brain, and malfunction of specific neuronal types in different networks contributes to diverse pathological symptoms. Taking memory as an example, specific memories are held in a subset of neurons, referred to as engram cells. Conditional genome manipulation in the heterogeneous brain would provide a powerful tool for investigating the encoding and storage of specific memories. Recently, Sun *et al.* [1] developed a clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 system for precise genomic perturbations in specific neuronal subpopulations with high temporal and spatial specificity, which can be widely applied in revealing brain functions including memory.

Substrate for Memory Storage

Memory refers to the storage of learned information in the nervous system; it is vital to adaptive behavior in mammals. Pharmacological research has largely relied on the impairment of a broad brain region, rather than a specific subset of neurons relevant to a given memory. It is now known that only a small number of neurons, the engram cells, is necessary to encode a memory. Selective ablation or inhibition of engram cells erases the memory response, while direct activation can induce the associated behavioral output [2]. Engram cells were first identified by the combination of immediate early gene (IEG) labeling with a doxycycline-inducible system [2]. The expression of IEGs, including *arc* and *c-fos*, are frequently used as biomarkers of neuronal excitation [3, 4]. During a given learning experience, the promoter of an IEG can be co-opted to label activated neurons with exogenous protein, such as fluorescent protein, β-galactosidase, or an optogenetic component [2, 5].

For decades, memory encoding has been hypothesized to involve structural changes at the synaptic junctions of neuronal ensembles, and strengthening of synaptic connections of existing neurons might underlie memory storage [6]. However, recent results have demonstrated that memory information is still retained in engram cells even in the absence of engram cell-specific reinforcement of synaptic strength, showing a stark dissociation between memory content and synaptic plasticity [7]. It was recently speculated that the connectivity pattern of engram cells encompassing multiple brain regions might be a potential substrate for memory storage, and this functional connectivity is established during memory encoding and retained in subsequent consolidation [8].

Limitations of Current Methodology

As engram cell connectivity is the putative mechanism for a given memory, it is necessary to fully decipher the logic of complex engram networks and their role in memory

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storage, and comprehensive mapping of engram circuits at the holistic level is required. However, there are several technical limitations to study engram circuits among multiple brain regions with precise spatial and temporal resolution. Further, optogenetics merely allows transient activation or inhibition of engram cells at the cell level, highlighting the need for tools for conditional genome perturbation to explore the molecular underpinnings of engram cells.

Methods relying on RNA interference and DNA antisense oligonucleotide have been widely used for gene silencing in the brain, and also have potential in clinical therapeutics for brain disorders. However, neither strategy allows site-specific gene modification or generation of stable gene knockout. Conditional recombination models are commonly used to decipher brain functions with spatiotemporal accuracy, while the construction of animal strains is time-consuming and labor-intensive, especially transgenic rats and non-human primates.

Conditional Genome Editing in Engram Cells

Emerging in 2013, gene editing based on CRISPR-Cas9 provides a powerful platform for efficient, convenient, and rapid manipulation of endogenous genes in diverse organisms [9]. The CRISPR-Cas9 system is composed of two units, the guide RNA (gRNA) that is made to match the DNA target sequence by forming Watson–Crick base pairs, and the Cas9, an endonuclease enzyme that induces DNA double-strand breaks at the target location. Such breaks can initiate non-homologous end-joining, an error-prone DNA repair machinery, resulting in a frame-shifting insertion/deletion (indel) mutation, thus disrupting the translational reading frame and enabling functional analysis of defined genes in the central nervous system.

Cre–loxP tools that allow conditional control of the spatiotemporal expression of Cas9 have been established in rodents [10], enabling precise gene manipulation in defined neuronal types, even engram cells. Combining CRISPR-Cas9 and activity-dependent cell-labeling technique, Sun *et al.* achieved controlled genome perturbation in engram cells bearing memory storage or extinction, thereby impairing remote memory or extinction learning, respectively, in rats (Fig. 1).

The connectivity of engram cells putatively contributes to memory formation [8]; this highlights the need for conditional genome editing in engram cells identified by specific afferent/efferent connections. Anterograde and retrograde adeno-associated viruses (AAVs) have been broadly applied to map the output and input connectivity of defined neuronal subpopulations. Retrograde viruses target axonal terminals and retrogradely transport their transgenes to the projection neurons, while anterograde viruses allow axonal tracing and the manipulation of postsynaptic neurons. Using retrograde rAAV2-retro, Sun *et al.* [1] restricted the Cas9-mediated gene knockdown in amygdala-projecting infralimbic cortical neurons, and blocked extinction learning in rats. Gene perturbation in postsynaptic engram cells was further achieved by Cas9 and anterograde trans-synaptic AAV1 [1]. Integrated with fluorescence-activated cell-sorting (FACS) and deep sequencing approaches, the transcription dynamics of neurons edited by Cas9 can also be investigated within intact biological contexts.

Perspectives of Genome Editing in Neuroscience

In addition to harnessing endonuclease Cas9 for editing genomic sequences, it also allows non-mutagenic, sequence-specific gene regulation *via* transcription activation or repression. Engineered nuclease-deficient Cas9 (dCas9), which contains mutations in its HNH (H841A) and RuvC1 (D10A) domains, retains the gRNA-guided ability to bind to the complementary genomic sequence without cleaving it, thus allowing the recruitment of various transcription factors for gene modulation at the transcription level. For instance, when fused with the transcriptional repressor Krüppel-associated box (KRAB) and guided by a gRNA targeting the transcription start site, the dCas9-KRAB fusion proteins stop transcription elongation *via* RNA polymerase II blocking, thus repressing endogenous genes in mammalian cells [11]. In contrast, when tethered to the transcription activators, such as the herpes simplex virus VP16 activation domain (VP64), the dCas9 complex can achieve efficient transcription activation, and several protein engineering approaches have been adopted to enhance the activation efficiency [12]. As indel formation is avoided, the CRISPR-dCas9 system can induce a highly homogeneous genotype in engram cells, but not the genetic mosaicism induced by nuclease-active Cas9.

Several inducible systems have been developed for CRISPR-Cas9 *in vitro*. One is photoactivatable Cas9, in which the Cas9 protein is split into two fragments and fused with photoinducible dimerization domains without nuclease activity. Blue light allows split Cas9 fragments to re-associate, thus restoring gRNA-guided nuclease activity [13]. In addition, chemically inducible Cas9 can become active upon addition of small molecules, such as rapamycin [14] and tamoxifen [15]. Combining these inducible systems with the promoters of IEGs can achieve higher temporal control of gene perturbation in target cells.

Multiple genes can be modified simultaneously by different gRNAs, which is useful for investigating genetic

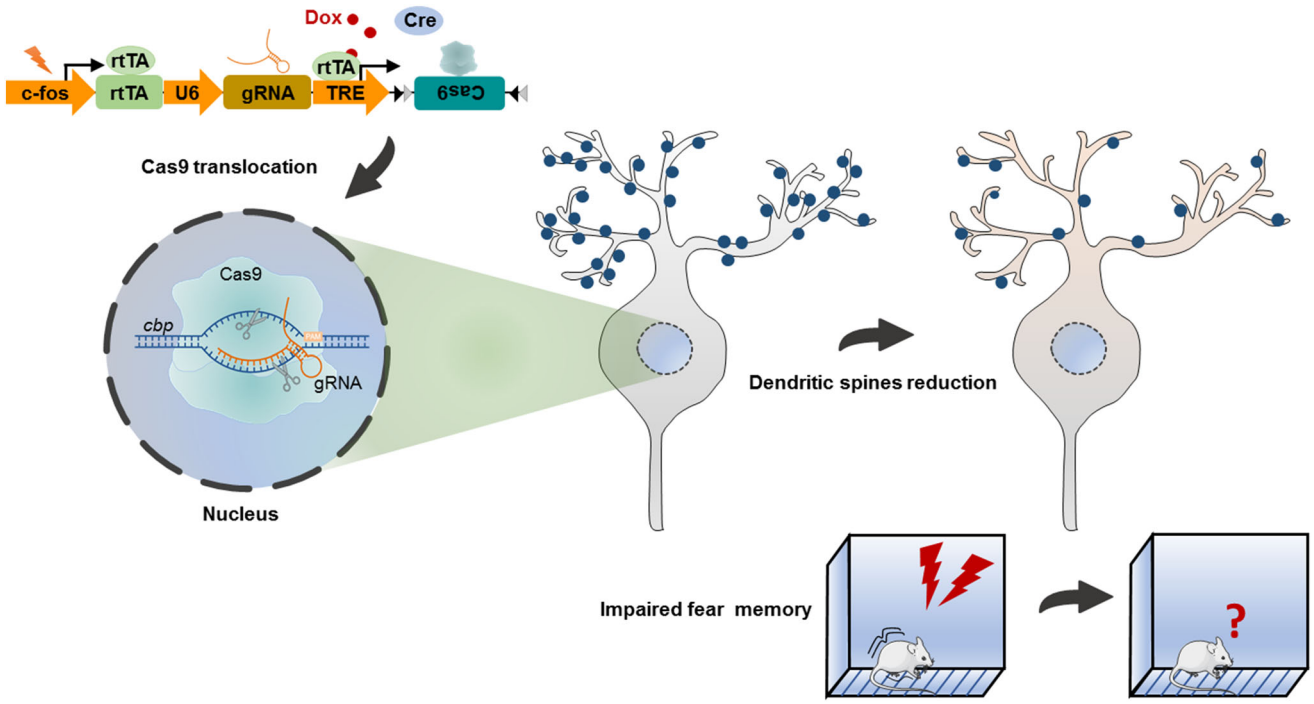

Fig. 1 A schematic of the experiment [1]: taking contextual fear conditioning as an example, with doxycycline (Dox), training induces the expression of rtTA, which binds to TRE, drives the expression of SaCas9, and induces indel generation in the *cbp* (CREB-binding protein) locus of engram cells. Dendritic spines decrease after *cbp* knockout, and rats show less freezing during the recall test. Spatiotemporal expression of Cas9 can also be achieved using anterograde or retrograde AAVs expressing Cre recombinase.

interactions among engram networks. Furthermore, as AAV-PHP.eB and AAV-PHP.S are able to cross the blood-brain barrier [16], these shuttle vectors allow intravenous administration, thus enabling systemic delivery of Cas9 and manipulation of neuronal ensembles in the whole brain. With safety and ethical concerns addressed, promoting memory extinction *via* genome editing in engram cells bearing the traumatic event might be a potential therapy for post-traumatic stress disorder.

There are still several challenges for broad application of the CRISPR-Cas9 system in mammalian brains. First, off-target effects of Cas9 need to be controlled for *in vivo* and in therapeutic applications. This can be partially resolved by paired nickases in which adjacent off-set nicks are induced by two Cas9 nickases [17]. Fusion of dCas9 to FokI nuclease can also improve the specificity of genome editing [18]. Second, persistent overexpression of bacterial Cas9 can evoke host immune responses and neurotoxicity [19]. Reducing the half-life of Cas9 by tagging with geminin [20] and direct delivery of Cas9 proteins rather than the DNA encoding it could be useful approaches to alleviate the potential toxicity and immunogenicity of Cas9 *in vivo*.

CRISPR-Cas9 has been at the forefront of life science since its emergence, and would be widely applied in neuroscience to elucidate the brain function including memory. The molecular mechanisms of the enduring changes among engram cells and their connections during memory encoding and subsequent consolidation require further exploration, where projection- and function-specific genome editing is a powerful tool.

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