Applications of CRISPR–Cas systems in neuroscience

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Applications of CRISPR–Cas systems in neuroscience

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Abstract | Genome-editing tools, and in particular those based on CRISPR–Cas (clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated protein) systems, are accelerating the pace of biological research and enabling targeted genetic interrogation in almost any organism and cell type. These tools have opened the door to the development of new model systems for studying the complexity of the nervous system, including animal models and stem cell-derived *in vitro* models. Precise and efficient gene editing using CRISPR–Cas systems has the potential to advance both basic and translational neuroscience research.

Functional genomics
The study of gene functions and interactions in relationship to RNA transcripts and protein products using genome-wide data, and often involving high-throughput methods.

RNA interference
(RNAi). A technique used to knock down the expression of a specific gene by introducing a double-stranded RNA molecule that complements the gene of interest and triggers the degradation of the target mRNA.

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Our understanding of brain function at the cellular and circuit level has been greatly advanced by functional genomics and the availability of various genetic tools to decipher neuronal diversity and function and model human brain disorders in non-mammalian and mammalian organisms. Just as the development of chemical DNA mutagens[1] and RNA interference (RNAi)[2] led to huge leaps in the fields of genetics and developmental biology — mainly as a result of research in non-mammalian organisms such as flies, worms and fish[3–5] — precise genetic modifications introduced by homologous recombination (HR) in embryonic stem cells (ESCs)[6] paved the way for studying the mammalian brain and modelling human diseases in mice and rats. For example, many neurological disorders, such as Alzheimer disease, are associated with genetic risk factors that can be introduced and studied in animal models[7]. In addition, novel approaches based on human ESCs and induced pluripotent stem cells (iPSCs) are changing the way that we model cellular processes under normal and pathological conditions *in vitro*. For example, human stem cells can be differentiated into neurons or glia to genetically dissect the molecular mechanisms of complex brain disorders *in vitro*[8–12]. Genome-editing technologies are allowing researchers to take full advantage of both animal and cellular models and to work more easily with non-traditional model organisms for neuroscience research.

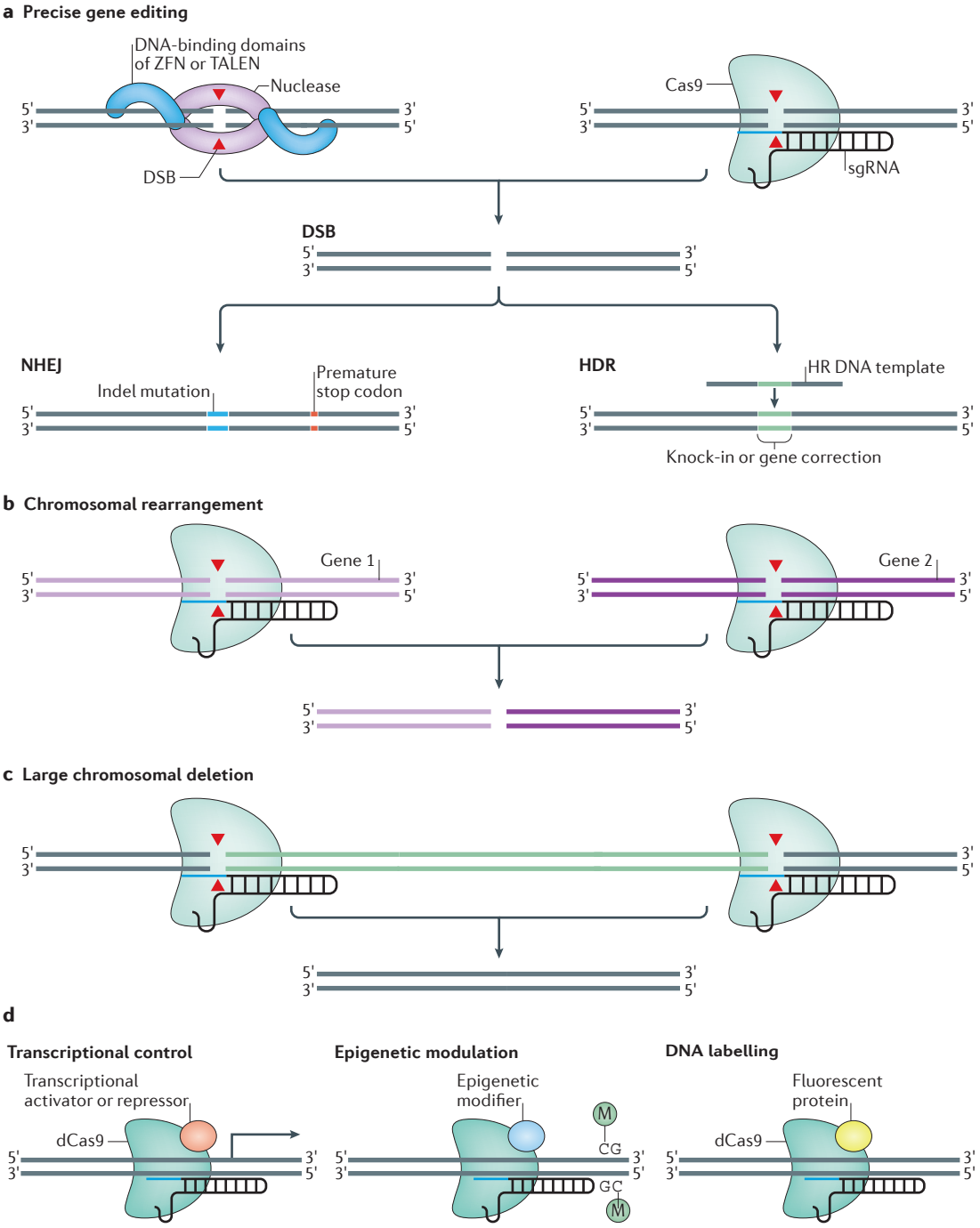
Genome-editing tools based on site-specific DNA nucleases, including zinc-finger nucleases (ZFNs)[13–15], transcription activator-like effector nucleases (TALENs)[16–19] and the CRISPR-associated (Cas) effector proteins of clustered regularly interspaced short palindromic repeat (CRISPR) systems, such as Cas9 (REFS 20–25) and Cpf1 (REFS 26,27), have been developed to facilitate site-specific genomic modifications. In addition, ZFs[28], TALEs[29] and enzymatically inactive versions of Cas9 (known as dead Cas9 (dCas9))[30] can be coupled to functionally different enzymatic domains[30–35] or fluorescent proteins[36] to achieve targeted transcriptional control, epigenetic modification and DNA labelling (FIG. 1).

ZFNs and TALENs recognize specific DNA sequences through protein–DNA interactions, whereas the DNA-specificity of Cas proteins is RNA-guided. To target Cas proteins to specific genomic loci, dual-guide RNAs or single-guide RNAs (sgRNAs)[24,25,27,37,38] can be designed and generated quickly. Another key advantage of Cas proteins is that multiple sgRNAs can be used simultaneously to edit multiple genes, which can be useful for studying genetic interactions and modelling multigenic disorders, something that previously required multiple cloning and complex protein engineering steps to achieve using ZFNs and TALENs.

The benefits of using CRISPR–Cas systems to study the nervous system are highlighted by several successful applications in different animal species and cell types to study synaptic and circuit function[39–41], neuronal development[42–45] and diseases[41,46]. Here, we describe how genome-editing tools, and in particular those based on CRISPR–Cas enzymes, are opening new avenues for neuroscientific and biomedical research through the generation of new model systems, both *in vivo* and *in vitro*, and discuss the challenges and possible future applications of this technology for understanding the brain.

Overview of genome-editing strategies

Site-specific nucleases, including ZFNs, TALENs and Cas proteins, enable precise genetic modifications by inducing double-strand DNA breaks (DSBs) at target locations in the genome. Two highly conserved DNA-repair machinery pathways typically repair DSBs that would otherwise result in cell death: non-homologous end joining (NHEJ) and homology-directed repair



Homologous recombination (HR). The exchange of homologous DNA strands between similar DNA molecules, an event that occurs naturally during meiosis to generate genetic variation. HR is used to direct error-free repair of DNA double-strand breaks induced by DNA nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) proteins.

Embryonic stem cells (ESCs). Totipotent cells derived from embryos that can be genetically manipulated *in vitro* to generate transgenic, knock-in and knockout mice. ESCs can also be directed to differentiate into various cell types *in vitro*, including neurons and glial cells.

Induced pluripotent stem cells (iPSCs). Pluripotent cells derived from reprogrammed differentiated adult cells; iPSCs have properties similar to those of embryonic stem cells and therefore can, in principle, be differentiated into all cell types of the body.

Figure 1 | **Genome-editing applications of CRISPR–Cas9. a** | Non-homologous end-joining (NHEJ) and homology-directed repair (HDR) after a DNA double-strand break (DSB) is induced by zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9). ZFNs and TALENs recognize their DNA-binding site via protein domains that can be modularly assembled for each DNA target sequence. Cas9 recognizes its DNA-binding site via RNA–DNA interactions mediated by the short single-guide RNA (sgRNA), which can be easily designed and cloned. The error-prone NHEJ repair pathway[53] can result in the introduction of insertion or deletion (indel) mutations that can lead to a frame shift, the introduction of a premature stop codon and, consequently, gene knockout. The alternative repair pathway, HDR[14,47–53], can be used to introduce precise genetic modifications if a homologous DNA template is present. **b** | Two different sgRNAs guide Cas9 to induce DNA cleavage at two different genes, resulting in chromosomal rearrangements[116,117]. **c** | Two proximate sgRNAs guide Cas9 to induce DNA cleavage at two different loci of the same gene, introducing large deletions[118,119]. **d** | The nuclease-inactivated version of Cas9 (dead Cas9 (dCas9)) can be fused to different functional enzymatic domains to mediate transcriptional control, epigenetic modulation or fluorescent DNA labelling of specific genetic loci[30–36]. HR, homologous recombination; M, methyl group.

(HDR)[14,47–55] (FIG. 1a). The highly error-prone NHEJ pathway induces insertions and deletions (indels) of various lengths that can result in frameshift mutations and, consequently, gene knockout. By contrast, the HDR pathway directs a precise recombination event between a homologous DNA donor template and the damaged DNA site, resulting in accurate correction of the DSB. Therefore, HDR can be used to introduce specific mutations or transgenes into the genome. Because ZFNs and TALENs achieve specific DNA binding via protein domains, individual nucleases have to be synthesized for each target site. By contrast, Cas9 is guided by a specificity-determining guide-RNA sequence (CRISPR RNA (crRNA)) that is associated with a *trans*-activating crRNA (tracrRNA) and forms Watson–Crick base pairs with the complementary DNA target sequence, resulting in a site-specific DSB[22,23,37,56]. A simple two-component system (consisting of Cas9 from the bacterial species *Streptococcus pyogenes*[24,25] or *Staphylococcus aureus*[57] and a fusion of the tracrRNA–crRNA duplex to a sgRNA)[37] has been engineered for expression in eukaryotic cells and can achieve DNA cleavage at any genomic locus of interest. More recently, Cpf1, a single-RNA-guided nuclease that does not use tracrRNA, has also been adapted for genome editing[27]. Hence, different Cas proteins can be targeted to specific DNA sequences simply by changing the short specificity-determining part of the guide RNA, which can be easily achieved in one cloning step.

Gene editing across species

Non-human animal models provide an experimental platform to dissect the complexity of the brain and study the cellular and molecular underpinnings of brain disorders. Neuroscience in particular benefits from exploiting a wide range of species, including worms, flies, fish and mammals, as well as non-traditional model systems, such as birds and amphibians[58]. Disrupting gene expression is a common approach to study gene function and understand loss-of-function disease mutations. For many years, RNAi was the 'gold standard' for gene silencing and studying gene function *in vitro* and *in vivo*[59,60]; however, genome editing based on engineered designer nucleases offers several advantages over RNAi (TABLE 1). For example, genome-editing tools can be modified to allow for more refined control of gene expression beyond simple gene knockdown, adding to their versatility (FIG. 1d).

Multiplying the power of simple model organisms. At the molecular level, non-mammalian model systems can provide important information about fundamental features of the nervous system as a result of their well-characterized genetic and cellular organization and amenability to a range of genetic tools. For example, many evolutionarily conserved genes involved in human neurological disorders such as Alzheimer disease and Parkinson disease have been extensively studied

Table 1 | **Comparison of approaches for gene knockdown or knockout**

	Approach				
	RNAi	**ASO**	**ZFN**	**TALEN**	**CRISPR–Cas**
Molecular target	RNA	RNA	DNA	DNA	DNA
Result of targeting	Reversible knockdown	Reversible knockdown	Irreversible knockout	Irreversible knockout	Irreversible knockout
Ease of generating target specificity	Easy: simple oligo synthesis and cloning steps and limited chemical modifications to enhance RNA degradation	Easy: simple oligo synthesis and cloning steps; often chemical modification is required to enhance RNA binding and ASO stability	Difficult: substantial cloning and protein engineering required	Moderate: substantial cloning steps required	Easy; simple oligo synthesis and cloning steps
Off-target activity	High	High	Moderate	Low	Low
Ease of multi-plexing	High	High	Low	Moderate	High
Transcriptional and epigenetic control	Direct control not possible	Direct control not possible; TSOs can interfere with protein translation	DNA-binding ZF domains can be fused to new functional domains	DNA binding domains can be fused to new functional domains	Enzymatically inactive dCas9 can be fused to new functional domains
Ease of delivery into the mammalian CNS	High: delivered by nanoparticles, bioconjugates, cell-penetrating peptides or viral vectors	High: delivered by nanoparticles, bioconjugates, cell-penetrating peptides or viral vectors	Moderate: delivered by viral vectors	Moderate: delivered by viral vectors but large size makes packaging into viral vectors challenging	Moderate: delivered by electroporation, PEI-mediated transfection, nanoparticles and viral vectors
Ease of generating large-scale libraries	High: simple oligo synthesis and cloning required	High: simple oligo synthesis and cloning required	Low: complex protein engineering required for each gene	Moderate: technically challenging cloning steps	High: simple oligo synthesis and cloning required
Costs	Low	Low	High	Moderate	Low

ASO, DNA antisense oligonucleotide; Cas, CRISPR-associated protein; CRISPR, clustered regularly interspaced short palindromic repeat; dCas9, dead Cas9; PEI, polyethylenimine; RNAi, RNA interference; TALEN, transcription activator-like effector nuclease; TSOs, translation-suppressing oligonucleotides; ZF, zinc-finger; ZFN, ZF nuclease.

using flies, worms and fish[61–63]. For years, studies using these simple model organisms relied mainly on genetic screens using chemical mutagenesis and RNAi[3–5] or imprecise methods for transposon excision and retroviral insertion[64–66]. More-precise genetic modifications have been achieved using ZFNs[67–69], TALENs[70–73] and Cas proteins (reviewed in REF. 74). In the case of Cas proteins, large numbers of RNA guides can be easily synthesized to study gene function on a large scale. By contrast, generating large libraries based on ZFNs and TALENs is challenging owing to difficulties in designing and synthesizing these proteins with varying DNA binding specificities (TABLE 1). In a proof-of-concept study, approximately 50 genes were screened with Cas9 and novel loci involved in electrical synapse formation in zebrafish were identified[43]. Such *in vivo* screening approaches in small model organisms offer an accessible platform to identify the genes involved in various aspects of nervous system function and dysfunction.

Rapid generation of mammalian models. The development of methods facilitating HR in ESCs[6] enabled neuroscientists to study the effects of gene knockouts, mainly in mice. This approach has been significantly enhanced by genome-editing technologies (FIG. 2a,b). Genome editing in single-cell embryos has been used to generate mouse[75], rat[76] and primate models[77,78] that can be used to study the role of specific proteins in nervous system function. Mouse and rat models provide a bridge between our understanding of the molecular underpinnings of the nervous system gleaned from studies in non-mammalian systems and the complex phenotypes observed in human brain disorders. However, in some cases, a comprehensive understanding of the human brain will require primate models, which have brains that are more similar to the human brain in terms of neuroanatomical, physiological, perceptual and behavioural characteristics.

Transgenic approaches in primates are generally very inefficient. However, successful insertion of transgenic alleles in primates, including macaques[79,80] and the common marmoset[81], has been achieved using retroviral and lentiviral approaches in early embryos. For example, the viral insertion of a disease-related version of the human gene huntingtin (*HTT*) into the macaque genome recapitulated clinical features of Huntington disease[80], representing an important step forward for genetic-disease modelling in non-human primates. TALENs have also been successfully used in monkeys to model mutations in methyl-CpG-binding protein 2 (*MECP2*), an X-linked Rett syndrome gene[77], and genome-engineered primates have been generated by precise disruption of single and multiple genes with Cas9 (REF. 78). The simplicity of the use of Cas proteins relative to that of ZFNs and TALENs, and the ability to modify multiple genes simultaneously, is a breakthrough that is already catalysing molecular interrogation of neurological and psychiatric dysfunctions in disease-relevant brain circuits using primate models[78,82]. The ability to examine brain function in genetically modified non-human primates has the potential to contribute significantly to our understanding of higher cognitive functions and to the development of new therapeutic strategies for diseases that cannot be adequately modelled in rodents. However, such research raises important bioethical questions and requires careful consideration of the costs and benefits before moving forward.

In vivo gene editing in the brain

In vivo gene editing allows the systematic genetic dissection of neuronal circuits and the ability to model pathological conditions while bypassing the need to engineer germline-modified mutant strains. This experimental approach is fast, independent of genetic background, animal species and availability of ESCs, and can be applied to existing disease models and transgenic strains, as well as to aged animals to study age-related neurological changes (FIG. 2c). *In vivo* methods based on RNAi have been commonly used to reduce the expression of genes in the brain[83]. In addition, alternative methods based on DNA antisense oligonucleotides (ASOs) can be used for gene silencing and have been shown to be promising therapeutic molecules for suppressing pathogenic protein aggregates in the brain[84,85]. However, neither strategy allows the generation of stable gene knockouts or site-specific epigenetic modifications (TABLE 1). In the mouse brain, histone modifications and transcriptional control have been achieved using ZFs[86] and TALEs[32], and Cas9 has been used to induce indel mutations in neurons to achieve stable gene knockouts in living animals[39,41]. This demonstrates the capacity for spatial and temporal control of gene expression in fully developed circuits and also opens the door to probing epigenetic dynamics[30–33,35] in the brain. Epigenetic control is of particular interest, as there is increasing evidence that epigenetic mechanisms, such as histone modifications and DNA methylation, play a part in learning, memory formation and the pathology of neuropsychiatric disorders[87]. Using Cas proteins, functional domains of DNA-methylation or -demethylation enzymes or histone modifiers can be easily targeted to specific DNA sequences to edit the epigenome with high spatial and temporal specificity *in vivo* (FIG. 1d).

Delivery to the brain. Viral vectors are a promising mode for delivery of Cas proteins to the brain. Viral vectors have defined, tissue-specific or cell type-specific tropism and can be admitted either locally to the brain or through the bloodstream to achieve more-systematic tissue penetration[88]. The most-attractive gene-delivery vectors are adeno-associated viruses (AAVs), which afford long-term expression without genomic integration, are relatively safe and are non-pathogenic[89,90]. However, AAV vectors have limited transgene capacity, and the large size of the commonly used *S. pyogenes* Cas9 variant poses a significant challenge for AAV-mediated delivery[41,91]. AAV-mediated delivery may become even more challenging when Cas9 is enlarged by the fusion of additional functional domains. Smaller Cas9 orthologues, such as those derived from *S. aureus*, are easier to pack[57], making them an attractive option for *in vivo* genome editing in the brain.

Epigenetic mechanisms
Multilayered cellular processes that modulate gene expression and function in response to interoceptive and environmental stimuli during development, adult life and ageing, including DNA methylation, post-translational histone modifications, ATP-dependent nucleosome and higher-order chromatin remodelling, non-coding RNA deployment and nuclear reorganization.

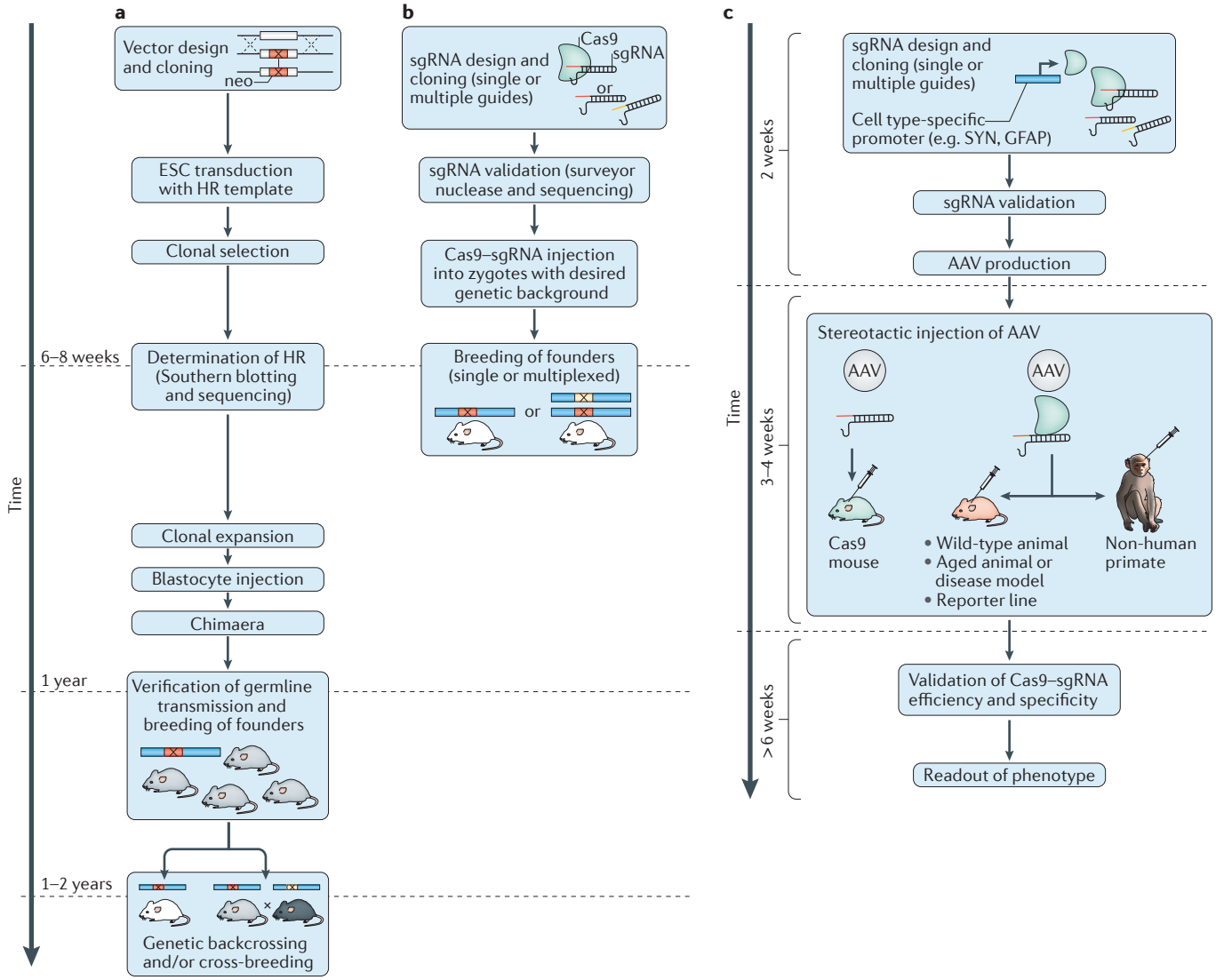


Figure 2 | **Using Cas9 to generate genetically modified rodents and for *in vivo* genome editing. a,b** | Comparison of the timelines of traditional gene targeting using classic homologous recombination (HR) in embryonic stem cells (ESCs; part **a**) and clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) gene targeting in one-cell embryos (part **b**). There are two main time- and cost-intensive phases of the HR approach. First, the design and cloning of the targeting vector, ESC transduction and selection, and generation of chimaeras. Second, the backcrossing of mice to a desired background and/or cross-breeding to generate multiple genetically modified animals. By contrast, cloning of short single-guide RNA (sgRNA) into a targeting vector, verification of sgRNA on-target efficiency (through the surveyor nuclease assay or sequencing), Cas9–sgRNA microinjection and founder identification are relatively easy and fast[120]. Because embryos can be obtained from any mouse strain and multiple genes can be targeted simultaneously, genetic backcrossing and cross-breeding are not required. **c** | Cas9 nucleases also enable precise *in vivo* genome editing of specific cell types in the mammalian brain on a relatively short timescale. Cas9 is cloned under the control of cell type-specific promoters, and sgRNA efficiency is validated *in vitro* before being packaged into viral vectors, such as adeno-associated viruses (AAVs). sgRNA can then be stereotactically delivered into the brains of mice that have endogenous Cas9 expression (Cas9 mice)[91], or the sgRNA can be delivered together with Cas9 into wild-type mice[41] or rats, aged animals, disease models or reporter lines. *In vivo* genome editing in the brain is not limited to rodents and can theoretically be applied to other mammalian systems, including non-human primates. GFAP, glial fibrillary acidic protein; Neo, neomycin anitibiotic selection marker; SYN, human synapsin promoter.

Other techniques have been also used to deliver Cas9 and RNA guides to the brain, including *in utero* electroporation[39] and polyethylenimine (PEI)-mediated transfection[46]. In rodents, electroporation and PEI-mediated transfection are easy to use, fast and efficient at delivering large plasmid DNA into a high number of neurons. However, two drawbacks of these techniques are their low spatial accuracy of transgene expression and the necessity of prenatal intervention, which often results in low viability and targeting of mitotic neuronal precursors instead of postmitotic, differentiated neurons.

Liposomes
Lipid vesicles artificially formed by sonicating lipids in an aqueous solution. Liposomes can be packed with negatively charged molecules to deliver them into cells and are therefore promising vehicles for therapeutic applications.

Cre–*loxP* recombination
A site-specific recombination system derived from *Escherichia coli* bacteriophage P1. Two short DNA sequences (*loxP* sites) are engineered to flank the target DNA. Activation of the Cre recombinase enzyme catalyses recombination between the *loxP* sites, leading to excision of the intervening sequence.

Alternatively, Cas9 protein itself, rather than the DNA or RNA that encodes it, could be delivered, an approach that is particularly interesting for protein-based therapeutics. The anionic nature of sgRNA allows the integration of Cas9–sgRNA complexes into cationic liposomes, a commonly used DNA-, RNA- and protein-delivery tool. Liposome Cas9–sgRNA complexes have already been successfully used to achieve genome editing in the mouse inner ear[92]. Therefore, lipid-mediated delivery of Cas9 may also serve as powerful tool for genome editing in the brain in the future.

Cell type-specific genome editing. In the mammalian brain, there are probably several hundred neuronal subtypes, each with distinct morphological, biophysical, biochemical and computational functions. Thus, cell type-specific tools are required to dissect this heterogeneous tissue. Research has shown that the malfunction of specific cell types in different brain regions contributes to diverse symptoms usually connected with neuropsychiatric symptoms, such as hallucinations, depression and repetitive motor behaviour[93]. This highlights the need to pinpoint causal relationships between cell types within the context of relevant neuronal networks, genetics and behavioural dysfunction, which will require precise genome editing in specific cellular subtypes. Site-specific Cre–*loxP* recombination elements that enable the control of the spatiotemporal expression of Cas9 have been introduced in fish[94] and mouse embryos[91], and similar approaches could achieve precise gene editing in defined cell types *in vivo*. The vast number of established Cre-driver mouse lines[95] and inducible Cas9 systems[96–98] can, when combined with conditional gene-targeting strategies, provide enormous combinatorial power to decipher the logic of complex neuronal networks and their role in neurological disorders *in vivo*.

In vivo efficiency and specificity. In postmitotic neurons, Cas9 has been successfully used to introduce single[39–41,46,91] and multiple DSBs[41,46] resulting in NHEJ and efficient formation of indel mutations. For example, AAV delivery of Cas9 and sgRNA targeting *Mecp2* in the adult mouse brain resulted in the local loss of more than 70% of MECP2, which was sufficient to recapitulate phenotypes observed in classic *Mecp2*-mutant mouse models and patients with Rett syndrome[41]. In another study, Cas9-mediated deletion of common tumour-suppressor genes, such as patched homologue 1 (*Ptch1*), *Trp53* (also known as *Tp53*), phosphatase and tensin homologue (*Pten*) and neurofibromin 1 (*Nf1*), in the cerebellum or forebrain efficiently induced the formation of medulloblastoma or glioblastoma tumours, respectively[46]. Despite this success, the validation of Cas-mediated gene editing in the brain is still challenging, and sensitive methods are required for analysing Cas efficiency and specificity in targeted brain regions (BOX 1).

Although NHEJ in postmitotic neurons has been demonstrated to be active, it remains unclear how efficient HDR is in postmitotic cells. It is commonly believed that HDR predominantly occurs in the S and G2 phases of the cell cycle[99,100], and HDR is therefore thought to be rare in non-dividing cells, such as neurons. Introduction or correction of precise genetic mutations via HDR in the brain would validate disease mutations *in vivo* and open the door to therapeutic applications of genome editing in brain disorders. Thus, future work should focus on identifying and activating signalling pathways required for triggering HDR in differentiated cells. However, it should also be noted that gene insertion has been achieved through NHEJ pathways, which may allow us to insert DNA into neurons and glia[101].

In contrast to precise gene knockout and insertion, genome editing aimed at transcriptional regulation and epigenetic modulation may be less challenging in the brain, as these approaches are independent of DNA-repair pathways. Achieving epigenetic and transcriptional control in neurons can aid in the study of the molecular mechanisms of natural gene silencing in the nervous system and can help us to better understand neurological disorders associated with gene imprinting, such as Angelman syndrome[102].

Gene editing in human iPSCs

Combinatorial approaches based on iPSC technology and genome editing offer another approach to model human neurological disorders *in vitro*. A key advantage of this approach is that genetic modifications can be studied in different human genetic backgrounds, because iPSCs retain all of the individual donor's genetic information. This is particularly important for complex

Box 1 | **Validating Cas nuclease efficiency and specificity in the brain**

Validating clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) nuclease efficiency and specificity is particularly challenging in the mammalian brain because of its complex architecture and cellular diversity. To precisely validate nuclease efficiency and specificity, targeted cells first have to be identified and sorted out from the heterogeneous cell population in the brain. Recently, an easy and efficient method for this was developed in which fluorescent-activated cell sorting (FACS) is used to isolate fluorophore-tagged nuclei of targeted cells to purify and analyse genomic DNA and nuclear RNA with high resolution and sensitivity[41].

Cas efficiency

Cas nuclease efficiency can be validated using enzymatic DNA cleavage assays (such as surveyor nuclease technology[110]) or DNA sequencing[41,46,91]. DNA-sequencing analysis provides a complete picture of insertion and deletion (indel) frequency, types of frame-shift and in-frame mutations, length and exact sequence of indels, and information about mono- and bi-allelic modifications when applied to single cells[41]. In addition, RNA levels of the targeted gene can be determined using quantitative PCR (qPCR) or RNA-sequencing methods. Depending on the targeted exon (that is, whether it is an early or late exon), truncated transcripts might be expressed from the target gene and should also be considered when qPCR probes are designed. Ideally, effective protein knockdown should also be measured using histological, biochemical and/or functional (for example, electrophysiology and enzymatic activity assays) readouts.

Cas specificity

Similarly to zinc-finger nucleases and transcription activator-like effector nucleases, Cas proteins can cleave off-target sites in the genome. Many software tools predict potential off-target effects and help to choose optimal target sequences to reduce off-target activity (see Further information for a non-comprehensive list of online tools). On-target specificity can be further improved by using double-nicking[111,112] or truncated single-guide RNA approaches[113]. In addition, sensitive readout methods for identifying genome-wide Cas9 off-target activity have been developed that provide useful tools for evaluating specificity and safety of Cas9 in basic and clinical research (see Further information)[57,114,115].

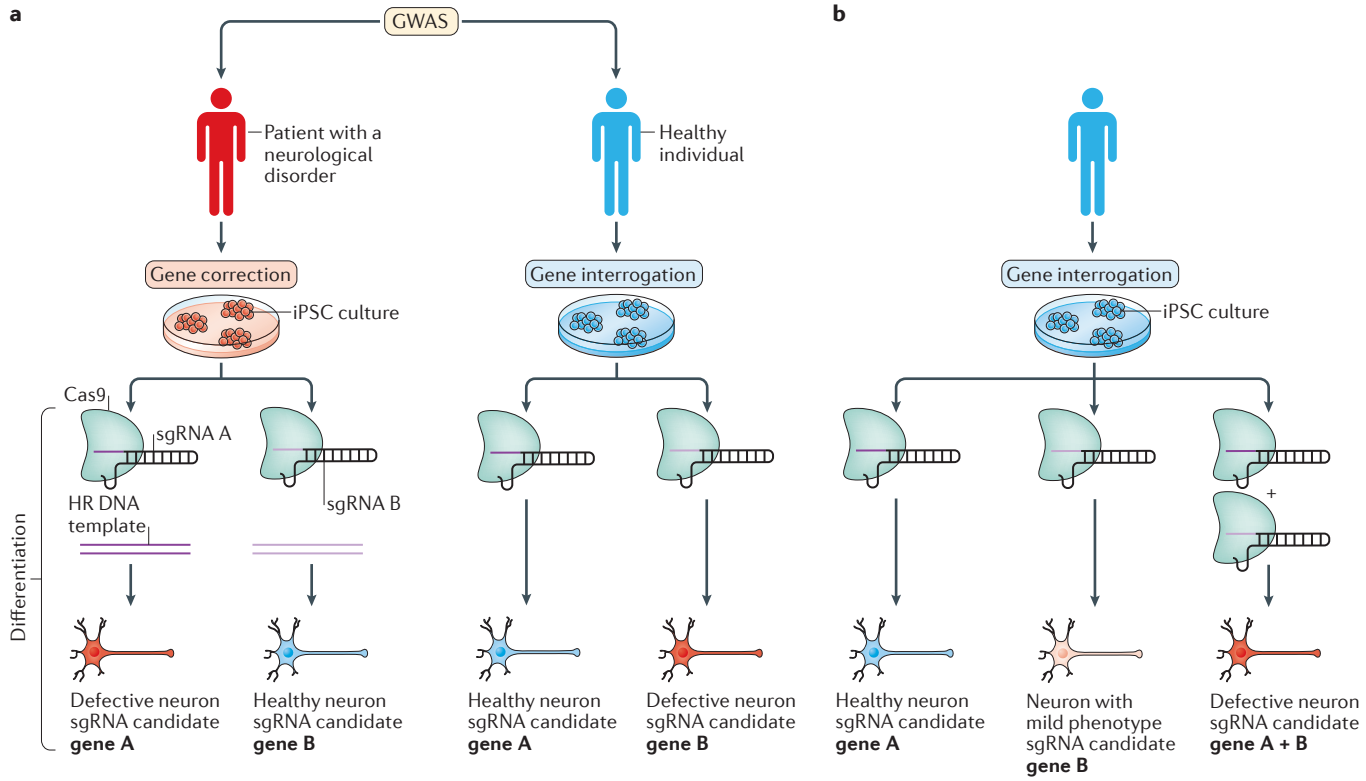


Figure 3 | ***In vitro* applications of Cas9 in human iPSCs. a** | Evaluation of disease candidate genes from large-population genome-wide association studies (GWASs). Human primary cells, such as neurons, are not easily available and are difficult to expand in culture. By contrast, induced pluripotent stem cells (iPSCs) derived from somatic cells (such as fibroblasts) of healthy individuals or patients with neurological disorders can be differentiated into neurons and cultured *in vitro*[8–12]. Disease candidate genes can be examined in two ways. Site-specific homologous recombination (HR) of the candidate gene using clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) nucleases can be applied in disease-affected cells (left). If this rescues disease phenotypes (as for candidate gene B in the example shown), the validity of the candidate gene is confirmed. Alternatively, candidate genes can be mutated in healthy cells (right). Where this recapitulates disease pathogenesis *in vitro* (as in the case of candidate gene B), the validity of the candidate gene is confirmed. **b** | The contribution of specific genetic loci to multigenic disorders, such as Alzheimer or Parkinson diseases, can also be systematically evaluated using Cas-mediated single and multiplex genome editing. This may enable dissection of possible synergistic effects (as shown for candidate genes A and B) and screening for functional correlations between disease phenotypes and distinct gene mutations. sgRNA, single-guide RNA.

neurological disorders, because genetic variants associated with such diseases act in concert with many other alleles. Another advantage is that the genetically modified cells can be differentiated into almost any cell type, including those that are not easily accessible in patients, such as neurons and glia.

iPSC-based disease models have been generated for several neurological disorders, including Parkinson[10,11], Alzheimer[9] and Huntington[8] diseases, and they have been proven to closely mimic cellular and molecular features of human diseases. Genome-editing tools applied to these models can be used to examine the genetic link between risk variants and cellular pathways involved in multigenic neurological disorders in a high-throughput manner (FIG. 3). Furthermore, specific signalling pathways involved in the pathogenesis of the disease can be precisely dissected to gain insight into the molecular mechanisms of the disease and to identify new drug targets[10]. Gene editing may be performed either in iPSCs or induced later in differentiated cells[96,98], allowing for the investigation of phenotypes that arise during cell differentiation, which may be relevant when studying neurodevelopmental aspects of a disease such as Rett syndrome[103–105]. In addition, inducing or rescuing a phenotype in differentiated cells will be useful for validating potential therapeutic applications.

Future perspectives

Genome-editing technologies allow for the introduction of genetic modifications into almost any cell type and organism. For example, Cas9 has already been used to alter genes in species such as killifish[106] and salamander[107], which are commonly used to study ageing and tissue regeneration, respectively. It may also open up the possibility of developing models in other species of interest to neuroscience research, such as social insects or songbirds[58], which have thus far been intractable to genetic modification. In addition to the generation of new model systems, including iPSC-derived *in vitro* models, genome editing in combination with single-cell

transcriptomics[108] provides a route to understanding cell type-specific gene function within a heterogeneous tissue, allowing for precise dissection of genetic networks in the brain. Furthermore, together with genome-wide association studies, *in vivo* genome editing holds potential for personalized therapeutic applications for brain disorders[109]. However, to realize these advances, several open challenges have to be addressed. First, existing methods for delivering Cas proteins and RNA guides to the brain must be optimized and new methods must be developed to achieve sufficient levels of specificity and efficiency. Second, new methods for stimulating efficient gene insertion and correction in postmitotic cells have to be established. Third, safety and ethical concerns have to be carefully addressed. Nevertheless, we believe that novel genome-editing technologies based on CRISPR–Cas systems, together with powerful readout methods, will help us better understand the logic of neuronal circuits and unravel some of the mysteries of complex neurological disorders in the near future.

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Competing interests statement
The authors declare competing interests: see Web version for details.

FURTHER INFORMATION
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CasOT (CRISPR/Cas system (Cas9/gRNA) Off-Targeter): http://eendb.zfgenetics.org/casot/
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