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Translation potential of current preclinical techniques for gene therapy of neurological diseases in clinic. A critical review.

Translační potenciál současných preklinických technik pro genovou terapii neurologických onemocnění v klinice. Kritické zhodnocení.

Bachelor's thesis

Supervisor: Mgr. Ondřej Novák, Ph.D

Prague, 2023

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Paulína Žideková

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Abstract

Research in the field of gene therapy has potential to become a revolutionary way to the existing treatment for a wide spectrum of neurological diseases. To treat these disorders causally, by specific substituting, deleting, silencing or editing faulty genes could be a privilege of gene therapy. The concept of translational medicine is to facilitate the transfer of working principles in preclinical research into treatment in humans. Its key issue is to overcome limitations associated with the gap between the tremendous variety molecular biology tools of preclinical research and the lack of simple corresponding options in humans. Clinical implementation of most of the preclinical approaches is still considered to be limited. The main focus of this thesis is to summarize latest advancements of molecular and genetic engineering tools that themselves or in combination have the potential to promote most preclinical gene therapy of neurological diseases to clinical use. Based on that, this study aims to suggest perspective methods of treatment for selected neurological diseases.

Keywords: gene therapy, translational medicine, neurological diseases, immune response, vector delivery, epilepsy

Abstrakt

Výzkum v oblasti genové terapie má potenciál se stát revolučním přístupem k léčbě širokého spektra neurologických onemocnění. Léčit tyto poruchy kauzálně, specifickou substitucí, delecí, umlčením nebo úpravou vadných genů, by mohlo být výsadou genové terapie. Koncept translační medicíny je soustředěn na přenos principů fungování v překlinickém výzkumu do léčby u lidí. Jeho klíčovým problémem je překonat omezení spojená s velkou mezerou mezi obrovskou rozmanitostí nástrojů molekulární biologie překlinického výzkumu a nedostatkem odpovídajících možností u lidí. Klinickou implementaci většiny předklinických přístupů je stále možné považovat za omezenou. Hlavním záměrem této práce je shrnout nejnovější pokroky nástrojů molekulárního a genetického inženýrství, které samotné nebo v kombinaci mají potenciál posunout většinu preklinické genové terapie neurologických onemocnění do klinické praxe.

Klíčová slova: genová terapie, translační medicína, neurologická onemocnění, imunitní odpověď, vektorové doručení, epilepsie

CONTENTS

LIST OF ABBREVIATIONS

5HT3	serotonin receptor 3
AAV	adeno-associated virus
ABE	adenine based editor
AD	(trans)activation domain
BBB	blood-brain barrier
BGH	bovine growth hormone
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
Cas	CRISPR-associated protein
CBE	cytosine based editor
CIB1	cryptochrome-interacting basic-helix-loop-helix protein 1
CIBN	N-terminal variant of CIB1
CIRCLE-seq	circularization for in vitro reporting of cleavage effects by sequencing
CNO	clozapine-N-oxide
CNS	terminal resolution site
CRISPR	clustered regularly interspaced short palindromatic repeats
CRISPR GUARD	CRISPR guide RNA assisted reduction of damage
crRNA	CRISPR RNA
Cry2	cryptochrome circadian regulator 2
CSF	cerebrospinal fluid
DBD	DNA-binding domain
dCas9	nuclease-deactivated Cas9
DREADD	designer receptor exclusively activated by designer drug
EEG	electroencephalogram
FAD	flavin adenine dinucleotide
FKF1	flavin-binding kelch domain F box 1
FMN	flavin mononucleotide
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLP	good laboratory practice
GlyR	glycine receptor
GPCR	G-protein-coupled receptor
gRNA	guide RNA
GtACR	tuillardia theta anion channelrhodopsin

HDR	homology-directed repair
HEK293	human embryonic kidney 293
hGFAP	human glial fibrillary acidic protein
HNH nuclease domain	named after the HNH motif, which is a conserved sequence of amino acids present in the domain
hSYN	human synapsin-1
ChR	channelrhodopsin
IPD	ion pore domain
ITR	inverted terminal repeat
KORD	kappa-opioid receptor
mGluR6	metabotropic glutamate receptor 6
miRNA	micro RNA
MRI	magnetic resonance imaging
mRNA	messenger RNA
nAChR	nicotinic acetylcholine receptor
NGS	next-generation sequencing
NHEJ	non-homologous end joining
NLS	nuclear localization sequence
Opto-mGluR6	optogenetic metabotropic glutamate receptor 6
PAM	protospacer adjacent motif
PBS	primer binding site
PCB	phycocyanobilin
PCR	polymerase chain reaction
pegRNA	prime editing guide RNA
PhyB	phytochrome B
PIF	phytochrome-interacting factor
poly(A)	polyadenylation signal
pre-crRNA	precursor CRISPR RNA
PSAM	pharmacologically selective actuator module
PSEM	pharmacologically selective effector molecule
PV	parvalbumin
rAAV	recombinant AAV
RT	reverse transcriptase
RTT	RT template
rtTA	reverse tetracycline-controlled transcriptional activation
RuvC domain	named after the RuvC protein, which is a DNA repair endonuclease in E. coli

scAAV	self-complementary adeno-associated virus
SCNA1	voltage-gated channel alpha subunit 1
sgRNA	single guide RNA
SMA	spinal muscular atrophy
SMN	survival motor neuron
SMRT	single molecule real-time
SpCas9	Cas9 from Streptococcus pyogenes
SST	somatostatin
SV40	simian virus 40
tracrRNA	trans-activating crRNA
TRE	tetracycline response element
TRS	terminal resolution site
tTA	tetracycline-controlled transcriptional activation
vGAT	vesicular gamma-aminobutyric acid transporter
VIP	vasoactive intestinal peptide
VIVO	verification of in vivo off-targets
VVD	vivid protein
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
wtAAV	wild-type adeno-associated virus
ZMW	zero-mode waveguides

1. Introduction

Gene therapy is a therapeutic approach that involves modifying patient's genetic composition. It utilizes the delivery of therapeutic genes, gene editing tools, or transcription modulating tools to specific cell populations in order to correct for the underlying genetic defects, or promote the proper function or survival of the target cells. By restoring or modulating the expression of key genes, gene therapy can provide long-lasting therapeutic benefits and improve the quality of life.

Gene therapy has potential to revolutionize the treatment of various neurological diseases. One of the most promising applications of gene therapy in neurology is the treatment of genetic disorders by addressing the underlying pathophysiology mechanisms of the disease. For example, in Huntington's disease, gene therapy aims to silence or remove the mutant huntingtin gene [1], while in spinal muscular atrophy, gene therapy delivers a functional copy of the survival motor neuron 1 gene [2]. Another application of gene therapy in neurology is the treatment of late-onset diseases by delivering therapeutic genes that can replace lost or damaged cells, restore function, or reduce inflammation. In Parkinson's disease, therapy targets non-dopaminergic neurons to produce dopamine and improve motor function [3]. In Alzheimer's disease, gene therapy regulates the production of beta-amyloid or tau proteins, reducing their accumulation and preventing neuronal damage [4]. Gene therapy can also be used to deliver genes for neurotrophic factors that promote the survival and growth of neurons, which can be beneficial in diseases such as amyotrophic lateral sclerosis [5] and spinal cord injury [6]. In addition, gene therapy has potential in precision medicine, where treatments can be customized based on a patient's unique genetic makeup. In diseases such as epilepsy [7], in which genetic factors can play a significant role, gene therapy can already help via targeting specific mutations or variations that contribute to the disease.

The potential of gene therapy to spread widely as a causal treatment for neurological diseases is significant but it requires careful translation from preclinical studies to clinical trials. Preclinical studies provide proof-of-concept and test safety and efficacy in animal models. Clinical translation requires identifying the genetic cause of the condition, developing efficient methods to manipulate the genome or specific activity, and designing effective delivery methods. Cost, logistics, and regulatory hurdles must also be addressed before therapy can be approved for clinical use.

Fortunately, recent technological advancements, such as the decreased cost of next-generation sequencing (NGS), have made it possible to conduct genetic analysis on every patient who visits a neurological clinic, which is crucial for developing effective gene therapies. Once the problem has been identified, targeted gene therapies can be used to identify causal treatments.

Advanced molecular tools, such as clustered regularly interspaced short palindromatic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) or opto/chemogenetics, have ponential to revolutionize the field of gene therapy by allowing for more precise and efficient manipulation of the neuronal genome or specific neuronal activity. CRISPR-Cas9 has emerged as a powerful tool for targeted gene editing, using RNA-guided nucleases to cut and modify specific DNA sequences [8]. Opto/chemogenetics, on the other hand, allows for the specific manipulation of neuronal activity using light or chemical signals to activate or inhibit specific

neural circuits. The selectivity of these tools makes them ideal to be used in gene therapies that can target specific causal mechanism related to genetic mutations or variations.

Developing effective delivery methods for therapeutic genes is another key challenge for translating preclinical gene therapy studies into clinical trials. In neurological diseases, the targeted cells, often located in the brain or spinal cord, are protected by the blood-brain barrier (BBB). Developing a successful delivery method for gene therapies must be able to cross the BBB, provoke only a low immune response, and show some selectivity to target the specific cells. The use of adeno-associated virus (AAV) vectors evolves as a delivery method for gene therapy in neurological diseases [9]. AAV vectors in conjunction with short promoters and gene regulatory elements have been shown to successfully deliver therapeutic genes to the nervous system while eliciting a minimal immune response.

The use of NGS, CRISPR-Cas9, opto/chemogenetics and AAV vectors with short promoters and gene regulatory elements have already helped to better understand the genetic mechanisms underlying neurological diseases. Proper combining of the mentioned advanced methods can be used to treat many more neurological disorders and create causative and highly personalized therapies.

In this thesis, the latest molecular and genetic engineering tools for preclinical gene therapy for neurological diseases are thoroughly examined. The study focuses on the strengths, limitations, and potential for clinical translation of these tools. The identification of tools that can be translated to clinical practice is crucial in the development of safe and effective gene therapies. Moreover, potential treatment methods for specific neurological disease using these tools, either alone or in combination, is proposed in the later chapter. This study's evaluation of the potential of these tools for clinical translation could aid in bridging the gap between preclinical research and clinical trials.

Overall, this thesis seeks to describe molecular and genetic engineering tools that have the potential for clinical translation, propose potential treatment method for specific neurological disease, and ultimately contribute to the development of effective and safe gene therapies for these debilitating disorders.

2. Next-generation sequencing

Next-generation sequencing (NGS) methods enable investigation of the genetic basis of diseases and provide insights into disease prognosis and potential treatment options[10]. The main difference between first-generation sequencing and NGS (second and third generation) is the method and its large degree of parallelization used to sequence the DNA. First-generation sequencing methods, such as Sanger sequencing, are laborious and time-consuming, involving separation of DNA fragments by size using gel electrophoresis. Second-generation sequencing, including Illumina, uses a sequencing-by-synthesis approach that involves fragmenting DNA, attaching adapters, amplifying, and sequencing in parallel. Third-generation sequencing technologies, such as Pacific Biosciences and Oxford Nanopore, reads long DNA stretches in real-time via single-molecule sequencing without polymerase chain reaction (PCR) or cloning.

NGS methods have advantages over first-generation sequencing, including high throughput, accuracy, and lower cost. These advancements have led to faster and more cost-effective sequencing of entire genomes and transcriptomes, resulting in the discovery of new genetic variants and a better understanding of the genetic basis of disease. In clinical settings, targeted gene panels, whole-exome sequencing, and whole-genome sequencing are used [10]. Whole-exome sequencing uses short DNA fragments while whole-genome sequencing uses long reads. Combining both methods overcomes their limitations, with short-read sequencing used to correct errors introduced by long-read sequencing during de novo genome or transcriptome assembly [11].

2.1 Illumina

Illumina sequencing involves creating a library of DNA fragments. All fragments are flanked by either of two specific oligonucleotide adapters. These adapters noncovalently anchor the DNA fragments to a glass flow cell. For one fragment the process is depicted in (Figure 1).

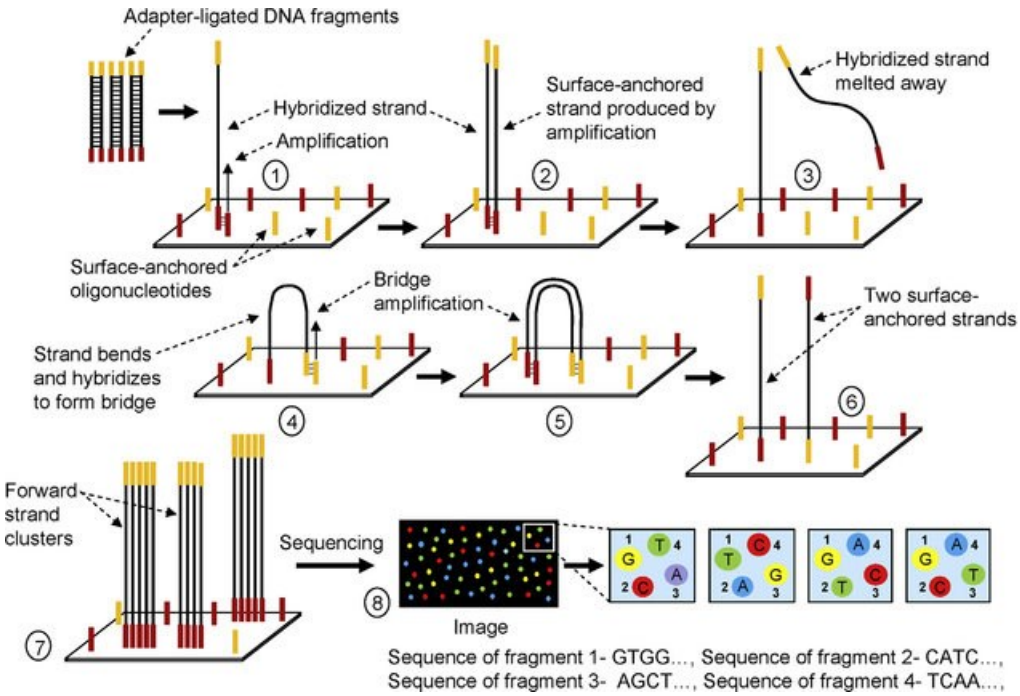

Figure 1. Mechanism of Illumina sequencing. (1) Fragmented DNA into short sequences with adapters is hybridized onto surface-anchored oligonucleotides. (2) The single-stranded fragment is initially amplified by PCR to create identical DNA fragments. (3) The double-stranded PCR product is denatured, resulting in single-stranded DNA fragment. (4) The single-stranded DNA fragment is allowed to hybridize to complementary oligonucleotides on a solid surface, forming a bridge between the surface and the DNA fragment. (5) The bridge is amplified through PCR, resulting in multiple copies of the DNA fragment. (6) The double-stranded DNA is denatured, and each strand is anchored to the surface at a different location, resulting in two surface-anchored strands. (7) The forward strand clusters are sequenced using fluorescent reversible terminator chemistry. (8) The fluorescent signals generated during sequencing are recorded and analyzed to determine the nucleotide sequence of the DNA fragment. Adopted from[12].

Single-stranded DNA fragment is attached to the first type of oligonucleotides on a glass flow cell, and a complement of the hybridized fragment is synthesized by DNA polymerase. Clonal amplification of strands is given by repeated hybridization of the unattached ends to the second type of oligonucleotide on a flow cell, complementary strand synthesis and denaturation of the two strands. This, so called bridge cloning mechanims, results in the formation of highly localized clusters of identical DNA fragments. The clusters are sequenced using fluorescent dyes, with each nucleotide added during DNA synthesis modified with a cleavable dye of different color and cleavable next nucleotide blocker. When a specific nucleotide of the complementary strand of templates within the cluster is synthesized, a fluorescent signal with a specific wavelength of the last added nucleotide is recorded, and subsequently the dye and the blocker are chemically cleaved. The repetition number of this process, with each cycle adding a new nucleotide to the growing DNA strand, equals the read length. Sequence of letters read out from one fragment is given by the sequence of the fluorescent colors read out in consecutive steps from the particular cluster. Finally, the resulting sequences from all clusters are analyzed using specialized software that aligns the sequence reads to a reference genome, identifies genetic variations and mutations, and determines the sequence of the original DNA before fragmentation.

2.2 Single molecule real-time sequencing by Pacific Biosciences

Single-molecule real-time (SMRT) sequencing, developed by Pacific Biosciences [13], begins with library construction, which involves ligation of hairpin adaptors to double-stranded DNA, generating a single-stranded circular DNA template. This template is then loaded into a SMRT Cell chip containing zero-mode waveguides (ZMWs), which are nanophotonic structures that enable the detection of fluorescent signals from individual nucleotide (Figure 2).

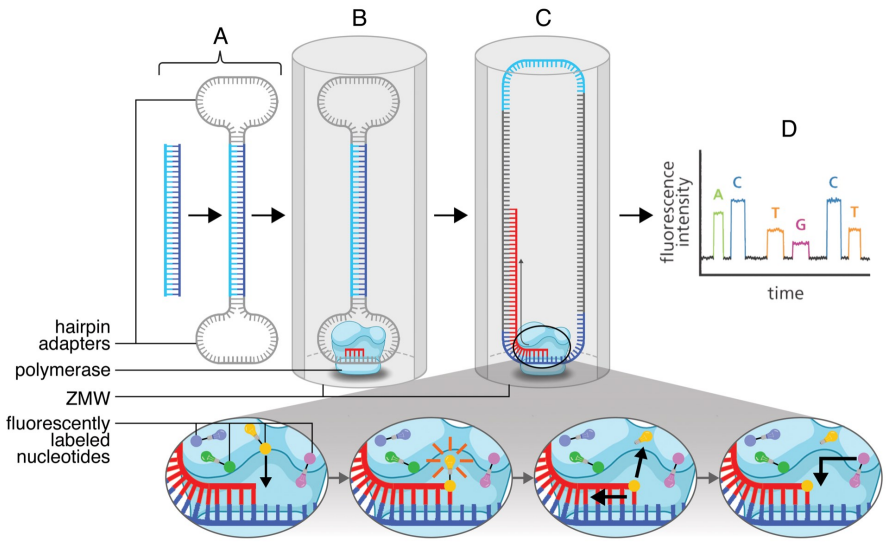

Figure 2. Mechanism of single molecule real-time sequencing by Pacific Biosciences. (A) Double-stranded DNA is prepared as a library with hairpin adapters. (B) The library is loaded onto a SMRT Cell with nanoscale observation chambers. (C) Fluorescently labeled nucleotides are incorporated by polymerase in the vicinity of ZMWs. (D) The emitted fluorescence is recorded in real time. Created in Adobe Photoshop with help of Biorender.com.

During SMRT sequencing, DNA polymerase molecules selectively bind to the bottom of ZMW in the detection region. As nucleotides are incorporated into the growing DNA strand, the formation of phosphodiester bonds by DNA polymerase results in the release of fluorophores from the incorporated nucleotides. The emitted light signals from replications across all ZMWs are interpreted in real-time to generate a continuous long-read nucleotide sequence.

SMRT sequencing has a unique advantage in detecting epigenetic modifications in DNA. The interpulse duration, which is the time between the incorporation of nucleotides by DNA polymerase, can be used to identify these modifications. For example, methylation of cytosine can cause a delay in the incorporation of the next nucleotide, which increases the interpulse duration. By analyzing these values, it is possible to estimate epigenetic modifications and their locations within the genome [14].

2.3 Nanopore sequencing by Oxford Nanopore Technologies

Nanopore sequencing uses a biological membrane separating two ionic solutions to identify different bases passing through a designed membrane protein (Figure 3), allowing the detection of both single-stranded RNA and DNA [15].

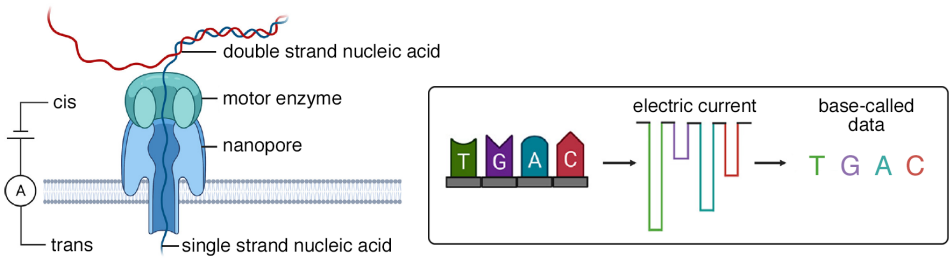

Figure 3. Mechanism of nanopore sequencing. Two chambers (cis and trans) filled with ionic solutions are separated using a membrane containing a nanopore. A nucleic acid is electrophoretically driven through the pore in a controlled manner due to the presence of a motor protein. Only one strand of nucleic acid passes through the pore being unwound upon translocation. Current shifts are recorded in real time as single nucleotides of DNA or RNA chain translocates through a pore. Adopted from [16].

The sequencing process uses a flow cell containing a nanopore that only allows single-stranded nucleic acids to pass through the membrane (Figure 3). The hairpin adapter is applied between the forward and reverse strands to enable 2D sequencing, which allows for sequential sequencing of both strands of double-stranded DNA. A processive helicase motor enzyme is used to control the movement of a single strand through the pore in the 5'-to-3' direction [17]. During sequencing, electric amplifiers record any changes in the ion current across the membrane, which are then analyzed by a base-calling algorithm to identify the nucleotide sequences [18].

2.4 Translational potential and relevance to clinic

The decreasing cost of NGS (Figure 4) is making genomics more accessible for clinical applications[19]. NGS technologies, such as Illumina, SMRT, and nanopore sequencing, are promising due to their low cost and ability to generate large amounts of genetic data quickly.

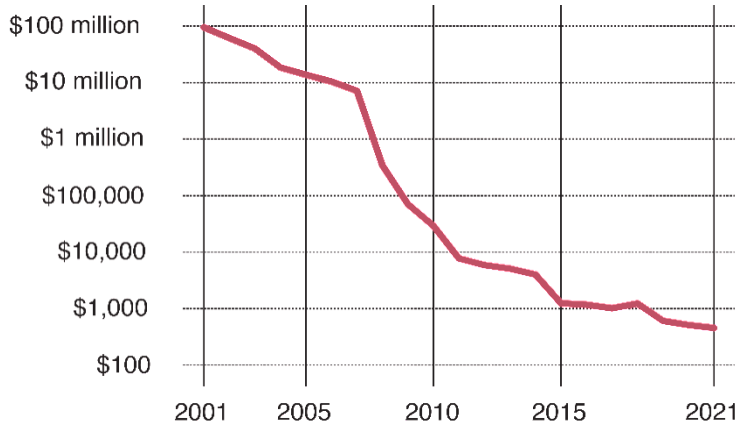

Figure 4. Whole genome sequencing cost per human genome over time. Adopted from[19].

In the context of neuronal diseases, NGS can be used to sequence the genome or transcriptome of patients to identify the genetic mutations that may be responsible for the disease. This enables the development of personalized treatments that target specific genetic mutations and the identification of genetic markers that predict disease progression, helping to monitor and adjust treatment as necessary [20]. Additionally, NGS can be useful for identifying potential drug targets involved in the disease, leading to the development of new drugs or repurposing existing ones [21]. However, standardization of protocols for data analysis and interpretation remains a challenge that needs to be addressed.

3. CRISPR-Cas9

The clustered regularly interspaced short palindromatic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) system is a naturally occurring adaptive immune response in prokaryotes that allows bacteria and archaea to defend against bacteriophages intercepted as foreign DNA [22]. When a cell is invaded by foreign DNA, a small portion of the DNA called the protospacer is incorporated into an array of CRISPR that is located on prokaryotic chromosome. This protospacer becomes a spacer when a palindromic repeat is added to the primary transcript, thereby separating the spacers (Figure 5) [23]. The CRISPR array is transcribed into a precursor CRISPR RNA (pre-crRNA), which is then attached to the non-coding trans-activating CRISPR RNA (tracrRNA) present in the cell through the stereotype repeat regions [24]. After RNase type III processing, crRNA and tracrRNA form a complex known as a guide RNA (gRNA). The gRNA includes a spacer RNA that is linked by hydrogen bonds to tracrRNA and plays a crucial role in defining the target sequence to be modified by the connected Cas9 protein. During the interference stage of the CRISPR-Cas system, the gRNA directs the Cas9 endonuclease to cleave foreign DNA, but only in the presence of a specific shot sequence called the protospacer adjacent motif (PAM).

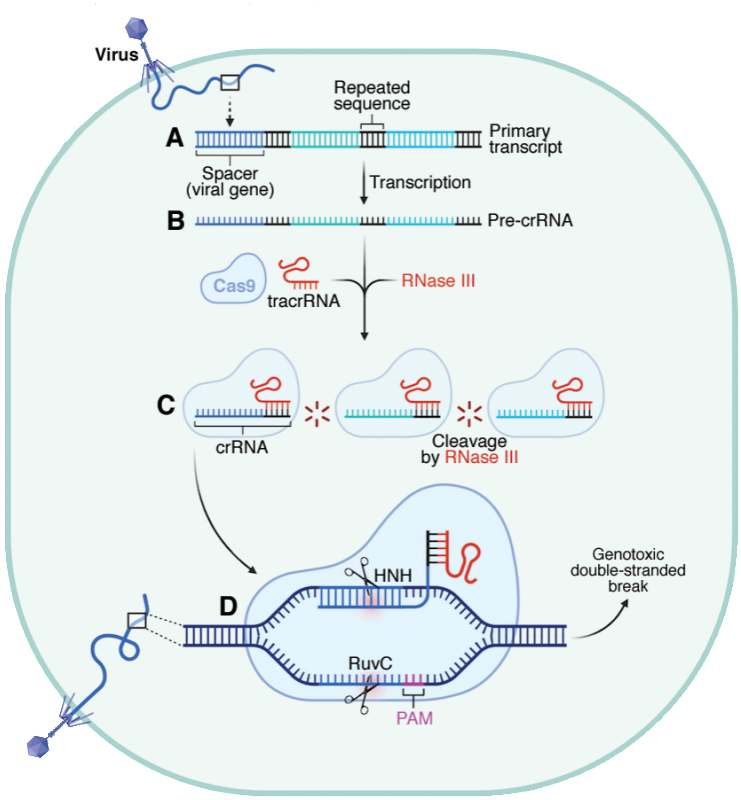

Figure 5. CRISPR-Cas system as adaptive immune response in prokaryotes. (A) Viral DNA is incorporated into the prokaryotic genome in the form of spacers separated by repeats. (B) Transcription occurs to form pre-crRNA that contains both the repeats and spacers. (C) tracrRNA forms a duplex with crRNA and targets Cas9 to crRNA. The complex is then cleaved by RNase III. (D) In the case of reinfection by the same virus, the virus genome with a PAM is recognized by the cr:tracrRNA complex with Cas9. This complex then cleaves the viral DNA by two Cas9 nuclease domains (RuvC and HNH) causing double stranded break, preventing the virus from replicating and causing an infection. Adopted from [25].

The required PAM sequence varies with the type of Cas protein used. To ensure effective CRISPR-Cas9 editing, the target DNA sequence must match the PAM sequence of the Cas protein utilized. For the widely used Cas9 from Streptococcus pyogenes (SpCas9), the PAM sequence is 5‘-NGG-3‘, where N represents any nucleotide [26]. However, a modified version of SpCas9 called SpRY nuclease has been engineered to recognize nearly all DNA sequences and thus minimize the requirement for specific PAM sequences [27].

The PAM sequence is located immediately downstream of the target DNA sequence and is not complementary to the gRNA (Figure 5). When the Cas9 endonuclease recognizes the PAM sequence, it triggers the formation of a complex between the gRNA and Cas9, resulting in the cleavage of the target DNA sequence. This precise mechanism enables DNA cutting and editing using the CRISPR-Cas system [28].

The Cas9 protein consists of two nuclease domains that cleave one strand of double-stranded DNA upstream of PAM and induce a blunt-ended double-stranded break (Figure 5). The RuvC domain (named after the RuvC protein, which is a DNA repair endonuclease in E. coli) splits non-complementary DNA strands, whereas the HNH nuclease domain (named after the HNH motif, which is a conserved sequence of amino acids present in the domain) cuts the complementary DNA strand.

The prokaryotic immune defense system has been adapted as a gene-editing tool in eukaryotic cells using a 20-nt guide sequence fused to chimeric single-guide RNA (sgRNA) (Figure 6**Chyba! Nenašiel sa žiaden zdroj odkazov.**). This enables the specific and precise editing of genes in eukaryotic cells, which was previously difficult to achieve.

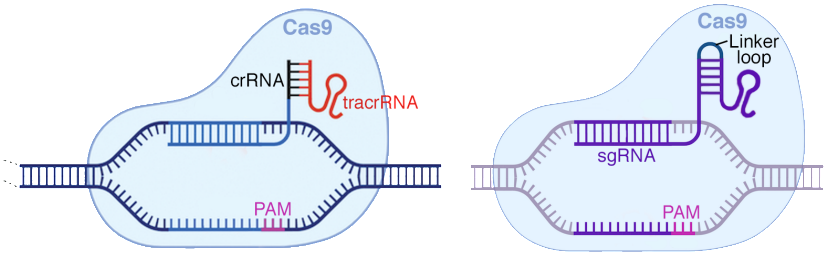

Figure 6. Comparison of Cas9 protein structures programmed by different RNA constructs. The left-side image shows the structure of the Cas9 protein that recognizes the PAM, which is programmed by a duplex of crRNA and tracrRNA. On the right side, there is a different structure of the Cas9 protein also recognizing PAM, but in this case, it is programmed by a sgRNA that is formed by a duplex of crRNA and tracrRNA linked by a loop. Adapted from [25].

3.1 CRISPR-Cas9 applications

Using the DNA targetting, CRISPR-Cas9 can induce specific changes in DNA sequences. This approach can be used to knockout genes that cause or contribute to neurological diseases using non-homologous end joining (NHEJ) or to knock-in or replace genes that can treat or cure these diseases using homology-directed repair (HDR). Single-stranded breaks in DNA offer the opportunity for single-base or prime editing, which enables precise correction of point mutations associated with neurological diseases. Another potential application of the CRISPR-Cas9 system is to control gene expression by epigenome editing.

One important consideration when using the CRISPR-Cas9 system for gene therapy is the potential for off-target effects, which can result in unintended changes to DNA sequences. However, there are several methods being developed to increase the specificity and reduce off-target effects of CRISPR-Cas9.

3.1.1 Gene silencing

In gene therapy, the CRISPR-Cas9 system has the potential to target and eliminate undesirable genes by inducing mutations in the DNA sequence.

One method involves using of two targeted sgRNAs along with a double Cas9 nickase, which has only one of the two nuclease domains (Figure 7). The double Cas9 nickase is capable of creating nicks on both strands of the double-stranded DNA, using only one of its nuclease domains. This process produces single-stranded overhangs that are removed, allowing the double-stranded DNA to be ligated without the undesirable genes [29]. By utilizing two sgRNAs, this technique enhances specificity and minimizes off-target effects by ensuring that Cas9 only binds to the intended target site, decreasing the likelihood of binding to off-target sites.

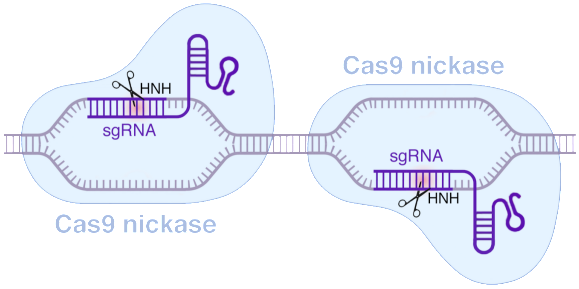

Figure 7. Restriction sites of double Cas9 nickase. Using a sgRNA, two Cas9 nickases can direct their restriction sites to opposite strands of a DNA target site. The HNH domain of each nickase creates a nick on each strand of the DNA, resulting in a double-stranded break at the target location. Adapted from [25].

Another approach involves using Cas9 to create a blunt double-stranded break in the DNA sequence, which then undergoes repair through the error-prone mechanism of NHEJ (Figure 8). During this process, random conversions can result in frameshift mutations or stop codons, ultimately leading to the knockout of the targeted gene [30].

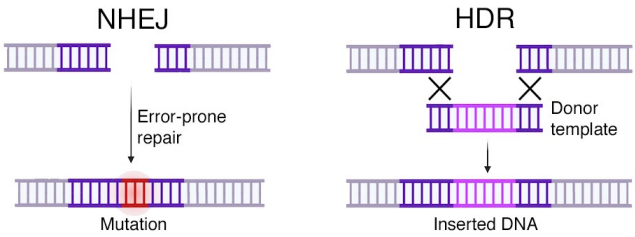

Figure 8. The role of double-strand break repair in promoting gene editing. (A) The NHEJ pathway, which is prone to errors, results in small deletions or insertions. (B) The HDR pathway, allows for the introduction of a repair template, which facilitates assisted recombination and can lead to gene correction or insertion. Adopted from [25].

3.1.2 Gene replacement

Gene replacement involves the use of high-fidelity HDR to knock down the desired sequence (Figure 8). During HDR, a donor template containing the desired mutation is introduced and is surrounded by homology arms that match the target site. The Cas enzyme then creates a double-stranded break at the target site, which is repaired by the cell using the donor template as a repair template. This results in the desired sequence being inserted at the target site with a high accuracy [30].

3.1.3 Base editing

Base editing enables the precise modification of single DNA bases, offering a potential cure for a wide range of point-mutation genetic diseases [31]. One strategy for base editing involves the use of cytosine-based editors (CBEs) composed of a cytidine deaminase enzyme fused with Cas9 nickase. CBEs can convert cytidine to uridine or guanine to adenine depending on the target site. Another type of base editor is adenine-based editor (ABEs), formed by fusing adenine deaminase enzyme with Cas9 nickase. ABEs can convert A-T base pairs to G-C base pairs [32].

3.1.4 Prime editing

The prime editing method involves the use of RNA-programmable Cas9 nickase to induce single-stranded breaks, which are then preserved while adding reverse transcriptase and prime editing guide RNA (pegRNA) (Figure 9). The pegRNA contains an sgRNA chain associated with a primer-binding site that is linked to the reverse transcriptase template containing the desired edits [33]. The DNA mutation is copied at the nicking point from an edit-encoding extension of the pegRNA using reverse transcriptase activity. The resulting 5‘ flap, which contains unedited DNA, is washed away, and the DNA repair process ensures the permanent insertion of the genetic information into the double-stranded DNA sequence, thereby creating precise editing.

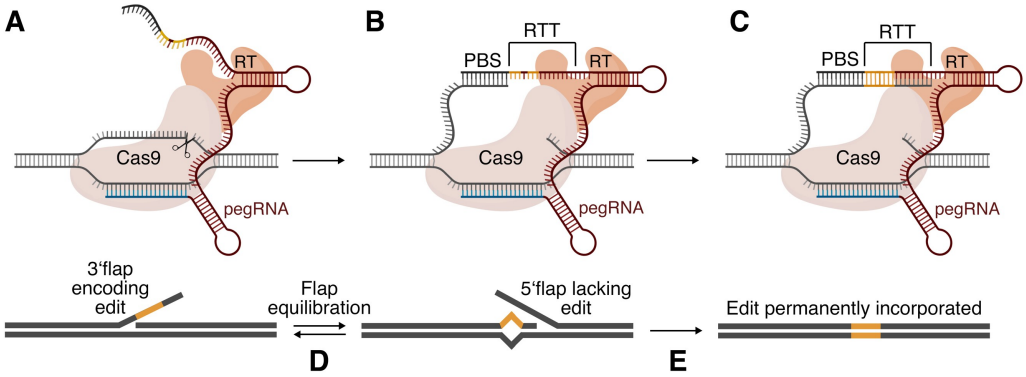

Figure 9. The preservation of RNA-programmable Cas9 nickase-induced single-stranded breaks with the addition of reverse transcriptase (RT) and pegRNA. (A) PegRNA binds to the target DNA sequence, and Cas9 nickase makes a nick. RT is also recruited to the nick site. (B) The pegRNA's primer binding site (PBS) hybridizes with the genomic DNA at the nick site, and RT template (RTT) on pegRNA includes the edit sequence. (C) The RT copies the edit sequence into a 3' flap on the genomic DNA. (D) The 3' flap and 5' end of genomic DNA undergo an equilibration process to form a flap structure. (E) The 5’ flap is cleaved and the nick site is ligated, repairing the genomic DNA with the edited sequence. Adapted from [34].

3.1.5 Epigenome regulation

Epigenetic markers control gene expression, and their manipulation can be used to regulate gene function. Nuclease-deactivated Cas9 (dCas9) can be fused with epigenetic modifiers to manipulate methylation and acetylation [35], enabling targeted changes to DNA [36] or histone complexes [37]. These modifications can either silence or activate genes.

3.1.6 Transcription regulation

Gene expression can also be effectively silenced or enhanced by attaching the dCas9 protein to a transcriptional repressor [38] or activator domain [39](Figure 10).

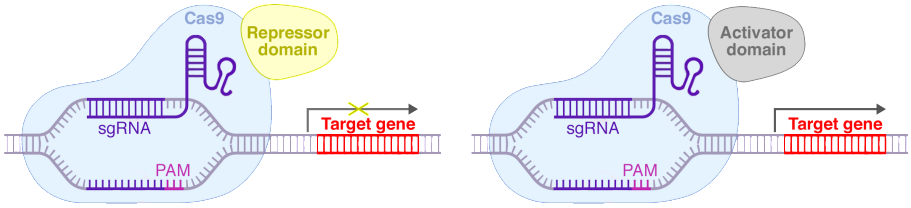

Figure 10. Regulating gene expression with Cas9 programmed by a sgRNA and recognizing a PAM. The left image shows the Cas9 protein fused with a repressor domain that binds to the target gene and prevents its transcription, while the right image shows the Cas9 protein fused with an activator domain that activates the transcription of the target gene. Created with Biorender.com in Adobe Photoshop.

3.2 Off-target effects

Off-target effects can be caused by factors such as incomplete base-pairing between the gRNA and the DNA target, as well as the presence of similar sequences elsewhere in the genome. They can result in unintended changes to genes and other genomic regions, which can have unpredictable and potentially negative consequences, such as disrupting normal gene function or even causing diseases like cancer, taking into account many oncogenes and tumor suppressor genes [40]. Off-target effects are a significant challenge for CRISPR-Cas9 technology in clinical applications, but there are various strategies to minimize them.

The verification of in vivo off-targets (VIVO) strategy can be used to identify off-target effects of the CRISPR-Cas9 system [41]. In this method, the genome of cells treated with the CRISPR-Cas9 system is sequenced in vivo and compared to the patient's reference genome. Any unintended alterations that may have arisen can be detected, providing insight into the off-target effects of the system.

Another technique used to identify off-target cleavage sites is circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq) [42]. This method involves using synthetic oligonucleotides to circularize genomic DNA fragments that have been cleaved by the CRISPR-Cas9 system in vitro. The circularized DNA fragments are then sequenced, and any off-target cleavage sites can be identified by comparing the resulting sequences to the reference genome.

Limiting the delivery of CRISPR-Cas9 components solely to the intended cells is an alternative approach to minimize unintended modifications. This can be accomplished using cell-specific promoters that restrict the expression of the Cas9 enzyme to the target cells (as discussed in 6).

To further minimize off-target effects, a process called CRISPR gRNA-assisted reduction of damage (CRISPR GUARD) can be used [43]. CRISPR GUARD involves using a sgRNA to recruit a dCas9 protein to the target site. The dCas9 protein lacks the nuclease activity necessary for DNA cleavage, but it can still bind to DNA and block other nucleases, including Cas9. This technique allows for precise genome editing by limiting the activity of Cas9 to the target site, reducing the likelihood of unintended alterations.

3.3 Translational potential and relevance to clinic

The CRISPR-Cas9 system has shown great promise for clinical applications in gene therapy, but the risk of off-target effects has been a significant obstacle to its widespread use. Recent progress in reducing off-target effects has significantly improved the potential of CRISPR-Cas9 as a therapeutic tool.

One of the approaches to minimize off-target effects involves inducing single-stranded breaks at the site of a double-stranded break. This can be achieved through various techniques, such as using double Cas9 nickase to gene silencing [29] and employing Cas9 nickase in base editors [44] or prime editing.

New techniques, including CRISPR GUARD, VIVO strategy and CIRCLE-seq, have further improved the specificity and accuracy of CRISPR-Cas9. CRISPR GUARD uses a modified sgRNA to recruit dCas9 to the target site and block the activity of Cas9, thereby reducing the chance of off-target effects [43]. The VIVO strategy entails sequencing the genome of cells treated with the CRISPR-Cas9 system in vivo and comparing it to the patient's reference genome to identify unintended changes, providing insight into the off-target effects of the system [41]. CIRCLE-seq allows for the detection of off-target effects by sequencing circularized DNA fragments [42].

Moreover, improvements in delivery methods, such as the use of AAV with short human promoters (as seen in 6), have also significantly increased the translational potential of CRISPR-Cas9.

4. Optogenetics and chemogenetics

Advances in molecular biology have opened new ways to manipulate the activity of cells, particularly excitable cells, including neurons. Two such techniques are optogenetics and chemogenetics, which can directly stimulate or inhibit cells or modulate their activity by regulating their signaling pathways, transcription, or translation. The potential applications of these techniques extend beyond excitable cells and could have significant implications for the treatment of neurological diseases in humans as a component of gene therapy.

4.1 Optogenetic tools

Optogenetic tools, such as opsins with light-sensitive ion channels or pumps, have greatly impacted the field of neuroscience, enabling precise control over the activity of specific neurons and neural circuits. These tools are capable of regulating the flow of ions across the cell membrane in response to light, allowing for direct stimulation or inhibition of neuronal activity. Optogenetic metabotropic glutamate receptor 6 is another type of optogenetic tool that can activate downstream signaling pathways and alter neuronal activity. To improve the specificity and control of these tools, they can be combined with light-sensitive domains like phytochrome B, cryptochrome 2, and light-oxygen-voltage. With the ability to target specific areas of the brain and cells, these optogenetic tools hold immense potential for treating neurological disorders with high accuracy.

4.1.1 Opsins with coupled light-sensitive ion channels or light-driven pumps

Opsins are a type of naturally occurring light-sensitive protein that have undergone extensive optimization for various characteristics, including conductivity, speed, excitation spectrum shift, light sensitivity, and trafficking.

Channelrhodopsins (ChRs) [45] are proteins that enable the flow of positively charged ions such as sodium or potassium across the cell membrane when exposed to blue or green light [46,47]. Influx of kations depolarizes neurons, triggering an action potential and leading to increased neural activity. ChR2_H134R is the most commonly used variant and has significantly increased conductivity compared to the wild-type variant [48]. Chronos, the fastest variant, has a response time in the order of single milliseconds, allowing for precise and fast control of neuronal activity [49]. Chrimson and ChRmine are prominent constructs with a red-shifted excitation spectrum, allowing for deeper penetration into brain tissue [50]. Using red-shifted and highly sensitive ChRmine, experiments using excitation of structures as deep as 7mm in intact brain have been shown feasible. Guillardia theta anion channelrhodopsin (GtACR) 1 and 2 are long-seeked naturally occurring light-driven chloride channels that are highly sensitive and conductive. Thus, they can be used as strong inhibitory actuator that is driven by green, or blue light, respectively [51]. Chloride channels such as stGtACR2 have been substantially optimized, showing the importance of optimized membrane protein trafficking [52].

Original ion pumps such as halorhodopsin and archaerhodopsin, which respond to green or yellow light, respectively [53], have also been optimized. ArchT3.0 and NpHR3.0 are examples of these pumps that can induce hyperpolarization in a cell, making it less likely to fire an action potential and consequently inhibit neurons [54].

4.1.2 Optogenetic metabotropic glutamate receptor 6

The natural mechanism of metabotropic glutamate receptor 6 (mGluR6) involves its activation by glutamate, a neurotransmitter released by photoreceptor cells in the retina in response to light stimuli. This activation triggers a signaling cascade that ultimately leads to the hyperpolarization of the photoreceptor cell and the inhibition of neurotransmitter release [55].

Optogenetic metabotropic glutamate receptor 6 (Opto-mGluR6) is a modified form of mGluR6 that is engineered to be light-sensitive [56]. It is composed of the extracellular domain of mGluR6, which is a G protein-coupled receptor (GPCR), and a light-sensing domain such as LOV or PhyB that is attached to its intracellular domain. When exposed to light, the attached light-sensing domain undergoes a conformational change that activates the intracellular domain of Opto-mGluR6. The activated G protein signaling pathway leads to the inhibition of the enzyme adenylate cyclase and a decrease in the intracellular concentration of cyclic adenosine monophosphate (cAMP). This decrease in cAMP concentration ultimately hyperpolarizes the neuron's membrane potential, resulting in the inhibition of neuronal activity in a reversible and controlled manner.

4.1.3 Light-sensitive photoreceptors

Phytochrome B (PhyB) is a light-sensitive protein that can be activated by red or near-infrared light through its N-terminal photosensory domain containing the phycocyanobilin (PCB) chromophore [57]. This activation induces reversible structural changes in the chromoprotein, leading to the binding of a protein-interacting factor (PIF) (Figure 11). This mechanism involves the dissociation of the PhyB-PIF complex under far-red light [58]. To control gene expression, PhyB is fused with a DNA-binding domain (DBD), while the transcription factor PIF is tagged with a (trans)activation domain (AD). These associated domains allow for the activation of gene expression when the PhyB-PCB complex is bound to PIF in the presence of red light.

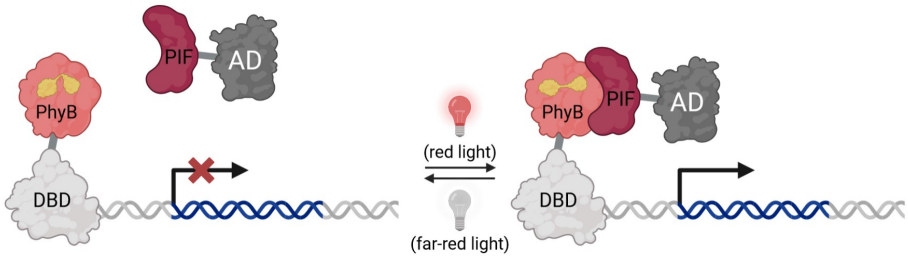

Figure 11. The process of stimulating gene expression through the co-localization of the AD and DBD. PhyB and PIF are dimerized through the activation of red light. PCB coupled with PhyB undergoes structural changes upon exposure to light, leading to the formation of a complex between PhyB and PIF. This complex activates gene expression through transcription activation. On the other hand, far red light dissociate the PhyB-PIF complex, leading to transcription repression. Created with BioRender.com.

Cryptochrome-based systems exhibit blue-light responsiveness through two distinct mechanisms.

In the first mechanism (Figure 12), cryptochrome circadian regulator 2 (Cry2) is attached to an AD, while cryptochrome-interacting basic-helix-loop-helix protein 1 (CIB1) is attached to a DBD [59]. Upon exposure to blue light, the flavin adenine dinucleotide (FAD) associated with Cry2 undergoes reduction, leading to

dimerization of Cry2 with CIB1. This in turn causes the transcription factor to activate gene expression, resulting in downstream effects.

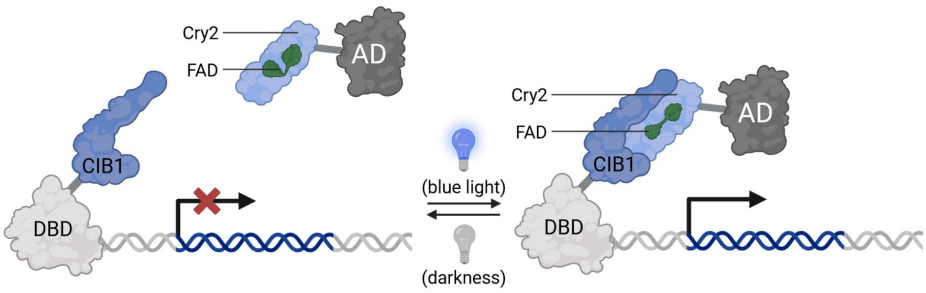

Figure 12. Gene expression activated through blue light-activated dimerization of Cry2 and CIB1. This requires bringing the AD and DBD close together. Upon exposure to blue light, FAD coupled with Cry2 is reduced and a complex between Cry2 and CIB1 is formed, resulting in transcription activation. In the dark, the Cry2-CIB1 complex is dissociated, leading to transcription repression. Created with BioRender.com.

In the second mechanism (Figure 13**Chyba! Nenašiel sa žiaden zdroj odkazov.**), Cry2 and CIB1 co-localize to reconstitute the active protein. Upon exposure to blue light, Cry2 undergoes reduction via FAD, leading to dimerization with CIB1. This dimerization event results in the reconstitution of the active protein, which is then able to activate gene expression and produce downstream effects.

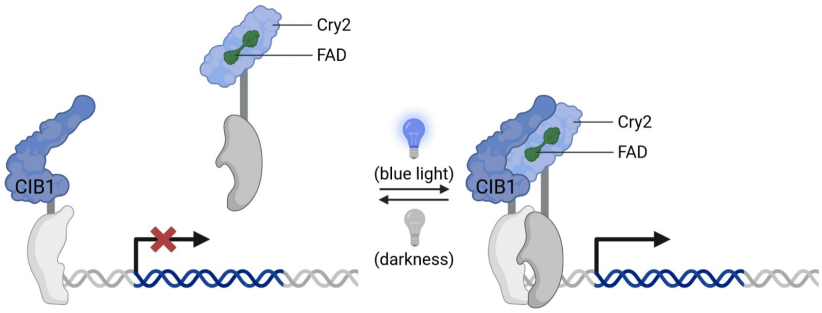

Figure 13. Dimerization of Cry2 and CIB1 via blue light-induced FAD reduction leads to the formation of an active protein that induces transcription. Transcription is repressed in the dark. Created with BioRender.com.

In addition, there is the possibility of light-inducible targeted gene manipulation using the CRISPR-Cas9-based transcription system (Figure 14) [60,61]. This system consists of two fusion proteins and sgRNA. The first fusion protein is the genomic anchor probe containing dCas9 and CIB1. The second fusion protein is the activator probe, which includes the CRY2 and the transcriptional activator domain. Upon blue light irradiation, CRY2 and CIB1 heterodimerize, and the transcriptional activator domain is recruited to the target locus to activate gene expression [60].

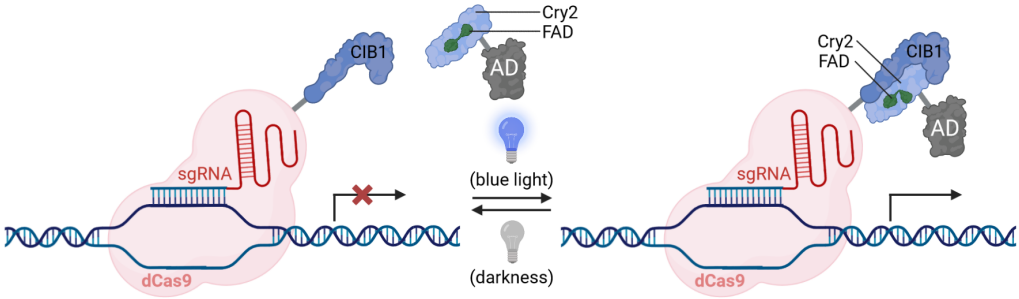

Figure 14. Mechanism of CRISPR-Cas9-based photoactivatable transcription system. Cry2 linked with a transcription activator, binds after blue light irradiation CIB1, leading to dimerization and bringing the activator in close proximity to DNA to initiate transcription. dCas9, guided by sgRNA, allows for sequence-specific targeting. Created with BioRender.com.

The Light-oxygen-voltage (LOV) domain is a type of photoreceptor that senses blue light and contains flavin mononucleotide (FMN) or FAD chromophores. Photon absorption results in a reversible covalent bond between the cysteine of the LOV domain core and the flavin cofactor [62]. The LOV domain is found in various proteins, such as AsLOV2 from Avena sativa, EL222 from Erythrobacter litoralis, flavin-binding kelch domain F box 1 (FKF1) protein, and Vivid (VVD).

The LOV domain regulates gene expression through dimerization/dissociation with other proteins. An example of this is how AsLOV2 and EL222 undergo a conformational change upon light stimulation, which causes the release of an alpha helix. This results in the repositioning of their DBD and AD, allowing for the initiation of gene transcription (Figure 15) [63,64]. To enhance the system's accuracy by importing it directly into the nucleus, a nuclear localization signal (NLS) can be inserted into the alpha helix of AsLOV2 or the N-terminus of EL222 [65]. By forming various complexes with different proteins, the LOV domain can either initiate or inhibit gene transcription, depending on the specific application.

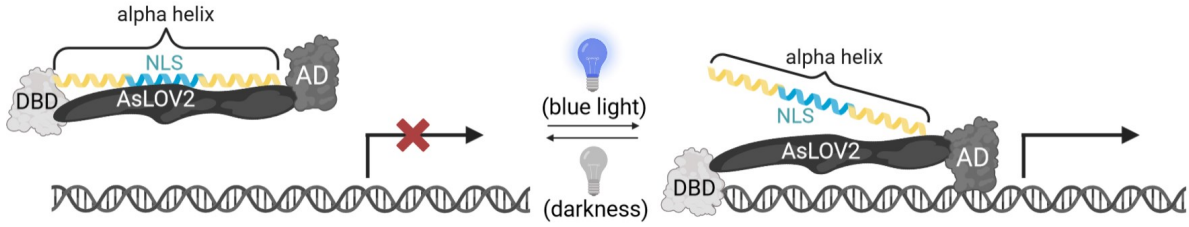

Figure 15. The reaction of the alpha helix in AsLOV2 triggered by blue light induction, which results in the uncaging of the α helix and the exposure of the previously hidden NLS. This allows for the nuclear localization of the AsLOV2 protein, and the repositioning of the DBD and AD to initiate gene transcription. Created with BioRender.com.

Similarly, light-sensitive proteins such as FKF1 and VVD undergo a conformational change when exposed to blue light. FKF1 is connected to AD and forms a heterodimer with the GIGANTEA protein, which is linked to the DBD [66]. This interaction results in transcription initiation of the gene. On the other hand, VVD interacts with other proteins to form a complex that has the ability to impede gene transcription [67]. As soon as these

transcription factors dimerize with their partner proteins, they are moved closer to the promoter region of the gene, which results in gene activation for FKF1 or gene transcription inhibition for VVD.

4.2 Chemogenetic tools

Chemogenetic tools are a type of molecular tool that utilizes receptors associated with channels or G proteins. These receptors can be activated by a specific chemical or a designed ligand, which can be endogenous compounds or synthetic molecules. When activated by the ligand, these receptors can modulate the activity of the associated ion channels or G proteins, leading to a variety of downstream effects such as neuronal depolarization or inhibition, and ultimately allowing the control of specific cellular pathways.

4.2.1 Designer receptors exclusively activated by designer drugs

Designer receptors exclusively activated by designer drugs (DREADDs) are engineered GPCRs that are capable of regulating neuronal excitation and inhibition.

Different types of DREADDs have been introduced, including a modified Gq-coupled human M3 muscarinic receptor [68] for the activation of neuronal cells and a modified Gi-coupled human M4 muscarinic receptor [69] for neuronal inhibition. Both types can be activated by clozapine-N-oxide (CNO), which can be easily administered orally. However, CNO is converted to bioactive clozapine, which can cause undesirable behavioral effects [70]. To overcome this issue, a new Gi-coupled chemoreceptor called kappa-opioid receptor DREADD (KORD) has been developed [71]. KORD can be activated by a pharmacologically inert ligand salvinorin B, which causes neuronal silencing through the same mechanism like hM4Di [71]. Introduction of a second chemogenetic system with a chemically different ligand also allows for bidirectional chemogenetic control, which means that the same neuron can be both activated and inhibited.

4.2.2 Chimeric ligand-gated ion channels

Chimeric ligand-gated ion channels are a type of chemogenetic tool that can be activated by synthetic pharmacologically selective effector molecules (PSEMs) [72]. These tools consist of pharmacologically selective actuator modules (PSAMs) and ion pore domains (IPDs) derived from different ion channels. The modified ligand-binding domains in PSAMs selectively bind PSEMs, allowing for the control of specific cellular pathways. PSAMs derived from modified nicotinic acetylcholine receptors (nAChRs) can cause either neuronal depolarization or hyperpolarization depending on their associated IPDs, which are derived from the serotonin type 3 receptor (5-HT3) and glycine receptor (GlyR), respectively (Figure 16) [73].

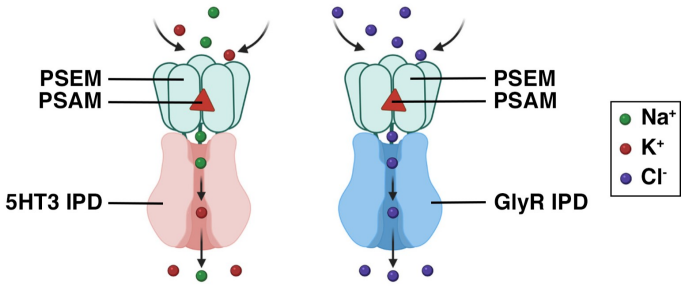

Figure 16. Diagram of modular chimeric channels that can activate or inhibit cells. The channel consists of three components: PSAM, IPD, and PSEM. When the PSAM is connected to 5-HT3 IPD, it opens the pore, allowing positively charged ions to flow into the cell, leading to depolarization. Conversely, when PSAM is connected to GlyR IPD, it opens a pore that allows negatively charged ions to flow into the cell, leading to hyperpolarization. Adopted from [73].

The designed PSEM for nAchR-derived PSAM is called varenicline, a drug prescribed in human medicine to support smoking cessation [74]. Therefore, its safety profile is well known. Interestingly, the ligand is not only highly selective but also has a high affinity for its target, which allows for effective control of neural activity.

4.3 Translational potential and relevance to clinic

The major concern when using opto/chemogenetic tools is their potential for immunogenicity. Photoreceptors like PhyB, Cry2, and LOV domains have low immunogenicity as they are completely intracellular. However, opsins like channelrhodopsins, halorhodopsins, and archaerhodopsins can lead to immune responses, due to their membrane-tethered activation. Opto-mGluR6, a modified version of a protein found naturally in the human body, reduces the likelihood of triggering an immune response in humans, making it a promising tool for human gene therapy.

DREADDs are derived from naturally occurring human GPCRs, which lowers the likelihood of an immune response. However, when the ligand CNO is administered, it gets converted to bioactive clozapine. This is a concern because clozapine can have off-target effects on other receptors in the body. Chimeric ligand-gated ion channels, such as the nAChRs, are a type of chemogenetic tool that are derived from human proteins and have low immunogenicity. Varenicline is a drug that activates nAChRs, and it binds very tightly to its target with a dissociation constant on the order of nanomoles [75]. This tight binding allows even low concentrations of varenicline to have a significant effect on neural activity, which is beneficial for minimizing potential side effects and maximizing therapeutic efficacy.

In addition to the issue of immunogenicity, optogenetics faces the challenge of delivering light to specific neurons, particularly in certain areas of the brain, despite its ability to control the activity of genetically modified neurons with high temporal resolution in miliseconds using light. To address this challenge, implantable devices like subdermal magnetic loop antennas linked with light-emitting diodes [76] and upconversion nanoparticles [77] have been developed. These devices can be used to modulate specific neuronal populations and convert near-infrared light to various wavelengths to overcome the limited tissue penetration of blue and green light.

On the other hand, chemogenetics uses small molecules that can approach their specific receptors via humoral way. Although more convenient and non-invasive, the temporal resolution of chemogenetics is in the order of minutes in the best case, being determined by factors such as dose, route of administration, and metabolism [75].

5. Adeno-associated virus vector for gene therapy

Adeno-associated virus (AAV) is a member of the Parvoviridae family that is non-enveloped, has approximately 4.7 kbp single-stranded DNA genome enclosed in a ~25 nm icosahedral capsid [78]. The wild-type AAV (wtAAV) genome includes two genes. One for DNA replication proteins (*rep*) and the second one for a structure and membrane proteins (*cap*). The coding regions of wtAAV DNA are flanked with 145 bases-long inverted terminal repeats (ITRs) which are essential for transgene replication. At the beginning of the replication process (Figure 17), the self-primed hairpin structure of the ITR element in the wtAAV genome serves as a primer for the synthesis of the second strand. Rep proteins facilitate this process by their endonuclease- and ATP-dependent DNA helicase functions, which cause nicking at the terminal resolution site (TRS) [79] and generate a new primer for complementary strand synthesis [80]. The cellular DNA polymerase synthesizes the complementary strand, forming a blunt-ended duplex molecule. The ITR then transforms into a double hairpin structure, which becomes a template for directional strand displacement synthesis of the genome. This results in the packaging of a single strand and covalently closed duplex molecule, initiating a new replication cycle [81].

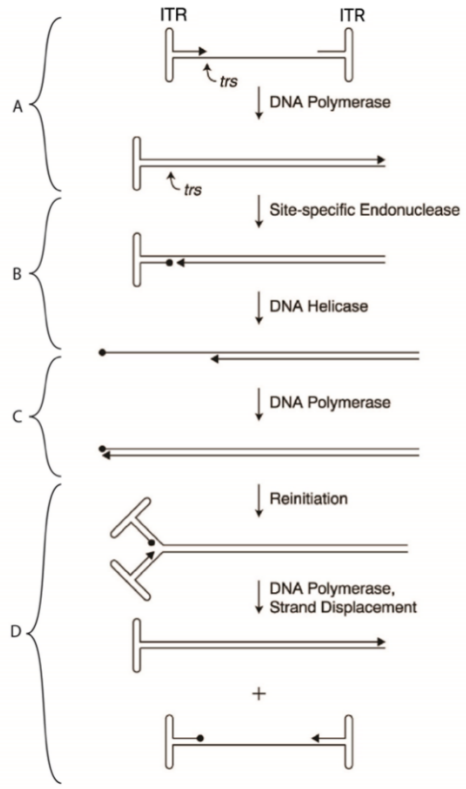

Figure 17. DNA replication of wtAAV. (A) ITR element in wtAAV genome serves as a primer for synthesis of the second strand. (B) Rep gene protein products that can act as DNA helicase and endonuclease cleave one strand at the TRS, creating a new primer for complementary strand synthesis. (C) Cellular DNA polymerase synthesizes the complementary strand, forming a blunt-ended duplex molecule. (D) The ITR reconstituted into a double hairpin structure creates a template for directional strand displacement synthesis of the genome by DNA polymerase. The process results in the packaging of a single strand while the duplex molecule is covalently closed at one end, initiating a new replication cycle. Adopted from [79].

5.1 Infection cycle

The diverse protein composition of the AAV capsid surface determines its ability to bind to cells, with different serotypes having specific preferences for receptors such as heparan sulfate proteoglycan [82] or sialic acid [83]. Co-receptor proteins, including growth factor receptors [84] and integrins [85], facilitate the endocytosis of AAV particles. Once bound to its receptor, the AAV virus undergoes endosomal processing and conformational changes that allow it to escape into the cytoplasm [86]. NLS on the outside of the capsid guides it to the nucleus, where the AAV genome undergoes uncoating and releases its single-stranded DNA. The second strand of the DNA is then synthesized (Figure 17). Successful transcription and replication of the wtAAV genome can be enhanced by the concurrent presence of a helper virus, such as adenovirus or herpes simplex virus. However, it is also possible for wtAAV to undergo productive replication in the absence of a helper virus, but it may not be as efficient. When a helper virus is not present, wtAAV may integrate its double-stranded DNA into the host genome, initiating a state of latent infection.

5.2 Recombinant adeno-associated virus vector

Recombinant AAV (rAAV) vectors (Figure 18) are created by replacing the rep and cap genes with foreign DNA while keeping the ITR sequences intact. This design enables efficient packing of the transgene into vector particles. In contrast to wtAAV, rAAV vectors lack the *rep* gene and are incapable of productive replication. Once the rAAV vector enters the nucleus of the host cell, the single-stranded transgene replicates into double-stranded DNA without integrating into the host cell genome. Transgene expression occurs through transcription of the concatemeric episomal DNA into RNA, which is then translated into a protein by the host cell machinery [87].

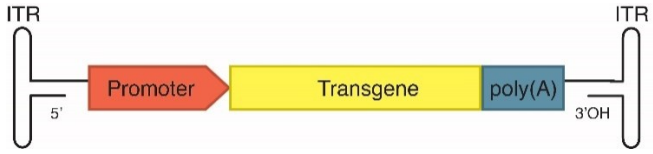

Figure 18. The basic composition of adeno-associated virus expression construct typically includes a promoter, one or more genes and conditioning regulatory sequences, for example a polyadenylation signal (poly(A)). ITRs remain intact at both sides. Created in Adobe Photoshop.

Two methods are used to produce recombinant AAV vectors: transient transfection-based and stable producer cell line-based methods. In transient transfection-based methods, three plasmids are introduced into human embryonic kidney 293 (HEK293) cells to produce the rAAV vector, and the cells are collected and the rAAV vectors are purified from the cell lysate [88]. This method is simple and fast but yields low titers. Stable producer cell line-based methods involve integrating the necessary plasmids into the genome of a host cell line. After culturing and adenovirus infection, the rAAV vectors are harvested from the culture medium [89]. This method is more complex and expensive but yields higher titers and is scalable for large-volume production.

5.3 Engineering rAAV vectors for improved nervous system-targeted gene delivery

The effectiveness of nervous system-targeted gene delivery using rAAV vectors can be improved by optimizing three main aspects: route of administration, a capsid engineering to improve blood brain barrier (BBB) crossing efficiency and cell attachment and entry, and regulation of transgene expression after nuclear entry by gene regulatory elements (as seen in 6). Despite these improvements, there are still obstacles to overcome, such as the limited size of AAV transgenes and immune responses to AAV vector-mediated gene therapy. Nevertheless, there are strategies available to address these challenges.

5.3.1 Routes of administration

The most common routes of administration for rAAV vectors into the nervous system are direct injection and systemic delivery.

Direct injection involves localized delivery to a specific region of the brain or spinal cord through different routes, including intraparenchymal, intracerebroventricular, and intrathecal injection. Direct intraparenchymal injection is useful for delivering rAAV vectors to specific nuclei, areas, or tracts within the brain [90]. Intracerebroventricular injection involves the injection of rAAV vectors into the cerebrospinal fluid (CSF) in the brain ventricles, which is suitable for targeting multiple regions of the brain, but only at a limited distance from the ventricle borders [91]. Intrathecal injection involves the injection of rAAV vectors into the CSF in the spinal canal, which primarily allows for delivery of the vectors to the entire spinal cord and lower brainstem[92].

Direct injection of rAAV vectors into the nervous system has precise targeting and controlled delivery advantages, which reduce the risk of unintended side effects and optimize therapeutic effects [93]. However, limitations include the limited reach of the vector due to diffusion of the AAV particles from CSF to neuronal parenchyma.

Systemic delivery via intravenous injection provides a fast route for the vector to enter the bloodstream and potentially reach the brain and spinal cord by crossing the BBB. Nevertheless, this approach has limitations due to rapid clearance by the liver and other organs, resulting in limited vector reaching the target cells in the nervous system. Moreover, the efficiency of BBB crossing is poor. In clinical trials and the first approved treatment protocols, this is compensated by a high total dose of the AAV vector [94]. However, such extreme titers increase the risk of off-target effects and potential side effects, especially the immune reaction.

In specific cases, the BBB can be temporarily and purposefully bridged by several strategies. One method is to use receptor-mediated transcytosis, which targets specific receptors on the surface of endothelial cells in the BBB to transport a peptide and any attached cargo molecules into the brain [95]. Another approach is to use certain drugs, such as mannitol, to temporarily disrupt the BBB via osmotic pressure in endothelial cells and allow AAV vectors to pass through [96]. Alternatively, focused ultrasound can be used to increase the BBB permeability [97].

5.3.2 Capsid engineering of rAAV vectors

Already the wild-type serotypes of AAVs have been shown to have somewhat specific tropisms for different cell types. For instance, AAV1 [98], AAV5 [99], AAV8 [100] and AAV9 [101] have been shown to efficiently transduce neurons in the brain and spinal cord when injected directly into brain parenchyma or CSF. Tropism to glial cells is less efficient. The only wt serotype with elementary ability to cross BBB is AAV9 [102].

Directed AAV capsid evolution involves preparing a library of random mutations in the capsid protein sequence of rAAV vectors and selecting variants with improved parameters. One notable success of the usage of direct evolution system was the AAV9 capsid variant called PHP.B, which was shown to have approximately 10x higher BBB crossing efficiency compared to wtAAV9 capsid [103]. This capsid variant was optimized by repeated sequence of generation of large library of capsid protein variants using an error-prone DNA polymerase, intravenous application of AAVs with various protein capsids, harvesting the targeted tissue and sequencing the DNA in the transduced cells. The trick was that the construct contained gene for EGFP and also the own sequence of the mutated capsid gene that encapsulated the particular AAV DNA. The harvested tissue was dissociated and sorted using fluorescence-activated cell sorting and the total DNA of the sorted fluorescent cells was checked and quantified for the specific capsid gene mutations. The second library was constructed from the most successful variants and after 2-3 repeats of this protocol, the variants with substantially improved BBB crossing efficiency were identified. Recently, a further improved variant called PHP.eB was developed by introducing additional mutations to PHP.B, leading to even greater (approximately 100x) BBB crossing efficiency compared to wtAAV9 [104]. However, later, PHP.eB was found to work only in certain mouse strains, specifically the C57 strains. This problem was solved using the optimization protocol performed in mouse crosses with highly heterogenous strain background. Concurrently, the BBB-crossing variants were also optimized to work in other species. The latest variant AAV.Cap-B10 have been shown to efficiently transduce neurons and glia cells in the central nervous system (CNS) of rats and marmosets after intravenous administration [105].

The next strategy uses rational design, which comprises structural information and knowledge of capsid biology. Ancestral AAV capsid engineering creates new AAV capsids by predicting their sequences through computation, synthesizing DNA sequences, and again, selecting for improved properties using directed evolution [106].

5.3.3 Limited rAAV transgene size

To use rAAV vectors for gene therapy, a major challenge is their limited cargo capacity. The AAV genome can accommodate only up to 4.7 kb of foreign DNA, the ITRs must be spared, which limits the size of the transgene that can be inserted and delivered. Several strategies have been developed to overcome these limitations. One possible approach is to use shorter promoters. By using smaller promoters, more space is available for the therapeutic gene itself (as mentioned in 6). Another strategy is to use multiple AAV vectors to deliver splitted larger transgenes and their reconstitution in the target cells. This can be accomplished using a "dual-vector" system, where two separate AAV vectors are used to deliver complementary parts of the transgene, or a "triple-vector" system in which three vectors are used to deliver the full-length transgene [107].

However, the CRISPR-Cas9 system, the main strategy for single-nucleotide diseases, is compatible with the limited AAV capacity [108].

5.3.4 Immune response against AAV vector-mediated gene therapy

AAV-mediated gene therapy faces the challenge of immune responses, especially at very high titers. To address this, optimization of transduction efficiency and BBB crossing can be utilized. Pre-existing neutralizing antibodies against AAV in the patient's bloodstream can also limit efficacy [109]. Solutions include developing new AAV vectors with reduced immunogenicity [110] and temporarily suppressing the immune system with drugs like corticosteroids or cyclosporine [111].

5.4 Translational potential and relevance to clinic

rAAV gene therapy has promising potential in treating neurological diseases [9] by efficiently transducing a wide range of cell types, including non-dividing cells in the nervous system, such as neurons and glial cells. Direct injection provides precise targeting but may require multiple injections, while systemic delivery allows for widespread distribution but lower transduction efficiency. Capsid engineering is another approach to improve specificity, potency, and safety of AAV vectors. Directed evolution and rational design have produced AAV9 variants with improved BBB crossing efficiency. Recent preclinical studies in non-human primates have revealed that AAV.CAP-B10 has an approximately 40-fold higher transduction efficiency in the CNS compared to AAV9 [105]. If this finding is replicated in humans, the fraction of AAV particles required for gene therapy using AAV.CAP-B10 could be reduced by 40 times in comparison to AAV9 while achieving the same level of transduction efficiency in the CNS.

To put this in perspective, the cost of producing 10^{12} particles of AAV is approximately $400; non-commercial producent, without the restriction of good laboratory practice (GLP). For example, in case of Zolgensma, an AAV9-based intravenous single-dose therapy for spinal muscular atrophy, the used dose is at $1x10^{14}$ vg/kg [94]. A child weighing 10 kg would require a dose of $1x10^{15}$ vg, which would cost around $400 000 for the sole production. Using GLP can be expected to double the price. However, if the dose of AAV.CAP-B10 needed to achieve the same therapeutic effect as Zolgensma is 40 times lower, the cost for the same treatment would be approximately $10 000 (20 000 with GLP) if whe use AAV.CAP-B10 instead of AAV9. The price of the Zolgensma treatment ivolves many components such as production, testing, handling, storage and, mainly, the intellectual property. However, analyzing the price, approximately $2 000 000, the production and handling can comprise up to the half of it. Such cost reduction can make the treatment more affordable and widely used. Widely used treatment can secondarily substantially reduce (dilute) the intellectual property price component per one patient.

Additionally, using more than one order lower dose of the vector may reduce the risk of adverse side effects associated with high doses of AAV vectors. Thus, developing PHP.eB and subsequently AAV.CAP-B10 as more efficient and effective AAV vectors for gene therapy has the potential to significantly reduce the cost and improve the safety of gene therapy treatments for CNS disorders.

6. Promoters and other gene regulatory elements

Neurological disorders are complex and multifactorial, and precise delivery of therapeutic genetic tools to specific cells in the nervous system is essential for successful treatment outcomes. Gene regulatory elements, DNA sequences that regulate gene expression, play a crucial role in conferring this specificity. By utilizing cell-specific shortened promoters, enhancers, transcription factors, micro RNAs and polyadenylation signals, gene expression can be limited to the intended cell types, thus minimizing any adverse effects due to influencing non-target cells. Precise targeting should result in safer and more effective therapeutic outcomes.

6.1 Shortened promoters

AAV vectors have proven effective in delivering therapeutic genes, but their limited capacity presents a challenge for gene therapy development. To address this, short natural or designed human promoters can be used to reduce the size of the mandatory DNA portion of AAV capacity, allowing for more space for the therapeutic gene itself.

To begin optimizing a shortened promoter sequence for gene therapy, the first step is to identify the full-length promoter sequence that initiates gene expression in the target cell type. Various methods such as deletion analysis, high-throughput screening, and in silico analysis can be employed. Deletion analysis involves progressively removing specific sections of the full-length promoter sequence to identify the minimum efficient sequence and gain insights into the functional organization of the promoter [112]. High-throughput screening uses large-scale screening techniques, such as NGS, to simultaneously test the activity of thousands of promoter fragments in the target cell type to identify the minimum sequence required for efficient gene expression [113]. In silico analysis involves using advanced computer algorithms and modeling to identify potential regulatory elements and transcription factor-binding sites that are essential for gene expression. This method can help to identify critical regions that can be further investigated [114].

The shortened promoter sequence is tested in vitro to evaluate its ability to drive gene expression in the selected cell type [115]. Promising sequences may undergo further optimization to enhance activity or specificity. The optimized promoter is then tested in vivo using animal models to evaluate its effectiveness in specific marker delivery to target cells, which may involve testing different dosages and delivery methods, in vivo imaging, or postmortem immunohistochemistry.

The human synapsin-1 (hSyn) promoter is a shortened promoter derived from the region upstream of the human synapsin-1 gene, which is expressed in neurons of the central nervous system [116]. It is optimized for small size, usually only 0.5 kb, and has strong and sustained gene expression [117]. The hSyn promoter has shown promise in promoting gene therapy delivery using AAV vectors for neurological diseases, including Parkinson's [118], Huntington's [119], and Alzheimer's disease [120]. In preclinical studies, AAV vectors containing the hSyn promoter effectively delivered therapeutic genes to neurons, leading to improvements in motor function, cognition, and memory in animal models.

The calcium/calmodulin-dependent protein kinase II (CaMKII) promoter is another short promoter useful for AAV gene therapy delivery [121]. It is derived from the upstream region of the CaMKII gene and

targets gene expression into excitatory neurons found in the central nervous system, particularly in the hippocampus and cortex. With a length of approximately 0.4 kb [122], the CaMKII promoter has been used in AAV gene therapy to treat epilepsy in mice [123].

The human glial fibrillary acidic protein (hGFAP) promoter, with a length of 2.2 kb [124], is a naturally short promoter that is highly specific to astrocytes, with minimal activity in other cell types in the brain [125]. Astrocytes substantially contribute to maintaining the extracellular composition, neurotransmitter clearance, modification of synapses, and other major task in healthy brain parenchyma Their dysfunction has been implicated in a range of neurological disorders. In preclinical studies, for example, the GFAP promoter has been already used to drive the expression of a gene encoding glial cell line-derived neurotrophic factor in a rat model of Parkinson's disease [126].This therapy led to significant improvements in the motor function of rats.

Additionally, short, naturally occurring promoters have been identified as potential candidates for gene therapy targeting specific interneurons in the brain. These promoters are derived from upstream regions of genes that are selectively expressed in interneurons, such as parvalbumin (PV), somatostatin (SST), vasoactive intestinal peptide (VIP), glutamic acid decarboxylase (GAD), and vesicular gamma-aminobutyric acid transporter (vGAT).

The PV promoter is approximately 2 kb in length and active in fast-spiking interneurons in the neocortex [127], which play a crucial role in regulating neuronal firing synchronization important for cognitive processes, such as attention and perception. It has shown promise in the treatment of neurological disorders such as epilepsy and autism [128].

The 2.5 kb long [129] SST promoter has potential in treating epilepsy [130] by targeting somatostatin-positive interneurons involved in feedback regulation of cortical activity.

The VIP promoter is active in VIP-positive interneurons, which regulate cortical activity and are implicated in neurological disorders [131]. VIP is a neuropeptide that acts as a neuromodulator and is involved in circadian rhythm, immune function, learning and memory [132], attention, and social behavior.

The GAD and vGAT promoters can target GABAergic interneurons for gene therapy, as GAD converts glutamate to gamma-aminobutyric acid (GABA) [133] and vGAT regulates GABA transporter transcription [134]. GAD promoter is 2.5 kb long while vGAT promoter is 1.8 kb long.

Short and specific promoters are an appealing approach for targeted gene expression in genetic engineering, as they can be engineered to activate gene transcription in specific cells or tissues, making them useful for therapeutic purposes. However, they may not be appropriate for genes requiring high levels of expression, particularly those involved in producing therapeutic proteins [135]. In contrast, in gene editing techniques like CRISPR-Cas, the need for strong promoters may not be as critical, since only a small number of copies of the sgRNAs and Cas enzymes are necessary for successful gene editing.

6.2 Transcription factors

Transcription factors are proteins that bind to DNA sequences in genes and regulate their expression. Adding binding sites for cell-specific transcription factors to an AAV vector carrying therapeutic genes enables controlled expression in specific cell types. One example is the tetracycline-controlled transcriptional

activation system (tTA) or its reverse (rtTA), in which the promoter driving transgene expression contains tetracycline response elements (TREs) that bind to either tTA or rtTA (Figure 19). In the absence of doxycycline, tTA binds to TRE and activates transgene expression. In the presence of doxycycline, rtTA binds to TRE and activates transgene expression. By introducing an AAV vector carrying the transgene under the control of a TRE promoter and expressing tTA or rtTA from the same construct in the target cell type, transgene expression can be tightly controlled in time by administration or removal of doxycycline [136]. This in principle enables the regulation of the treatment power on demand, i.e., corresponding to the actual disease severity.

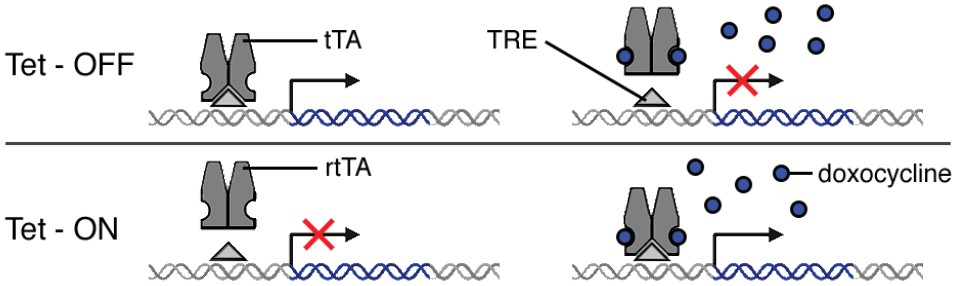

Figure 19. Diagram of the tetracycline-controlled transcriptional activation system. The promoter driving transgene expression contains TREs that bind to either tTA or rtTA. The upper part of the image shows the Tet-Off system, where in the absence of doxycycline, tTA binds to TRE and activates transgene expression, while in the presence of doxycycline, tTA is inhibited and the transgene expression is turned off. The bottom part of the image shows the Tet-On system, where in the absence of doxycycline, rtTA binds to TRE and represses transgene expression, while in the presence of doxycycline, rtTA is activated and transgene expression is turned on. Adapted from [137].

6.3 Enhancers

Enhancers are gene regulatory elements that can enhance cell type-restricted gene expression by increasing promoter activity through binding to transcription factors. They can be located upstream or downstream of the promoter, and can be close or far away from it. In AAV delivery, enhancers refer to any sequence that increases the amount of the final protein product.

Enhancers can be either naturally occurring or synthetic. The naturally occurring enhancers include the human cytomegalovirus enhancer and the simian virus 40 (SV40) enhancer, while the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is an example of a synthetic enhancer [138]. Combining these enhancers with specific promoters has been shown to significantly increase the expression levels of transgenes. For instance, the hSyn and CaMKII promoters can be combined with WPRE to enhance neuronal gene expression without compromising the specificity of the promoters [139,140]. Similarly, in astrocytes, combining the GFAP promoter with a human cytomegalovirus enhancer [141] or SV40 enhancer can increase gene expression.

6.4 Micro RNA

In gene therapy using AAV vectors, incorporating microRNA (miRNA)-binding sites into the messenger RNA (mRNA) sequence of the therapeutic gene can provide an additional level of specificity for controlling gene expression. MiRNAs are small non-coding RNA molecules that regulate gene expression by binding to complementary sequences on target mRNA molecules suppressing their translation or promoting their degradation. By incorporating miRNA-binding sites into the mRNA sequence of a transgene, the expression of the transgene can be regulated by endogenous miRNA expression in a cell type-specific manner. This allows for fine-tuned regulation of the level and timing of transgene expression, minimizing off-target effects and increasing the therapeutic efficacy of gene therapy [142].

6.5 Polyadenylation signal

In AAV vectors, polyadenylation signal (poly(A)) serves as a crucial gene regulatory element. During post-transcriptional processing, a stretch of adenine nucleotides is added to the 3' end of the mRNA, which extends the lifetime of the mRNA molecule by preventing early degradation. This leads to the production of multiple protein molecules from a single mRNA. The most commonly used type of poly(A) in AAV vectors is the bovine growth hormone (BGH) poly(A) sequence, which is a 225 bp fragment derived from the 3' untranslated region of the BGH gene. Its strong activity results in high levels of transgene expression [143]. Studies have demonstrated the effectiveness of the BGH poly(A) sequence across a broad range of cell types, making it a versatile tool for gene expression in AAV-based gene therapy [144].

6.6 Translational potential and relevance to clinic

Shortened human promoters can enhance AAV gene therapy for neurological diseases by reducing mandatory DNA sequences. Specific promoters can increase gene expression specificity for target cell types. Transcription factor binding sites, enhancers, miRNA binding sites, and poly(A) can also be included for stronger cell-specific expression. Precision is crucial for treating neurological diseases that selectively affect certain neuron populations. Temporal control of gene expression is possible, regulating therapy expression at specific disease stages and avoiding long-term overexpression complications [145].

7. Potential treatment approach for epilepsy

Epilepsy is a complex neurological disorder that affects approximately 1% of the global population [146]. It is a multifactorial condition that is treated primarily for its symptoms rather than its root cause. The main dogma of epilepsy is that it results from an imbalance between excitatory and inhibitory signaling in the brain. Children with Dravet syndrome provide an unfortunate illustration of the detrimental effects of epilepsy. This genetic disorder arises from a mutation in the sodium voltage-gated channel alpha subunit 1 (SCNA1) gene, particularly in inhibitory cells. Regrettably, despite this knowledge, there is no known causal treatment for Dravet syndrome. Instead, patients receive a combination of antiepileptic drugs, which cannot fully prevent seizures and can cause severe side effects, such as liver damage, depression, and cognitive and developmental delays. This condition can significantly reduce a patient's quality of life, affecting their ability to work, drive, and engage in daily activities.

Here I propose a solution, to conduct a multi-read NGS to identify the precise location of the problem in patients with epilepsy. If a known mutation is identified, the preclinical steps can be skipped, but if a new mutation is identified, the preclinical steps will be required to be carried out. If multiple new mutations are found, computer modeling will be utilized to determine which mutation could be responsible for the patient's epilepsy. This will be done through a series of simulations to determine the potential effects of each mutation on the patient's health. Once the simulation has identified the mutation most likely to be responsible for the epilepsy, it will be tested in two patient-specific preclinical steps.

To begin the process, a plasmid containing sgRNA and Cas will be created to introduce the specific mutation to healthy cells. This plasmid will then be transferred into mixed neuronal cultures of mice, which consist of both excitatory and inhibitory neurons. The neuronal activity of these cultures will then be analyzed using in vitro techniques such as patch-clamp, multielectrode array, and calcium imaging. The electrical and calcium signals produced by the cultures will be carefully monitored to determine whether the mutation has any detrimental effects on neuronal activity. Such effects are supposed to prove the potential involvement of the mutation in epilepsy.

To further investigate the causality of the mutation in epilepsy, the next step would involve inserting the plasmid with sgRNA and Cas9 into mouse pups using AAV-PHP.eB with a hSyn promoter. This will allow the plasmid to edit the genome of the mouse pups, potentially leading to the expression of the mutation in a more physiologically relevant setting. If the inserted mutation is causative of epilepsy, then the mice are supposed to develop seizures or other epileptic symptoms.

In case the particular mutation is confirmed to be responsible for the patient’s epilepsy, a radical treatment plan can be proposed to prevent the patient, often a child, from developing the full symptoms of the condition. My suggestion would be to design an sgRNA that specifically targets and corrects the identified mutation. Once the sgRNA has been prepared, I recommend creating an rAAV plasmid that contains the sgRNA and the Cas14 protein, the latest version of the Cas enzyme [147]. To ensure that the sgRNA is expressed effectively, I suggest using a truncated version of the GAD promoter. Additionally, I recommend adding WPRE and poly(A) sequences at the end of the plasmid to facilitate efficient transcription and processing of the RNA transcript.

As the identified mutation is a germline or early somatic mutation present throughout the brain, a systemic and BBB crossing treatment approach is necessary. To achieve this, I suggest using the AAV.CAP-B10 capsid to produce viral particles containing the proposed construct genetic material. The final step would be administration of the viral particles to a slightly immunosuppressed child with the hope that it will significantly improve their condition. A decade ago, this innovative approach would not be possible due to the lack of routine availability and affordability of DNA sequencing, as well as the absence of crucial components like a promoter, capsid, and an optimized Cas enzyme. However, with advancements in technology and research, these tools and resources are now readily accessible, making once impossible therapies a reality.

8. Conclusion

Recent advancements in molecular and genetic engineering have significantly enhanced the ability to translate research into clinical applications. By integrating these tools, personalized treatments that account for the unique genetic makeup of each patient can be developed. This will not only result in safer and more effective therapies, but mainly, a lot of new therapeutic designs can represent a causative treatment. To demonstrate the potential of these modern tools increasing the translatability of preclinical research towards patient-oriented treatment, we further outline the process of identifying the molecular genetic basis and designing gene therapy for an unidentified neurological disease, using spinal muscular atrophy (SMA) and epilepsy as examples.

The first step in identifying an unknown neurological disease is a comprehensive clinical evaluation. The patient should undergo a physical examination and medical history review, including neurological tests, imaging studies, and blood tests to rule out other conditions. In the case of SMA, the patient may present with muscle weakness, motor difficulties, and respiratory problems [148].

The next step is to perform genetic testing. Whole exome sequencing or whole genome sequencing can be used to identify mutations in the patient's DNA that may be causing the disease. In the case of SMA, genetic testing can identify mutations in the survival motor neuron (SMN) 1 gene, which is normally responsible for producing a SMN protein. When a genetic mutation is identified, confirmatory testing can be performed to confirm that the mutation is the cause of the neurological disease. In the case of SMA, confirmatory testing can be performed by analyzing the patient's SMN protein levels[149].

Once the genetic mutation is confirmed to be causing the neurological disease, gene therapy can be designed to address the underlying genetic defect. In the case of SMA, gene therapy can involve using an AAV to deliver a functional copy of the SMN1 gene to the patient's motor neurons [2]. The AAV can be engineered to use a shortened human promoter to ensure that the gene is expressed in the appropriate cells and at sufficient levels. If the genetic problem is identified as a point mutation or deletion, producing misfolded or truncated protein, CRISPR/Cas approach can be used with the hope that the necessary AAV titer will be lower.

Once the gene therapy is designed, it can be administered to the patient. In the case of SMA, the AAV can be injected directly into the patient’s spinal cord, where it can deliver the functional SMN1 gene to the appropriate cells. Due to new optimized capsids with higher BBB crossing efficiency, the titer can be more than one order of magnitude lower compared to the latest AAV-using therapies and the immunosuppressing pretreatment and treatment in general can be milder bringing less adverse effects.

To further describe such approach using epilepsy as an example, the first step would also involve a comprehensive clinical evaluation to determine the type and severity of seizures the patient is experiencing. This may include EEG (electroencephalogram) and MRI (magnetic resonance imaging) scans to identify any abnormalities in the brain that may be causing the seizures.

Genetic testing may be performed to identify any mutations in genes known to be associated with epilepsy. Let’s suppose that the NGS sequencing focused on such gene batteries shows that the cause is a newly identified point mutation in one of key voltage-gated ion channel, such as SCN1A.

Confirmatory testing can be performed by analyzing the mutation induced in neuronal cultures to show that mutation substantially changes the cellular excitability and network properties. With such knowledge the personalized gene therapy can be proposed.

Gene therapy design for epilepsy may involve using AAV vectors to deliver a functional copy of the mutated gene or using gene editing technologies such as CRISPR-Cas9 to correct the mutation [7]. The therapy may be designed to target specific brain regions or cell types to reduce the risk of off-target effects. Inhibitory neurons-selective promoters and gene regulatory sequences can be used to target the construct only to the problem causing cell types.

Gene therapy administration for epilepsy may involve injection of the viral vector or gene editing tool directly into the brain, or through other routes such as intravenous injection. Follow-up monitoring would involve regular EEG and MRI scans to evaluate changes in brain activity and identify any potential adverse effects of the therapy. Additionally, the patient's seizure activity and other symptoms would be monitored to assess the effectiveness of the therapy over time.

In conclusion, recent advancements in molecular and genetic engineering have revolutionized the ability to develop patient-oriented treatments based on individual genetic profiles. NGS with high parallelization extremely sped up reading of the entire genomes and decreased the price down to few hundreds dollars. The development of chemogenetics and optogenetics has been addressing the challenges related to foreign epitopes, as well as improving sensitivity and driving efficiency. CRISPR-Cas technology has gone a long way in terms of optimizing the efficiency and, mainly, the off-target site interactions. rAAV capsid engineering has already develop capsids with substantially improved BBB crossing efficiency and cell selectivity compared to original wild-type serotypes, which lowers the cost of AAV preparation and reduces the necessary titers to avoid immune system reactions. Short promotors that have been recently discovered and optimized together with gene regulatory sequences can even more focus the therapy selectively to cells involved in the pathophysiology and thus produce substantial lower adverse effect. Altogether, the already achieved improvements and the further ongoing research can push the causal treatment methods tested in preclinical research directly into clinical trials.

9. References

* secondary references

1. Yang, S. *et al. CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease*. *The Journal of Clinical Investigation* vol. 127 (2017).
2. Mercuri, E. *et al.* Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy type 1 (STR1VE-EU): an open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurology* (2021).
3. Christine, C. W. *et al.* Magnetic Resonance Imaging-Guided Phase 1 Trial of Putaminal AADC Gene Therapy for Parkinson's Disease. *Ann Neurol* (2019).
4. Griciuc, A., Federico, A. N., Natasan, J., Forte, A. M. & McGinty, D. Gene therapy for Alzheimer's disease targeting CD33 reduces amyloid beta accumulation and neuroinflammation. *Hum Mol Genet* **29**, 2920–2935 (2020).
5. Mòdol-Caballero, G. *et al.* Specific Expression of Glial-Derived Neurotrophic Factor in Muscles as Gene Therapy Strategy for Amyotrophic Lateral Sclerosis. *Neurotherapeutics* (2021).
6. Figley, S. A. *et al.* Delayed Administration of a Bio-Engineered Zinc-Finger VEGF-A Gene Therapy Is Neuroprotective and Attenuates Allodynia Following Traumatic Spinal Cord Injury. *PLoS One* (2014).
7. Tanenhaus, A. *et al.* Cell-Selective Adeno-Associated Virus-Mediated SCN1A Gene Regulation Therapy Rescues Mortality and Seizure Phenotypes in a Dravet Syndrome Mouse Model and Is Well Tolerated in Nonhuman Primates. *Hum Gene Ther* (2022).
8. Doudna, J. A. & Charpentier, E. *The new frontier of genome engineering with CRISPR-Cas9*. *Science* vol. 346 (2014).
9. Mendell, J. R. *et al.* Current Clinical Applications of In Vivo Gene Therapy with AAVs. *Molecular Therapy* **29**, 464–488 (2021).
10. Stödberg, T. *et al.* Epilepsy syndromes, etiologies, and the use of next-generation sequencing in epilepsy presenting in the first 2 years of life: A population-based study. *Epilepsia* (2020).
11. Miller, J. R. *et al.* Hybrid assembly with long and short reads improves discovery of gene family expansions. *BioMed Central Genomics* (2017).
12. Choudhuri, S. *Fundamentals of Molecular Evolution*. *Bioinformatics for Beginners* (Academic Press, 2014).
13. Eid, J. *et al. Real-time DNA sequencing from single polymerase molecules. Science (New York, N.Y.)* vol. 323 (2009).
14. Schadt, E. E. *et al.* Modeling kinetic rate variation in third generation DNA sequencing data to detect putative modifications to DNA bases. *Genome Res* **23**, 129–141 (2013).
15. Clarke, J. *et al.* Continuous base identification for single-molecule nanopore DNA sequencing. *Nat Nanotechnol* **4**, 265–270 (2009).

16. * Beckett, A. H., Cook, K. F. & Robson, S. C. A pandemic in the age of next-generation sequencing. *Portland Press Limited* **43**, 10–15 (2021).
17. Jain, M. *et al.* Improved data analysis for the MinION nanopore sequencer. *Nat Methods* **12**, 351–356 (2015).
18. Laszlo, A. H. *et al.* Decoding long nanopore sequencing reads of natural DNA. *Nat Biotechnol* **32**, 829–833 (2014).
19. * Cost of sequencing a full human genome. https://ourworldindata.org/grapher/cost-of-sequencing-a-full-human-genome.
20. N' Songo, A. *et al.* Comprehensive Screening for Disease Risk Variants in Early-Onset Alzheimer's Disease Genes in African Americans Identifies Novel PSEN Variants. *Alzheimers Disease* (2017).
21. Ibilibor, C. *et al.* RNA sequencing in a penile cancer cohort: an investigation of biomarkers of cisplatin resistance and potential therapeutic drug targets. *Clin Genitourin Cancer* **20**, 219–226 (2022).
22. Barrangou, R. *et al. CRISPR provides acquired resistance against viruses in prokaryotes*. *Science* vol. 315 (2007).
23. Brouns, S. J. J. *et al. Small CRISPR RNAs guide antiviral defense in prokaryotes*. *Science* vol. 321 (2008).
24. Deltcheva, E. *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* (2011).
25. A tool for genome editing: CRISPR-Cas9 - BioRender Blog. https://www.biorender.com/blog/nobel-prize-chemistry-2020.
26. Mali, P. *et al. RNA-guided human genome engineering via Cas9*. *Science* vol. 339 (2013).
27. Walton, R. T., Christie, K. A., Whittaker, M. N. & Kleinstiver, B. P. *Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants*. *Science* vol. 368 (2020).
28. Jinek, M. *et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity*. *Science* vol. 337 (2012).
29. Ran, F. A. *et al.* Double nicking by RNA-guided CRISPR cas9 for enhanced genome editing specificity. *Cell* **154**, (2013).
30. * Pickar-Oliver, A. & Gersbach, C. A. The next generation of CRISPR–Cas technologies and applications. *Nature Reviews Molecular Cell Biology* vol. 20 490–507 Preprint at (2019).
31. Komor, A. C., Kim, B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* (2016).
32. Gaudelli, N. M. *et al.* Programmable base editing of T to G C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471 (2017).
33. Anzalone, A. V *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149 (2019).
34. Prime editing mechanism. https://en.wikipedia.org/wiki/File:Prime_editing_mechanism.png.

35. Hilton, I. B. *et al.* Epigenome editing by a CRISPR/Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* **33**, 510–517 (2015).
36. Vojta, A. *et al.* Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res* **44**, 5615–5628 (2016).
37. Kearns, N. A. *et al.* Functional annotation of native enhancers with a Cas9-histone demethylase fusion HHS Public Access. *Nat Methods* **12**, 401–403 (2015).
38. Gilbert, L. A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647–661 (2014).
39. Maeder, M. L. *et al.* CRISPR RNA-guided activation of endogenous human genes. *Nat Methods* (2013).
40. * Wen, W. S., Yuan, Z. M., Ma, S. J., Xu, J. & Yuan, D. T. CRISPR-Cas9 systems: Versatile cancer modelling platforms and promising therapeutic strategies. *Int J Cancer* **138**, 1328–1336 (2016).
41. Akcakaya, P. *et al.* In vivo CRISPR editing with no detectable genome-wide off-target mutations. *Nature* (2018).
42. Tsai, S. Q. *et al.* CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods* **14**, 607–614 (2017).
43. Coelho, M. A. *et al.* CRISPR GUARD protects off-target sites from Cas9 nuclease activity using short guide RNAs. *Nat Commun* (2020).
44. Yeh, W.-H. *et al.* In vivo base editing restores sensory transduction and transiently improves auditory function in a mouse model of recessive deafness. *Sci Transl Med* **12**, (2020).
45. Nagel, G. *et al.* Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *PNAS* **100**, 13940–13945 (2003).
46. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* **8**, 1263–1268 (2005).
47. Han, X. *et al.* Millisecond-Timescale Optical Control of Neural Dynamics in the Nonhuman Primate Brain. *Neuron* **62**, 191–198 (2009).
48. Nagel, G. *et al.* Light activation of Channelrhodopsin-2 in excitable cells of caenorhabditis elegans triggers rapid behavioral responses. *Current Biology* **15**, 2279–2284 (2005).
49. Klapoetke, N. C. *et al.* Independent Optical Excitation of Distinct Neural Populations . *Nat Methods* **11**, 338–346 (2014).
50. Chen, R. *et al.* Deep brain optogenetics without intracranial surgery. *Nat Biotechnol* **39**, 161 (2021).
51. Govorunova, E. G., Sineshchekov, O. A., Janz, R., Liu, X. & Spudich, J. L. *Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics*. *Science* vol. 349 (2015).
52. Mahn, M. *et al.* High-efficiency optogenetic silencing with soma-targeted anion-conducting channelrhodopsins. *Nature Communication* (2018).
53. Gradinaru, V., Thompson, K. R. & Deisseroth, K. eNpHR: a Natronomonas halorhodopsin enhanced for optogenetic applications. *Brain Cell Biol* (2008).

54. Diester, I. *et al.* An optogenetic toolbox designed for primates. *Nat Neurosci* 387–397 (2011).
55. Kralik, J., van Wyk, M., Stocker, N. & Kleinlogel, S. Bipolar cell targeted optogenetic gene therapy restores parallel retinal signaling and high-level vision in the degenerated retina. *Commun Biol* **5**, (2022).
56. van Wyk, M., Pielecka-Fortuna, J., Löwel, S. & Kleinlogel, S. Restoring the ON Switch in Blind Retinas: Opto-mGluR6, a Next-Generation, Cell-Tailored Optogenetic Tool. *PLoS Biol* **13**, (2015).
57. Levskaya, A., Weiner, O. D., Lim, W. A. & Voigt, C. A. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature* **461**, (2009).
58. Müller, K. *et al.* A red/far-red light-responsive bi-stable toggle switch to control gene expression in mammalian cells. *Nucleic Acids Res* **41**, (2013).
59. Kennedy, M. J. *et al.* Rapid blue light induction of protein interactions in living cells. *Nat Methods* **7**, 973–975 (2010).
60. Nihongaki, Y., Yamamoto, S., Kawano, F., Suzuki, H. & Sato, M. CRISPR-Cas9-based photoactivatable transcription system. *Chem Biol* **22**, 169–174 (2015).
61. Polstein, L. R., Gersbach, C. A. & Chem Biol, N. A light-inducible CRISPR/Cas9 system for control of endogenous gene activation. *Nat Chem Biol* **11**, 198–200 (2015).
62. Crosson, S. & Moffat, K. Photoexcited Structure of a Plant Photoreceptor Domain Reveals a Light-Driven Molecular Switch. *Plant Cell* **14**, 1067–1075 (2002).
63. Harper, S. M., Neil, L. C. & Gardner, K. H. *Structural basis of a phototropin light switch*. *Science* vol. 301 (2003).
64. Motta-Mena, L. B. *et al.* An optogenetic gene expression system with rapid activation and deactivation kinetics. *Nat Chem Biol* **10**, 196–202 (2014).
65. Niopek, D. *et al.* Engineering light-inducible nuclear localization signals for precise spatiotemporal control of protein dynamics in living cells. *Nat Commun* (2014).
66. Yazawa, M., Sadaghiani, A. M., Hsueh, B. & Dolmetsch, R. E. Induction of protein-protein interactions in live cells using light. *Nat Biotechnol* **27**, 941–945 (2009).
67. Zoltowski, B. D. & Crane, B. R. Light Activation of the LOV Protein Vivid Generates a Rapidly Exchanging Dimer. *Biochemistry* (2008).
68. Alexander, G. M. *et al.* Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. *Neuron* **63**, 27–39 (2009).
69. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S. & Roth, B. L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *PNAS* **104**, 5163–5168 (2007).
70. Maclaren, D. A. A. *et al.* Clozapine N-Oxide Administration Produces Behavioral Effects in Long-Evans Rats: Implications for Designing DREADD Experiments. *eNeuro* **3**, 219–235 (2016).
71. Vardy, E. *et al.* A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior. *Neuron* **86**, 936–946 (2015).

72. Magnus, C. J. *et al. Chemical and genetic engineering of selective ion channel-ligand interactions*. *Science* vol. 333 (2011).
73. Boehm, M. A. *et al.* Translational PET applications for brain circuit mapping with transgenic neuromodulation tools. *Pharmacology Biochemistry and Behavior* vol. 204 Preprint at https://doi.org/10.1016/j.pbb.2021.173147 (2021).
74. Anthenelli, R. M. *et al.* Neuropsychiatric safety and efficacy of varenicline, bupropion, and nicotine patch in smokers with and without psychiatric disorders (EAGLES): a double-blind, randomised, placebo-controlled clinical trial. *The Lancet* **387**, 2507–2520 (2016).
75. Magnus, C. J. *et al.* Ultrapotent chemogenetics for research and potential clinical applications. *Science (1979)* **364**, (2019).
76. Zhang, Y. *et al.* Battery-free, lightweight, injectable microsystem for in vivo wireless pharmacology and optogenetics. *Proc Natl Acad Sci U S A* **116**, 21427–21437 (2019).
77. Wang, Z., Hu, M., Ai, X., Zhang, Z. & Xing, B. Near-Infrared Manipulation of Membrane Ion Channels via Upconversion Optogenetics. *Adv Biosyst* **3**, (2019).
78. Atchison, R. W., Casto, B. C. & McD Hammon, W. Adenovirus-Associated Defective Virus Particles. *Science (1979)* **149**, (1965).
79. Rodney, J. & Muzyczka, N. *Mechanism of Rep-Mediated Adeno-Associated Virus Origin Nicking*. *JOURNAL OF VIROLOGY* vol. 74 https://journals.asm.org/journal/jvi (2000).
80. Rodney, J. & Muzyczka, N. *Rep-Mediated Nicking of the Adeno-Associated Virus Origin Requires Two Biochemical Activities, DNA Helicase Activity and Transesterification*. *JOURNAL OF VIROLOGY* vol. 73 https://journals.asm.org/journal/jvi (1999).
81. Earley, L. F. *et al.* Adeno-Associated Virus Serotype-Specific Inverted Terminal Repeat Sequence Role in Vector Transgene Expression. *Hum Gene Ther* **31**, (2020).
82. Xie, Q., Lerch, T. F., Meyer, N. L. & Chapman, M. S. Structure-function analysis of receptor-binding in adeno-associated virus serotype 6 (AAV-6). *Virology* **420**, 10–19 (2011).
83. Wu, Z., Miller, E., Agbandje-McKenna, M. & Samulski, R. J. α2,3 and α2,6 N-Linked Sialic Acids Facilitate Efficient Binding and Transduction by Adeno-Associated Virus Types 1 and 6. *J Virol* **80**, 9093–9103 (2006).
84. Qing, K. *et al. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2*. *NATURE MEDICINE • VOLUME* vol. 5 http://medicine.nature.com (1999).
85. Summerford, C., Bartlett, J. S. & Samulski, R. J. aVb5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* **5**, (1999).
86. Venkatakrishnan, B. *et al.* Structure and Dynamics of Adeno-Associated Virus Serotype 1 VP1-Unique N-Terminal Domain and Its Role in Capsid Trafficking. *J Virol* **87**, 4974–4984 (2013).
87. Penaud-Budloo, M. *et al.* Adeno-Associated Virus Vector Genomes Persist as Episomal Chromatin in Primate Muscle. *J Virol* **82**, 7875–7885 (2008).

88. Chahal, P. S., Schulze, E., Tran, R., Montes, J. & Kamen, A. A. Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery. *J Virol Methods* **196**, 163–173 (2014).
89. Chadeuf, G. *et al.* Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus-induced amplification of the integrated rep-cap genome. *Journal of Gene Medicine* **2**, 260–268 (2000).
90. Rosenberg, J. B. *et al.* Safety of Direct Intraparenchymal AAVrh.10-Mediated Central Nervous System Gene Therapy for Metachromatic Leukodystrophy. *Hum Gene Ther* (2021).
91. Kim, J. Y., Grunke, S. D., Levites, Y., Golde, T. E. & Jankowsky, J. L. Intracerebroventricular Viral Injection of the Neonatal Mouse Brain for Persistent and Widespread Neuronal Transduction. *Journal of Visualized Experiments* (2014).
92. Gong, Y. *et al.* Intrathecal Adeno-Associated Viral Vector-Mediated Gene Delivery for Adrenomyeloneuropathy. *Hum Gene Ther* **30**, 544–555 (2019).
93. Taghian, T. *et al.* A Safe and Reliable Technique for CNS Delivery of AAV Vectors in the Cisterna Magna. *Molecular Therapy* **28**, 411–421 (2020).
94. * Kotulska, K., Fattal-Valevski, A. & Haberlova, J. Recombinant Adeno-Associated Virus Serotype 9 Gene Therapy in Spinal Muscular Atrophy. *Front Neurol* **12**, (2021).
95. Zhang, X. *et al.* Customized blood-brain barrier shuttle peptide to increase AAV9 vector crossing the BBB and augment transduction in the brain . *Biomaterials* **281**, (2022).
96. Carty, N. *et al.* Convection-enhanced delivery and systemic mannitol increase gene product distribution of AAV vectors 5, 8, and 9 and increase gene product in the adult mouse brain. *J Neurosci Methods* **194**, 144–153 (2010).
97. Felix, M.-S. *et al.* Ultrasound-Mediated Blood-Brain Barrier Opening Improves Whole Brain Gene Delivery in Mice . *Pharmaceutics* **13**, (2021).
98. Kim, S. *et al.* Protection of nigral dopaminergic neurons by AAV1 transduction with Rheb(S16H) against neurotoxic inflammation in vivo. *Exp Mol Med* (2018).
99. Spronck, E. A. *et al.* AAV5-miHTT Gene Therapy Demonstrates Sustained Huntingtin Lowering and Functional Improvement in Huntington Disease Mouse Models. *Mol Ther Methods Clin Dev* **13**, 334–343 (2019).
100. Masamizu, Y. *et al.* Efficient gene transfer into neurons in monkey brain by adeno-associated virus 8. *Neuroreport* **21**, 447–451 (2010).
101. Samaranch, L. *et al.* Adeno-Associated Virus Serotype 9 Transduction in the Central Nervous System of Nonhuman Primates. *Hum Gene Ther* (2012).
102. * Manfredsson, F. P., Rising, A. C. & Mandel, R. J. *AAV9: A potential blood-brain barrier buster*. *Molecular Therapy* vol. 17 (2009).
103. Deverman, B. E. *et al.* Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol* **34**, 204–209 (2016).

104. Chan, K. Y. *et al.* Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat Neurosci* **20**, 1172–1179 (2017).
105. Goertsen, D. *et al.* AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset. *Nat Neurosci* **25**, 106–115 (2022).
106. Ortiz, J. S. *et al.* AAV ancestral reconstruction library enables selection of broadly infectious viral variants Publication Date AAV ancestral reconstruction library enables selection of broadly infectious viral variants. *Nature* **22**, 934–946 (2015).
107. Tornabene, P. *et al.* Intein-mediated protein trans-splicing expands adeno-associated virus transfer capacity in the retina. *Sci Transl Med* (2019).
108. Schmidt, M. J. *et al.* Improved CRISPR genome editing using small highly active and specific engineered RNA-guided nucleases. *Nat Commun* (2021).
109. Fitzpatrick, Z. *et al.* Influence of Pre-existing Anti-capsid Neutralizing and Binding Antibodies on AAV Vector Transduction. *Mol Ther Methods Clin Dev* **9**, 119–129 (2018).
110. Giles, A. R., Govindasamy, L., Somanathan, S. & Wilson, J. M. Mapping an Adeno-associated Virus 9-Specific Neutralizing Epitope To Develop Next-Generation Gene Delivery Vectors. *J Virol* **92**, (2018).
111. Chowdary, P. *et al.* Phase 1–2 Trial of AAVS3 Gene Therapy in Patients with Hemophilia B. *New England Journal of Medicine* **387**, 237–247 (2022).
112. Xu, Y. Z., Kanagaratham, C., Jancik, S. & Radzioch, D. Promoter deletion analysis using a dual-luciferase reporter system. *Methods in Molecular Biology* **977**, 79–93 (2013).
113. Wu, M. R. *et al.* A high-throughput screening and computation platform for identifying synthetic promoters with enhanced cell-state specificity (SPECS). *Nature Communications* **10**, (2019).
114. Cole, S. W. *et al.* Computational identification of gene-social environment interaction at the human IL6 locus. *PNAS* **107**, 5681–5686 (2010).
115. Gray, S. J. *et al.* Methods Optimizing Promoters for Recombinant Adeno-Associated Virus-Mediated Gene Expression in the Peripheral and Central Nervous System Using Self-Complementary Vectors. *Hum Gene Ther* **22**, (2011).
116. Kügler, S., Kilic, E. & Bähr, M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Ther* **10**, 337–347 (2003).
117. Jin, L. *et al.* High-efficiency transduction and specific expression of ChR2opt for optogenetic manipulation of primary cortical neurons mediated by recombinant adeno-associated viruses. *J Biotechnol* **233**, 171–180 (2016).
118. Cheng, S. *et al.* Therapeutic efficacy of regulable GDNF expression for Huntington's and Parkinson's disease by a high-induction, background-free "GeneSwitch" vector. *Exp Neurol* **309**, 79–90 (2018).
119. Ekman, F. K. *et al.* CRISPR-Cas9-Mediated Genome Editing Increases Lifespan and Improves Motor Deficits in a Huntington's Disease Mouse Model. *Mol Ther Nucleic Acids* **17**, 829–839 (2019).

120. Zheng, L., Wang, Z., Liu, Y., Zhao, J. & Huang, S. Activation of the RMTg Nucleus by Chemogenetic Techniques Alleviates the Learning and Memory Impairment in APP/PS1 Mice. *Neuropsychiatr Dis Treat* **18**, 2957–2965 (2022).
121. May, F. J., Head, P. S. E., Venturoni, L. E., Chandler, R. J. & Venditti, C. P. Central nervous system-targeted adeno-associated virus gene therapy in methylmalonic acidemia. *Mol Ther Methods Clin Dev* **21**, 765–776 (2021).
122. Chandler, R. J. *et al.* Systemic AAV9 gene therapy improves the lifespan of mice with Niemann-Pick disease, type C1. *Hum Mol Genet* **26**, 52–64 (2017).
123. Colasante, G. *et al.* In vivo CRISPRa decreases seizures and rescues cognitive deficits in a rodent model of epilepsy. *Brain* **43**, 891–905 (2020).
124. Maubach, G., Lim, M. C. C., Zhang, C. Y. & Zhuo, L. GFAP promoter directs lacZ expression specifically in a rat hepatic stellate cell line. *World J Gastroenterol* **12**, 723–730 (2006).
125. Lee, Y., Messing, A., Su, M. & Brenner, M. GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia* **56**, 481–493 (2008).
126. Do Thi, N. A. *et al.* Delivery of GDNF by an E1,E3/E4 deleted adenoviral vector and driven by a GFAP promoter prevents dopaminergic neuron degeneration in a rat model of Parkinson's disease. *Gene Ther* **11**, 746–756 (2004).
127. Tkatch, T. *et al. An efficient rAAV vector for protein expression in cortical parvalbumin expressing interneurons*. *Nature* (2022).
128. Barnes, S. *et al.* Disruption of mGluR5 in parvalbumin-positive interneurons induces core features of neurodevelopmental disorders. *Mol Psychiatry* **20**, 1161–1172 (2015).
129. * Duba-Kiss, R., Niibori, Y. & Hampson, D. R. GABAergic Gene Regulatory Elements Used in Adeno-Associated Viral Vectors. *Front Neurol* **12**, (2021).
130. Drexel, M., Rahimi, S. & Sperk, G. Silencing of Hippocampal Somatostatin Interneurons Induces Recurrent Spontaneous Limbic Seizures in Mice. *Neuroscience* **487**, 155–165 (2022).
131. Goff, K. M. & Goldberg, E. M. A Role for Vasoactive Intestinal Peptide Interneurons in Neurodevelopmental Disorders. *Dev Neurosci* **43**, 168–180 (2021).
132. Bechtold, D. A., Brown, T. M., Luckman, S. M. & Piggins, H. D. Metabolic rhythm abnormalities in mice lacking VIP-VPAC2 signaling. *Am J Physiol Regul Integr Comp Physiol* **294**, 344–351 (2008).
133. Hoshino, C. *et al.* GABAergic neuron-specific whole-brain transduction by AAV-PHP.B incorporated with a new GAD65 promoter. *Mol Brain* (2021).
134. DeRosa, B. A. *et al.* HVGAT-mCherry: A novel molecular tool for analysis of GABAergic neurons derived from human pluripotent stem cells. *Molecular and Cellular Neuroscience* **68**, 244–257 (2015).
135. Wang, W. *et al. Impact of different promoters, promoter mutation, and an enhancer on recombinant protein expression in CHO cells*. *Nature* (2017).

136. Freundlieb, S., Schirra-Müller, C. & Bujard, H. A Tetracycline Controlled Activation/Repression System with Increased Potential for Gene Transfer into Mammalian Cells. *Journal of Gene Medicine* **1**, 4–12 (1999).
137. Das, A. T., Tenenbaum, L. & Berkhout, B. Tet-On Systems For Doxycycline-inducible Gene Expression. *Curr Gene Ther* **16**, 156–167 (2016).
138. Wang, L. *et al.* Enhancing Transgene Expression from Recombinant AAV8 Vectors in Different Tissues Using Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element. *Int. J. Med. Sci* **13**, 286–291 (2016).
139. Glover, C. P. J., Bienemann, A. S., Heywood, D. J., Cosgrave, A. S. & Uney, J. B. Adenoviral-mediated, high-level, cell-specific transgene expression: A SYN1-WPRE cassette mediates increased transgene expression with no loss of neuron specificity. *Molecular Therapy* **5**, 509–516 (2002).
140. Yang, Y. *et al.* Sonothermogenetics for noninvasive and cell-type specific deep brain neuromodulation. *Brain Stimul* **14**, 790–800 (2021).
141. Wang, C. Y. & Wang, S. Astrocytic expression of transgene in the rat brain mediated by baculovirus vectors containing an astrocyte-specific promoter. *Gene Ther* **13**, 1447–1456 (2006).
142. Muhuri, M. *et al.* Novel Combinatorial MicroRNA-Binding Sites in AAV Vectors Synergistically Diminish Antigen Presentation and Transgene Immunity for Efficient and Stable Transduction. *Front Immunol* **12**, (2021).
143. Bak, R. O. & Porteus, M. H. CRISPR-Mediated Integration of Large Gene Cassettes Using AAV Donor Vectors. *Cell Rep* **20**, 750–756 (2017).
144. Francis, J. S. *et al.* Preclinical biodistribution, tropism, and efficacy of oligotropic AAV/Olig001 in a mouse model of congenital white matter disease. *Mol Ther Methods Clin Dev* **20**, 520–534 (2021).
145. Bijlani, S., Anubhav, ·, Nahar, S. & Ganesan, · K. Improved Tet-On and Tet-Off systems for tetracycline-regulated expression of genes in Candida. *Curr Genet* **64**, (2018).
146. Beghi, E. *et al.* Global, regional, and national burden of epilepsy, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* **18**, 357–375 (2019).
147. Harrington, L. B. *et al.* Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science* **362**, 839–842 (2018).
148. * Ogbonmide, T. *et al.* Gene Therapy for Spinal Muscular Atrophy (SMA): A Review of Current Challenges and Safety Considerations for Onasemnogene Abeparvovec (Zolgensma). *Cureus* **15**, (2023).
149. Otsuki, N. *et al.* A new biomarker candidate for spinal muscular atrophy: Identification of a peripheral blood cell population capable of monitoring the level of survival motor neuron protein. *PLoS One* **13**, (2018).