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Engineered Viral Vectors for Functional Interrogation, Deconvolution, and Manipulation of Neural Circuits

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Abstract

Optimization of traditional replication-competent viral tracers has granted access to immediate synaptic partners of target neuronal populations, enabling the dissection of complex brain circuits into functional neural pathways. The excessive virulence of most conventional tracers, however, impedes their utility in revealing and genetically perturbing cellular function on long time scales. As a promising alternative, the natural capacity of adeno-associated viral (AAV) vectors to safely mediate persistent and robust gene expression has stimulated strong interest in adapting them for sparse neuronal labeling and physiological studies. Furthermore, increasingly refined engineering strategies have yielded novel AAV variants with enhanced target specificity, transduction, and retrograde trafficking in the CNS. These potent vectors offer new opportunities for characterizing the identity and connectivity of single neurons within immense networks and modulating their activity via robust delivery of functional genetic tools.

Introduction

Since herpes simplex virus was first used as a polysynaptic viral tracer for deciphering the input and output connectivity of the brain[1], a range of neurotropic viral vectors has been developed to achieve controlled unidirectional tagging of first-order presynaptic or postsynaptic neurons (Figure 1). These transsynaptic viral tracers have enabled pathway-specific gene delivery and forward screening of structural and functional connections of target neuronal populations with unprecedented precision, though their virulence often limits

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Conflict of Interest Statement

DVS is an inventor on patents involving AAV directed evolution and a co-founder of a company developing AAV vectors for clinical gene therapy.

studies to shorter terms[2]. In parallel, genetically encoded calcium and voltage indicators[3] and opsins[4], coupled with transgenic technologies to achieve cell-type-specific expression, have been exploited to monitor and modulate neural activity with high temporal and spatial control. However, the long-term study and manipulation of networks defined by their connectivity, rather than their genetic profile, have been thwarted by the challenge of developing viral vectors that mediate persistent and robust expression with minimal neurotoxicity.

Vectors based on adeno-associated virus (AAV) – which offer a favorable safety profile, low immunogenicity, and ability to transduce and persist as stable episomes in post-mitotic cells of the CNS – have emerged as safe and increasingly promising technologies for treating neurological disorders, yielding encouraging results in Phase I/II clinical trials for Parkinson's, Batten's, and Canavan's disease after a single vector administration[5, 6]. Furthermore, AAV capsid proteins are encoded by a single *cap* gene (2.2 kb), which the field has progressively shown can be modified to manipulate the virus's tropism and other infectious properties[7] (Figure 2a), leading to a strong interest in developing targeted AAV vectors for both enhanced therapeutics and long-term *in vivo* evaluation of neural circuits. AAV structural analyses[8–11], coupled with an understanding of mechanisms that determine biodistribution and delivery efficiency[12–14], can aid in the rational design of enhanced AAV vectors. In contrast, directed evolution is an approach that can achieve improvements in AAV vector performance without necessitating pre-existing structural and mechanistic knowledge of virus-cell interactions[15–20]. This technique involves genetic diversification of the *cap* gene[17, 21–26] (Figure 2b,c) and iterative rounds of phenotypic selection to drive convergence toward the fittest clones (Figure 2d).

This review focuses on strategies for engineering AAV variants with enhanced neurotropism and transport capabilities in the CNS. The resulting vectors, summarized in Table 1, can be coupled with a range of transgenes and gene regulatory elements, to visualize individual cells, track their activity, and direct their behavior.

Improving monosynaptic input mapping of neural circuits

Prior to the adoption of AAV for circuit mapping, replication-competent viral vectors capable of crossing synapses were widely employed for neuroanatomical studies. Glycoprotein (G)-deleted rabies virus (RV*dG*), pseudotyped with EnvA, was engineered to infect target cell types and restrict viral spread to direct presynaptic inputs[27]. Since the creation of RV*dG*, complementation with an optimized glycoprotein (oG) was found to improve efficiency of monosynaptic retrograde labeling by 20-fold[28]. Furthermore, another strain of rabies (RABV) called CVS-N2c was discovered to exhibit enhanced neurotropism and retrograde transfer with reduced immunogenicity, compared to the traditional SAD-B19 strain[29]. Vesicular stomatitis virus (VSV) can also be endowed with retrograde or anterograde polysynaptic tracing ability by pseudotyping with RABV-G or VSV-G, respectively, and made monosynaptic by supplying the G proteins *in trans*[30]. An analogous replication-conditional system has been devised for anterograde trafficking of herpes simplex virus (HSV), where thymidine kinase complementation rescues viral replication in

the infected cell and mediates expression in monosynaptically connected output populations[31].

These engineered transsynaptic viral tracers have offered more complete illumination of neurons and their dendritic arbors than their non-viral counterparts and thus enabled many advances; however, the rapid onset of neurotoxicity associated with their infection and replication hamper their application to physiological and behavior studies. That said, the recent development of a self-inactivating RVdG (SiR) that leaves a permanent genetic signature shortly before being transcriptionally silenced has extended the temporal window for optical imaging and functional interventions from 5–17 days to several months post-infection[32]. In contrast, lentiviruses[33] and AAVs[34] enable persistent, non-toxic gene expression and can provide local delivery of functional tools to analyze primarily efferent pathways originating from the injection site and to manipulate the biology of targeted neurons[2]. Furthermore, lentiviruses pseudotyped with fusion glycoproteins derived from VSV-G and RABV-G have enabled retrograde labeling of infected neuronal populations[35]. The remainder of this review delves into the diverse and growing toolkit of neurotropic AAV variants that merge viral vectors with functional and single-cell approaches.

Engineered AAV vectors with enhanced target infectivity and specificity

Most natural AAV serotypes preferentially transduce neurons following intraparenchymal administration[36–39], a property that has been harnessed for anatomical studies of bulk population connectivity[34]. AAVs have also been valuable tools for investigating retinal circuits in physiological and pathological conditions through *in vivo* imaging and electrical stimulation[40]. Furthermore, AAV-based delivery of donor templates has recently shown CRISPR-mediated targeted integration via homology-directed repair in mature post-mitotic neurons, enabling monitoring of endogenous proteins[41]. Nonetheless, infectivity and selectivity of natural AAVs for certain functionally relevant neuronal populations are suboptimal[34], and engineering approaches have been applied to the AAV capsid to create new vectors with improved neuronal tropism.

Photoreceptors have been the primary neuronal target in the retina, where intravitreal injections of AAV vectors have been widely pursued to avoid the adverse procedural effects of subretinal injections and engineering approaches have been applied to generate new AAVs that achieve robust pan-retinal distribution from this route[40]. For instance, surface-exposed tyrosines have been found to undergo intracellular phosphorylation and thereby mediate ubiquitylation and vector degradation, and tyrosine (Y) to phenylalanine (F) mutations have, in some cases, increased viral infectivity[42]. For example, intravitreal administration of AAV2-4YF, a quadruple Y-to-F mutant, mediates expression throughout the mouse retina, but only at high doses[43]. An additional tyrosine-to-valine mutation confers a three-fold improvement in photoreceptor transduction[44]. AAV2-4YF is also more effective than its parent AAV2 in reaching the outer layers of the canine retina but at lower efficiency than in the mouse eye[45]. Following these rational design efforts, the first application of *in vivo* selection in the CNS employed administration of large (~10^7) AAV capsid libraries, followed by isolation of rod photoreceptors and recovery of vectors that localized to these target cells[46]. This approach identified an AAV2 variant called 7m8, which mediates highly

efficient gene delivery to photoreceptors and retinal pigment epithelium across the entire murine retina from a simple intravitreal injection[46]. While 7m8 was also found to be the most efficient intravitreal vector to date in non-human primate, additional engineering is necessary to provide efficacious vectors in large animal models, illustrating that optimal vectors in one species may not translate to others[46].

AAV has also been engineered for enhanced neuronal tropism and spread in the brain. For example, tyrosine mutations on the AAV2 capsid improve neuronal transduction in the rat brain[47]. Altering known binding footprints of natural AAVs can also affect their biodistribution. For instance, ablation of heparan sulfate (HS) binding enhances volumetric spread of AAV2[47], while the HS and galactose binding domains of an AAV2/9 chimeric vector named AAV2g9 enable it to preferentially infect neurons over glia after intracerebroventricular (ICV) administration[48]. Fortuitously, a vector devised to present a polyalanine peptide as a control in one study[49], termed AAV-AS, yielded 15-fold greater transduction of the mouse brain from systemic administration relative to AAV9, with extensive neuronal delivery to the motor cortex, striatum, and spinal cord[50]. Interestingly, AAV-AS demonstrated significantly reduced neuronal infectivity in certain brain regions (e.g. thalamus), suggesting that it utilizes cell surface receptor(s) with varying expression levels across the CNS. Finally, a randomly shuffled chimeric vector called AAV-B1 delivered 8.6-fold more vector genome copies to the brain than AAV9 upon intravenous administration, facilitating higher neuronal transduction in the motor cortex, thalamus, and spinal cord[51].

Astrocytes have also become increasingly attractive targets for functional investigation, due to their emerging roles in instructing synapse formation and maturation during neural circuit development[52]. Directed evolution was conducted to engineer AAVs that, in contrast to natural serotypes, preferentially infect glia over astrocytes, and the resulting chimeric variants ShH19 and ShH13 both exhibited a pronounced shift in tropism toward astrocytes in the rat striatum[21]. Specifically, ShH19 transduces 5.5-fold more astrocytes than parental AAV2[21]. ShH13 also robustly infects Muller cells after subretinal injection, suggesting a common mechanism for enhanced entry into glial cells[21]. Interestingly, an AAV6 variant called ShH10 demonstrates >90% selectivity for rat Muller glia upon intravitreal injection and >60% greater infectivity than AAV2[21, 53]. Oligodendrocytes have also garnered interest in light of recent findings that they dynamically shape the myelin profile of neural networks[54]. An interesting variant generated from random capsid shuffling and directed evolution, Olig001, exhibits >95% selectivity for rat oligodendrocytes, which markedly distinguishes it from most natural AAVs[55].

In addition to genetic manipulation of post-mitotic cells in the CNS, interest in exploring and improving the regenerative capacity of the adult mammalian brain has fueled the generation of novel AAV vectors with improved *in vivo* delivery to adult neural stem cells (NSCs). In the subgranular zone (SGZ), a mutant of AAV2 that was evolved on cultured adult rat hippocampal NSCs named AAV r3.45[18] mediates up to five-fold more selective and three-fold more efficient transduction of NSCs than AAV2, 4, and 6 when administered directly into the rat dentate gyrus[56]. Similar properties were observed in the mouse hippocampus. To engineer an AAV variant for the subventricular zone (SVZ), a Cre-

dependent strategy that enables recovery of clones that transduce a target cell population, in this case NSCs, was used to isolate a chimeric AAV vector called SCH9 that infects 60% of NSCs in both hemispheres after a unilateral ICV injection[57]. These engineered vectors can potentially be exploited to gain a deeper understanding of the contextual cues and regulatory mechanisms that govern adult NSC fate decisions, as well as their contributions to adult brain function.

Evolved AAV vectors for functional circuit interrogation

Delivery of sensors and effectors to projection neurons through retrograde transport enables investigation of long-range connections that link circuit modules and coordinate the interplay between large neural networks[58]. AAV was first shown to enter axonal terminals and shuttle its payload to cell nuclei in mice[59] and more recently, to a greater extent, in non-human primates[60–63]. However, the utility of natural AAVs for circuit interrogation in the murine brain is limited by the inefficiency of their transport[59]. AAV libraries were injected into downstream targets of projection neurons, and viral genomes that were retrogradely transported to the cell bodies were recovered. A resulting vector, AAV2-retro, offered up to two orders of magnitude greater retrograde access in the corticopontine tract[64], and this high efficiency extended to numerous other pathways.

AAV-mediated retrograde transport to lower motor neurons from intramuscular administration has also been explored to access specific regions of the spinal cord, but this approach is again hampered by a low transport efficiency of less than 1%[65]. Tet1, a peptide with high affinity for the tetanus toxin GT1b receptor that undergoes retrograde transport in the spinal cord[66], was grafted onto AAV1 to target the capsid for enhanced axonal terminal binding and uptake[67]. Indeed, this rationally engineered vector exhibited a four-fold enhancement over AAV1 in DRG explants.

AAV's natural ability to undergo anterograde transport primarily in non-human primates[61, 68–70], coupled with its minimal neurotoxicity, has sparked exploration of its use as a trans-synaptic tracer. AAV1 was recently established as a superior anterograde tracer compared to other serotypes in the corticocollicular pathways of the murine brain, but required high titers and Cre-mediated amplification to unlock robust transgene expression in up to 40% of postsynaptic V1-recipient neurons[71]. In contrast with replication-competent transsynaptic tracers that spread uncontrollably across multiple serial synapses, AAV1 only tags first-order downstream structures, providing more precise labeling of neural circuits. This attractive feature compels the development of modified AAV vectors with enhanced transneuronal transduction, and a more comprehensive mechanistic understanding of anterograde transport[72, 73] may benefit these engineering efforts.

Engineered AAV variants for sparse neuronal labeling

To further dissect the architecture of neural networks and the mechanisms that govern their development, function, and plasticity, deconvolution of complex circuits has primarily been pursued with sparse, stochastic labeling to distinguish individual cells[74]. AAV-mediated delivery of multicolor expression cassettes, such as Brainbow[75], has conferred ease of

genetic manipulation, spatial and temporal control, and applicability to larger species, but non-uniform vector distribution from direct intracranial administration often renders cellular tracing and functional analysis of a target region challenging[76]. ICV administration may offer a route for stochastically labeling regions that border the cerebrospinal fluid[57]. Alternatively, systemic AAV administration to access the brain diffusely via its vasculature may reduce variability in vector copies delivered per cell, and vector dosage can be modulated to control labeling density[77].

The natural serotype AAV9 is capable of crossing the formidable blood-brain barrier (BBB) in the adult mammalian brain, resulting in widespread astrocytic transduction but sparse neuronal delivery[78]. AAVrh8 and AAVrh10 were subsequently shown to display similar biodistribution profiles in the CNS[79]. Moreover, mutation of surface-exposed tyrosine residues on AAV9 further increases infectivity[80]. Directed evolution has also recently been employed, in combination with a Cre-dependent selection strategy, to isolate AAVs capable of crossing the BBB[77, 81]. With this approach, AAV-PHP.B, an AAV9 variant containing a 7mer peptide insertion, was found to transduce the majority of astrocytes and neurons and a moderate fraction of oligodendrocytes in multiple regions after intravenous administration, resulting in at least 40-fold greater gene transfer throughout the CNS than AAV9 in mouse. AAV-PHP.A, another clone that emerged from the same selection, exhibited weaker but more selective astrocytic transduction than AAV-PHP.B[81].

Further evolution on promising engineered vectors has produced new third-generation AAV capsids that retain their parental tropism but show even more enhanced infectivity of a target cell population, mimicking the natural evolutionary search for local maxima in the protein fitness landscape[82]. AAV PHP.eB was generated through additional diversification of the heptamer and flanking amino acids of AAV PHP.B[81], followed by two more rounds of *in vivo* selection in mice[77]. At a relatively low systemic dose of 1×10^{11} viral genomes per mouse, AAV PHP.eB facilitated delivery to 69% of cortical neurons, 55% of striatal neurons, and a similar fraction of glia in these two regions compared to AAV PHP.B. This work also identified AAV PHP.S, which provided a two-fold increase in transduction of dorsal root ganglion (DRG) neurons over AAV9 and strong localization to cardiac and enteric ganglia. These potent vectors, which can deliver multiple genomes per cell, can potentially be harnessed for sparse multicolor labeling with high transgene expression to enable morphological studies at the single cell level. Alternatively, they can be utilized to record or perturb the activity of individual cells.

Conclusion

The engineered AAV viruses discussed here represent a diverse collection of vectors that have the potential to revolutionize the investigation of the mammalian connectome and the underlying functionalities of embedded neural circuits. Their ability to mediate robust expression in functionally relevant cell types of the CNS may also facilitate population-wide gene ablation, repression, or upregulation to generate improved mouse models that mimic postnatal onset of certain diseases. To further expand the available toolkit of AAV vectors, directed evolution strategies described above can be applied to other cell types of interest (e.g. oligodendrocytes), as well as other species to address the ongoing challenge that

vectors engineered in mouse rarely translate to large animals (including humans). Moreover, design of paradigms that select against transduction of antigen-presenting cells (APCs) in the brain can prevent cytotoxic immune responses and permit more extensive studies in non-human primates[83]. Advances in promoter engineering[84] will also aid in restricting expression to defined populations. Lastly, increasing AAV's packaging capacity, potentially via modulating residues on the capsid lumen that interact with its genome[85], will extend its applicability to larger transgenes and eliminate the need for less efficacious multi-vector systems. Such engineering approaches will further build momentum for AAV's application to both basic biological investigation and therapeutic translation.

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Highlights

- Recombinant AAV vectors are well suited for *in vivo* gene delivery to the CNS.
- AAV vectors can be engineered for enhanced target infectivity and specificity.
- New AAVs with improved retrograde access enable functional circuit interrogation.
- AAV variants capable of crossing the BBB may facilitate sparse neuronal labeling.

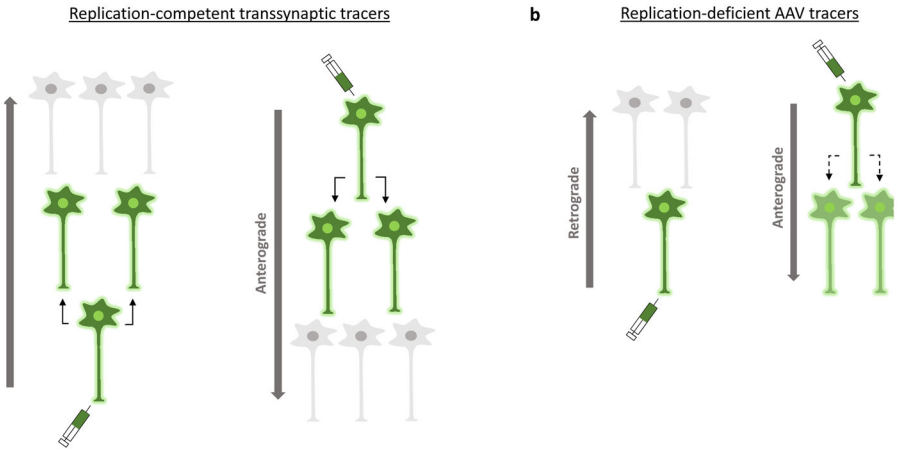

Figure 1. Retrograde and anterograde transport of viral tracers

a) Trans-synaptic labeling using replication-competent viral vectors. Co-delivery of viral tracers and the glycoprotein necessary for replication labels infected cells and their direct presynaptic inputs or postsynaptic outputs through viral transmission.

b) Axonal transport of replication-deficient AAV vectors. AAV-mediated retrograde access can mediate long-term transgene expression in projection neurons. Putatively, anterograde transport of AAV vectors can also occur when some intact virions escape the initial infected neuron and transduce first-order downstream populations, but high titers and catalytically active cargos are necessary to detect trans-synaptic labeling.

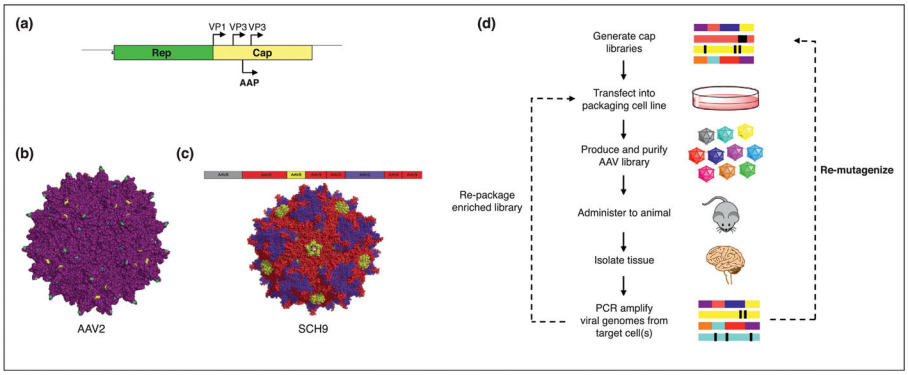

Figure 2. Directed evolution of AAV vectors

a) Schematic of the native AAV genome. The *rep* ORF encodes non-structural proteins that are necessary for viral replication, transcriptional regulation, and virion assembly. The *cap* ORF encodes three structural proteins (VP1, VP2, and VP3) that assemble into a 60-mer viral capsid with the aid of the assembly-activating protein (AAP) encoded by an alternate ORF. The *cap* gene can be modified to manipulate the virus's tropism and infectious properties.

b) Representative target sites for mutagenesis on the capsid surface of AAV2. Peptide ligands can be incorporated at R588 (yellow), a residue near the three-fold protrusions on the capsid surface. Alternate insertion sites include the N-terminus of VP2 (not shown) and residue G453 (green) located on the highest three-fold spike. Similar positions on other AAV serotypes can also be targeted for mutagenesis. Mutation of surface exposed tyrosines, such as Y272 (cyan) on the AAV2 capsid, can yield more potent vectors. Image was rendered in PyMOL using the AAV2 crystal structure (PDB ID: 1LP3).

c) 3D structure of SCH9, an example of a chimeric AAV vector created by recombining *cap* genes from natural AAV serotypes. Each parent serotype is represented by a different color (AAV9 red, AAV8 yellow, AAV2 purple) in a schematic of the *cap* gene and mapped onto the AAV9 crystal structure (PDB ID: 3UX1) in PyMOL.

d) Paradigm for directed evolution of AAV vectors. The *cap* gene is diversified to generate large *cap* libraries that are transfected into a packaging cell line to produce viral particles, such that each capsid variant surrounds the *cap* gene encoding it. Purified capsid libraries are administered to an animal, and a functional selective pressure is imposed by harvesting the tissue of interest. Viral genomes are amplified by PCR to recover *cap* variants that localized to the target cell(s). Variants are re-packaged into virions and enriched through iterative rounds of selection. Alternatively, recovered *cap* genes can be further mutagenized.

Table 1

Engineered AAV vectors and their tropism.

Variant	Route of administration	Tropism	Species	Additional notes	Reference
AAV2(4YF)	Intravitreal	Inner retina > PR > RPE	M, C	High doses required	*1, 2*
AAV2(4YF + T-V)	Intravitreal	Inner retina > PR > RPE	M	High doses required	*3*
7m8	Intravitreal	Pan-retinal	M, NHP		*4*
AAV2 T2 3Y+*dH*	Intracranial	Neurons	R	Improved spread	*5*
AAV2g9	ICV (neonates)	Neurons > astrocytes	M		*6*
ShH19	Intracranial	Enhanced astrocyte transduction	R		*7*
ShH13	Intracranial, subretinal	Enhanced astrocyte and Muller glia transduction	R		*7*
ShH10	Intravitreal	Muller glia ≫ inner retina	R		*8*
Olig001	Intracranial	Oligodendrocytes ≫ neurons	R		*9*
AAV r3.45	Intracranial (hippocampus)	Adult NSCs > neurons	M, R		*10*
SCH9	Unilateral ICV	Adult NSCs in SVZ	M	Similar transduction profile in both hemispheres	Ojala *et al*, in press
AAV2-retro	Intracranial	Retrograde access to neurons	M		*11*
AAV1-Tet1	N/A	Retrograde access to DRG neurons	R		*12*
AAV-AS	Intravenous	Neurons, astrocytes, endothelia	M, F	Variable neuronal transduction profile across brain regions	*13*
AAV-B1	Intravenous	Neurons, astrocytes, endothelia	M, F	Variable neuronal transduction profile across brain regions	*14*
AAV-PHP.B	Intravenous	Neurons, astrocytes, oligodendrocytes, endothelia	M	Majority of neurons and astrocytes transduced	*15*
AAV-PHP.A	Intravenous	Astrocytes	M		*15*
AAV-PHP.eB	Intravenous	Neurons, astrocytes, oligodendrocytes, endothelia	M	Enhanced neuron and Purkinje cell transduction relative to AAV-PHP.B	*16*
AAV-PHP.S	Intravenous	DRG, cardiac and enteric ganglia	M		*16*

PR, photoreceptors; RPE, retinal pigment epithelium; ICV, intracerebroventricular; SVZ, subventricular zone; NSC, neural stem cell; DRG, dorsal root ganglia; M, mouse; R, rat; C, canine; F, feline; NHP, non-human primate.

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