Adeno-Associated Virus Technologies and Methods for Targeted Neuronal Manipulation

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Abstract

Cell-type-specific expression of molecular tools and sensors is critical to construct circuit diagrams and to investigate the activity and function of neurons within the nervous system. Strategies for targeted manipulation include combinations of classical genetic tools such as Cre/loxP and Flp/FRT, use of cis-regulatory elements, targeted knock-in transgenic mice, and gene delivery by AAV and other viral vectors. The combination of these complex technologies with the goal of precise neuronal targeting is a challenge in the lab. This report will discuss the theoretical and practical aspects of combining current technologies and establish best practices for achieving targeted manipulation of specific cell types. Novel applications and tools, as well as areas for development, will be envisioned and discussed.

Introduction

Understanding neural networks as they relate to development, behavior, and learning is a critical objective of neuroscience. These questions can be addressed, in part, by understanding the role of specific neural cells and brain regions, as well as the impact of individual molecules in these circuits. The successful execution of these neurobiology studies requires methods that are highly targetable, efficient, and precise. In this regard, recombinant adeno-associated viral vectors (herein referred to as AAV) are powerful tools that can be used both to target and manipulate specific neuronal subtypes (defined based on gene expression, location, and connectivity) and non-neuronal cell types within the nervous system.

Scientists using AAV for gene transfer and/or neuronal targeting must consider various questions about experimental design, including (1) how to best deliver/administer AAV (Fig. 1A), (2) which AAV serotype to use (Fig. 1B), and (3) how to drive gene expression with gene regulatory elements (both within the AAV genome and the host animal or cell line) (Fig. 1C-F). These and many other factors can affect how efficiently cells of interest are targeted by AAV. Further, experimental parameters such as AAV titer and dosage can impact AAV efficiency, and these details are often omitted from experimental methods in the literature and can be expensive and timely to determine empirically for each study. Overall, designing an experiment with AAV is multifaceted and suboptimal experimental design can drastically reduce the quality of results. In this report, we will discuss practical aspects of using AAV and considerations for designing experiments.

Selecting the Route of Administration and Capsid

AAV tropism, as dictated by AAV capsid proteins, is an important factor affecting transduction efficiency and specificity across cell types. Since the mechanism of AAV transduction is through interaction of the AAV capsid with cell surface proteins and glycans, protein composition of the capsid (i.e., the AAV serotype) and the cell surface (i.e., based on cell type) determines transduction efficiency. Consequently, serotype and route of delivery should be carefully considered when designing experiments (Fig 1A, B). For an overview of the primary receptors for AAV serotypes, see (Schultz and Chamberlain, 2008).

Direct intraparenchymal delivery

When injected directly into the brain, many of the naturally-occurring AAV capsids, which share homology ranging from 65-99% (Drouin and Agbandje-McKenna, 2013), have distinct but significantly overlapping tropisms and distribution characteristics. AAV1, AAV2, AAV5, AAV8,

AAV9 and the engineered variant AAV-DJ are commonly used to target local populations of neurons after direct injections (Table 1). There are important differences in how far different capsid variants spread from the injection site – AAV2 and AAV-DJ diffusion is more confined and, therefore, these capsids are often chosen for applications that require precise targeting. While expression from AAV2 is mostly neuronal, several serotypes, including AAV1, AAV5, AAV8 and AAV9, also transduce astrocytes and oligodendrocytes.

Systemic delivery

Several natural AAV capsids cross the blood brain barrier (BBB). In contrast to direct injections, intravenous injections of AAV can provide central nervous system (CNS)-wide gene delivery. This activity is present across several species and is most pronounced when AAV is administered to the neonate. Neonatal injections of AAV9 and rh.10 have been used to transduce neurons broadly across the CNS. However, when delivered at later developmental stages including in the adult, transduction is more limited and primarily restricted to endothelial cells and astrocytes, with transduction occuring in 1-2% of neurons in the forebrain (Deverman et al., 2016; Dufour et al., 2014; Foust et al., 2009). In this context, engineered AAV capsids have provided new and dramatically more efficient options for widespread gene delivery to the CNS. The first of these vectors, AAV-PHP.B, enabled researchers to deliver genes to more than 50% of neurons and astrocytes across numerous brain regions with a single noninvasive injection (Deverman et al., 2016). Achieving this efficiency requires relatively high viral doses ($\sim 1 \times 10^{14}$ vector genomes/kg), thus requiring large volumes of high titer virus. A further-evolved AAV-PHP.B variant, AAV-PHP.eB, addresses this issue and can achieve >50% transduction of most neuron and astrocyte populations even with a 20-fold reduction in dose (Chan et al., 2017). While the activity of the PHP capsids is not universally observed across all species, the receptor engaged by the PHP capsids during AAV transduction has been identified and can be used to predict permissivities of cell or tissue types to these engineered capsids (Huang et al., n.d.). In addition, the same group has developed an additional AAV variant (the PHP.S variant) that can efficiently transduce dorsal root ganglia and other peripheral neuron populations following systemic administration, which should enable the study of these otherwise difficult to target peripheral neuron populations (Chan et al., 2017).

CSF delivery

A third option for gene delivery to the CNS is to inject vectors into the cerebral spinal fluid (CSF). Several access points can be used: the lateral ventricle (intracerebroventricular, ICV), the cisterna magna (CM), subpial (Miyanohara et al., 2016) or the intrathecal (IT) space along the spinal cord. When performed in neonates, ICV AAV administration can provide widespread gene delivery. In the adult, ICV and CM injections result in gene delivery in multiple brain regions, however, the expression is not uniform across all brain regions and superficial structures are preferentially targeted. Beyond neurons, ICV injections also provide access to periventricular cell populations. For example, after ICV injection, AAV4 can be used to transduce the ependymal cells (Liu et al., 2005), and two engineered AAV capsids, SCH9 and AAV4.18, enable transduction of subventricular zone neural progenitors (Murlidharan et al., 2015; Ojala et al., 2018). IT injection can be used to deliver genes to spinal cord motor neurons and dorsal root ganglions (Federici et al., 2012; Foust et al., 2013; Schuster et al., 2014).

Retrograde and anterograde transport for circuit studies

AAV vectors are also commonly used as part of circuit studies. Numerous natural AAV serotypes exhibit retrograde trafficking activity from their uptake at axon terminals. However, retrograde transduction with natural serotypes such as AAV1, AAV2, AAV6 and AAV9 requires

high vector doses due to the relative inefficiency of this transduction mechanism. More recently Tervo et al. (Tervo et al., 2016) and Davidsson et al. (Davidsson et al., n.d.) have created modified capsids AAV2-Retro and AAV MNM008, respectively, that provide efficient transduction of neurons that send axon projections into the injection site. Transduction efficiencies of both capsids are shown to circuit-dependent, and thus capsids should be validated for circuits of interest when planning experiments.

In summary, a consensus of opinion has not been reached regarding the best serotype for each cell type, brain region or application. Choosing the optimal serotype requires reviewing the literature most relevant to the planned experiment and performing pilot testing for new or at least for challenging applications. As new engineered capsids with unique features continue to be developed, the available options will become more numerous and more powerful (Chan et al., 2017; Davidsson et al., n.d.; Deverman et al., 2016; Ojala et al., 2018; Tervo et al., 2016)

Controlling Gene Expression with Regulatory Elements

Cre and Flp recombinase-dependent expression elements within AAV vectors remains the go-to system for restricting transgene expression to genetically defined cell types in model organisms. However, few Cre or Flp transgenic lines have been developed in other mammalian species. Furthermore, breeding multiple transgenic lines to generate the desired offspring can be time consuming and expensive. Therefore, there is significant interest in developing the means to achieve similar expression specificity in nontransgenic animals using flexible vector-based approaches that will translate across species.

Cis regulatory elements can be used to control transgene expression from AAV genomes. These elements include promoters and enhancers (Fig. 1C), as well as introns, micro-RNA recognition sequences, and internal ribosome entry sites (IRES) (Fig. 1D) that can be used to tailor RNA processing, stability, and translation to the experimental needs. Here we will discuss how these regulatory elements can be used to restrict AAV-mediated gene expression.

Enhancers and promoters

Enhancer and promoters (hereafter referred to as promoters for simplicity) can generally be divided into two classes: general/ubiquitous and cell type-specific. Typically, ubiquitous promoters provide high-level, long-term expression in most cell types, though some, such as the viral CMV promoter, have been shown to exhibit silencing in specific tissues over time (Klein et al., 1998; McCown et al., 1996; Paterna et al., 2000; Tenenbaum et al., 2004) (Table 2). High expression levels are not optimal for every application and alternative regulatory elements, such as those from the mammalian MeCP2 or PGK genes (Table 3) may be suitable for experiments where high-level viral enhancer driven expression is not desired. General recommendations for expression levels of various types of transgenes is summarized in Table 4.

Viruses evolved under constraints that drove the selection of short, strong promoters. In contrast, the regulatory elements that control mammalian gene transcription are often distributed over thousands to hundreds of thousands of bases. Due to the limited packaging capacity of the AAV genome, identifying AAV-compatible promoters has been challenging and the development of shortened promoters is an area of active study (de Leeuw et al., 2016; Nathanson, 2009). A list of cell type-specific promoters compatible with AAV vectors is provided in Table 3.

Multicistronic vectors

Although AAV vectors have a limited packaging capacity, it is possible to express multiple short transgenes from a single vector using one of several approaches: (1) separate translational units where each cDNA is controlled by separate 5' and 3' regulatory elements, (2) using IRES sequences to insert two separate translational units into a single mRNA, or (3) the use of viral 2A sequences to generate separate proteins from the same translational unit (Fig. 1D). Table 5 highlights considerations for choosing between the use of IRES sequences and 2A "self-cleaving" peptides.

One important consideration when evaluating expression strategies and determining specificity is that the expression levels required for reporter detection may not match what is necessary for the activity of an opsin, DREADD or recombinase. For example, fluorescent proteins are commonly used to evaluate gene regulatory elements and vector design. However, fluorescent reporter assays may give the false impression of specificity if high levels of expression are seen in target cell types and low-level expression goes undetected in off-target populations. If these same regulatory elements are then used to drive DREADDs or Cre, which can mediate their effects at low expression levels, then the specificity may appear reduced. If this goes unexamined, then the interpretation of experimental results could be compromised. Moreover, though they are commonly used, fluorescent proteins are not necessarily inert and can lead to immune responses in larger animals, and over-expression related toxicities in mice.

Post-translational regulatory elements

Transgene expression can also be controlled post-transcriptionally through the use of elements impacting RNA splicing, nuclear export, stability, and translation into proteins (Fig. 1E). Inclusion of an intron can have positive impacts on expression levels. Introns have also been combined creatively with recombinase sites and partially inverted transgenes to achieve tight intersectional control of transgene expression (Fenno et al., 2017, 2014). Many recombinant AAV genomes also include a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), which can dramatically enhance expression. For several examples of how inclusion of a WPRE affects expression from AAV vectors please see (de Leeuw et al., 2016).

Complementary miRNA target sites (TS) are frequently engineered into the 3' untranslated region (3' UTR) of AAV genomes to mitigate off-target transgene expression. These sequences are complementary to miRNAs expressed within off-target cell types but not within the target population. miRNA binding to the perfectly complementary miRNA TS results in degradation of the RNA. Inclusion of multiple copies of the short miRNA TS sequences can dramatically lower off-target transgene expression. For example, by incorporating three copies each of miR-1 and miR-122, which are specifically expressed in muscle and liver, respectively, Xie et al. reduced transgene expression from intravenously administered AAV9 in muscle, heart, and liver, while maintaining brain expression (Xie et al., 2011). miRNAs that enhance the restriction of lentiviral mediated gene expression to GABAergic neurons have also been identified (Keaveney et al., 2018; Xie et al., 2011). Given their short lengths, miRNA TS can be multiplexed within the same genome, making them attractive elements for reducing expression outside of the cell type of interest.

Knock-in driver Cre and Flp mouse lines

The mammalian central nervous system contains extremely diverse types of neurons. These neuronal cell types can be distinguished by their intrinsic gene expression profiles, which are differentially regulated throughout development. Binary expression systems of drivers and

reporters can be used to drive transgene expression in genetically-defined cells (reviewed in (Huang et al., 2014)).

There are two general strategies for using binary expression systems to access genetically-defined cell types: transactivation-based systems (based on tet-response elements, TRE) and recombination-based systems (based on lox-site recombination, lox) (described in (Taniguchi, 2014)) (Fig. 1F). Gene targeting techniques can be used to insert *Cre* or *Flp* site-specific recombinases in the mouse genome. These knock-in driver mouse lines express Cre or Flp under the activity of a target gene's endogenous promoter. Thus, Cre and Flp driver mouse lines constitute a genetic switch to turn on a recombinase-dependent reporter or effector. Since the recombinase is only expressed in cells defined by the target gene's endogenous promoter activity, this system allows labeling and manipulation of neurons defined by the targeted gene's expression pattern.

The reporter or effector whose expression is dependent on the driver's activity is introduced in vivo by either crossing the driver to a transgenic reporter mouse line, using a viral vector, or electroporating the DNA construct into the cells. For example, with the widely used Cre driver, the conditional reporter expression depends on Cre-dependent recombination of specific lox sites.

Targeting at random versus targeting to a specific gene's locus
Knock-in Cre and Flp recombinases can be inserted in the genome either randomly or at a particular gene locus (Fig. 1F). Conventional transgenic and BAC transgenic approaches are targeted at random into the genome. However, knock-in mouse lines targeted to a specific locus by homologous recombination have the advantage that expression of the inserted recombinase will recapitulate the expression pattern within cells of the endogenous gene of interest. There are several advantages to using targeted knock-in Cre driver mouse lines (Table 6). Targeted knock-in Cre or Flp mouse lines can be inserted at the gene's transcription/translation initiation site. When using this strategy, it should be noted that individuals can exhibit variable levels silencing. Typically, an optimal "non-silencing" male should be identified and used for genetic crosses. However, the offspring of this male may exhibit silencing and must be revalidated.

Temporal control with tamoxifen
To control the expression of a reporter in a subset of neurons that uniquely and transiently expresses a certain marker at a particular time point, tamoxifen-inducible recombinases (Feil et al., 2009), so-called CreER or FlpER, can be used as drivers. In CreER driver mice, activation of the expressed Cre recombinase requires administration of the estrogen receptor modulator drug tamoxifen to the animal, allowing for temporal control of the subpopulation.

The typical tamoxifen dose varies from 10-200 mg/kg, depending on the desired degree of recombination at the reporter allele. In effect, this controls the sparseness/density of the labeling of the targeted neuronal population. The timing for tamoxifen administration depends on the temporal characteristics of the promoter driving CreER in the specific population to be targeted. Importantly, the half-life of tamoxifen (approximately 48 hours) must be taken into consideration. Tamoxifen preparation is detailed in the published protocol in (Vaughan et al., 2015).

Tamoxifen can be administered in one of three ways, depending on the desired developmental time point for activating the CreER driver: oral gavage to the mother for embryonic induction; subcutaneous injection to offspring for early postnatal induction, or intraperitoneal injection in offspring for late postnatal and adult induction.

Best practices for induction of CreER or FlpER mouse lines
To generally improve the reliability of results obtained with CreER or FlpER mouse lines, tamoxifen dosage should be varied. If the level of recombination of the reporter achieved with a high dose of tamoxifen is low, administration of 4-hydroxytamoxifen (the native form of tamoxifen) can improve activation of CreER (Jahn et al., 2018). Tamoxifen needs to be metabolized in the liver to reach its active form so it can act on CreER. However, it can be preferred over 4-hydroxytamoxifen due to lower cost. Consider that the CreER or FlpER will be active for 48 hours following tamoxifen induction, which may impact induction during developmentally active time periods. Administration of tamoxifen by gavage to the pregnant mother to induce the pups at embryonic timepoints can lead to problems of miscarriage or poor mothering. When administering tamoxifen at embryonic timepoints, use of Swiss or CD1 compared to C57Bl6 mice can improve outcome in two ways: they produce larger litters and females are better mothers, which overall can improve pup survival.

Validating a knock-in driver mouse line
Knock-in Cre driver mouse lines are designed to control expression of reporter probes in genetically-defined cell-types. It is important to validate that the driver mouse line expressing a site-specific recombinase (e.g., Cre, CreER, Flp, FlpER) reflects the endogenous expression pattern of the gene targeted by the knock-in driver. Various approaches can be used separately or jointly to validate a knock-in driver mouse line: (1) Crossing the mice with a suitable reporter like Rosa26-CAG-LSL-td-tomato (Ai14) or Rosa26-CAG-LSL-h2B-GFP and assessing brainwide expression, (2) Immunostaining of the target regions, (3) dual fluorescent in situ hybridization (dual fISH) with probe for reporter (e.g., RFP or GFP for Rosa26-CAG-LSL-td-tomato (Ai14) or Rosa26-CAG-LSL-h2B-GFP, respectively). Note that assessing Cre lines by crossing to a reporter line gives an integrated view of Cre activity over the lifetime of the animal. To assess Cre activity in the target cell population at the particular age of interest, a viral vector with a Cre-dependent reporter in a Cre line can be used. Similarly, for CreER lines tamoxifen induction should be performed at different developmental timepoints to assess temporal specificity.

In addition, leakiness of both mouse lines and viruses must be assessed prior to interpretation of experimental results that rely on the complete restriction of off-target Cre and/or transgene expression. Leakiness of a mouse line can be assessed when validating the mouse line, ensuring that expression of the site-specific recombinase or activator (i.e., Cre, CreER, Flp, FlpER or tTA) is consistent with the gene of interest in its native form. Crossing the driver to a reporter (e.g., fluorescent reporter) and performing dual fluorescent in situ hybridization (dual fISH), with probes for the fluorescent reporter and for the gene of interest allows one to check that endogenous expression of the gene and activity of the driver are present in the same cells. Using knock-in driver mouse lines instead of BAC driver mouse lines allows for targeted expression, with a higher likelihood of expression within cells expressing the endogenous gene of interest. Leakiness of the tamoxifen-inducible driver mouse line can be assessed by crossing the inducible driver CreER or FlpER to a Cre or Flp dependent fluorescent reporter, and checking for expression of the fluorescent protein without administering tamoxifen. Finally, leakiness of the Cre- or Flp-dependent or tTA-activated AAV can be checked by injecting the virus in a mouse that has been crossed to a fluorescent reporter mouse line for the driver/activator. If expression from the virus and from the mouse reporter line match, this indicates that the AAV is specific to the driver/activator.

Viral Strategies for Targeting Defined Populations

Combining various experimental techniques can enable the precise targeting of specific neuronal populations of interest. The general advantage of combinatorial approaches is specificity of cell targeting can be improved with each additional technique. Using the natural expression patterns of AAV, neuronal populations can be specifically targeted based on their locations and connectivity. Here we will review several targeting strategies.

Axon Terminals
Axon terminals can be targeted by allowing robust expression of the AAV payload through to the axons of neurons at the injection site. Then, terminals in specific areas can be targeted optogenetically by only delivering light to the region harboring the terminals of interest (Fig. 2a). This technique can be further restricted such that only axons of a particular cell type are targeted by using cell-type specific promoters to drive the AAV expression (Fig. 2b). Thus, specific axonal targeting can be achieved by coupling anterograde expression from a local injection with optogenetic stimulation at an axon terminal (Nassi et al., 2015).

Projection Neuron Targeting
Targeting the cell bodies of projection neurons can be important for manipulating only those neurons that terminate at a particular site. To target these neurons, retrograde AAV can be delivered to projection sites and the cell bodies neurons terminating at that region will be targeted. This method alone will not give rise to pathway specificity. However, if used to deliver optogenetic tools, light can be delivered only to the region harboring the cell bodies of interest (Fig. 2c) (Nassi et al., 2015).

Finally, specific populations of projection neurons can be targeted by coupling local delivery of a Cre-dependent, inducible neuromodulator (e.g., a DREADD or opsin) with retrograde delivery of Cre to the site where the targeted projection neurons originate (Fig. 2d). Using this approach, a subset of projection neurons (Fig. 2d), rather than all the projection neurons (Fig. 2c), then express the Cre-dependent neuromodulator. This has the advantage that effectors (e.g., the DREADD ligand) can then be delivered systemically rather than locally and still only manipulate the subset of projection neurons that have been targeted (Nassi et al., 2015).

Areas for Development

The arsenal of new sensors, actuators, recombinases, genes, RNA and base editing enzymes, and other genetically encoded tools for studying the nervous system is rapidly growing. AAV vectors remain the most versatile and powerful approach for delivering these tools to the CNS. Nevertheless, delivery challenges remain and efforts are ongoing to develop new vectors that address several key needs including (1) improved widespread CNS gene transfer via IV and ICV routes, (2) AAV vectors capable of more efficient anterograde transport, (3) vector solutions for delivering transgenes too large to fit in a single AAV virus, (4) capsids that specifically target defined neural cell types and neuronal subtypes, and (5) viral vectors that enable transduction of microglia.

Overall, consistency and repeatability of both existing and newly developed AAV tools can be improved by following best practices and guidelines. While powerful technologies are being developed, each has technical limits which need to be considered both when designing experiments and when interpreting results. To this end, improved platforms for sharing

information, including technical guidelines and best practices, will serve the research community by enabling technologies to be used to their fullest capacities consistently across labs.
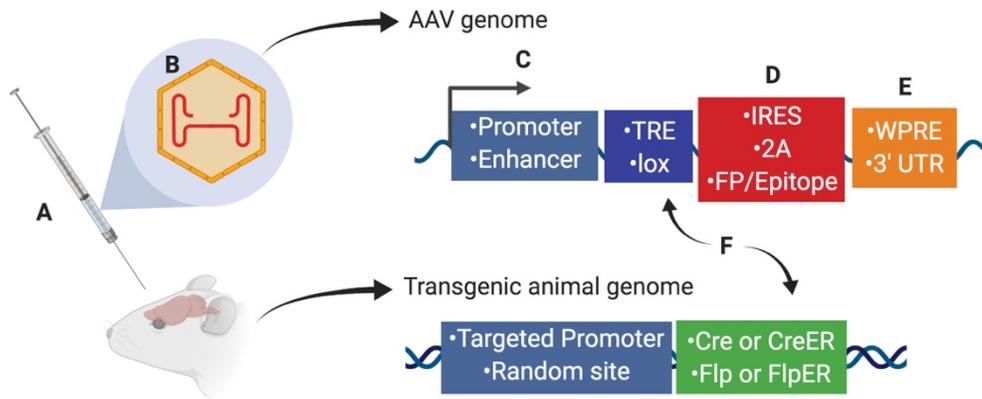
Figures and Tables

Figure 1. Several aspects of experimental design affect neuronal targeting and manipulation including (A) viral delivery method, (B) composition of viral capsid proteins, (C) promoters and/or enhancers driving transgene expression, (D) IRES or 2A elements for multicistronic expression coupled with fluorescent proteins (FP) or protein epitopes, (E) post-translational regulatory elements such as WPRE or 3' UTR, and (F) Recombinase (Cre, CreER, Flp, or FlpER) expression from transgenic driver lines (inserted genomically via targeted or random integration) and ligand-dependent or recombinase-dependent expression elements such as TRE or lox sites, respectively. Abbreviations: TRE, tetracycline-response element; lox, LoxP sequence; IRES, internal ribosomal entry site; 2A, 2A sequence for self-cleavage; FP, fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; 3' UTR, 3' untranslated sequence.
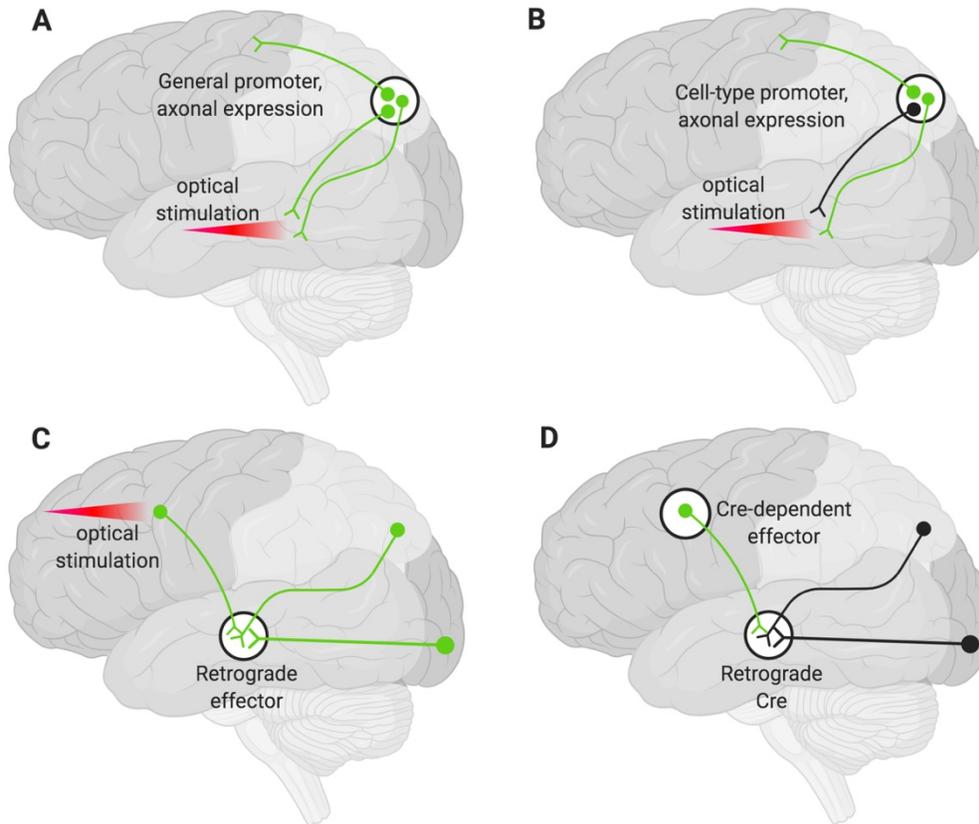
Figure 2. Various strategies for neuronal targeting using AAV. Delivery of neuronal effectors via AAV labels (green) axons and terminals with cell bodies at the injection site. (A) Effectors under a general promoter express in all transduced neurons with cell bodies at the injection site. Specific regions can be optically stimulated (red beam). (B) Effectors under cell-type promoters express only within a cell type. (C) Effectors delivered via a retrograde AAV express in all transduced neurons with axons that project into the injection site. Cell bodies in regions of interest can be optogenetically stimulated (red beam). (D) Delivery of a retrograde AAV expressing Cre recombinase (Retrograde Cre) to the projection site coupled with local delivery of a Cre-dependent effector limits expression to neurons within specific circuits.

Table 1: AAV administration routes for neuroscience

	Administration Route Expression		
	Direct	Intravenous	Delivery into the CSF (IT/ICV/CM)
Advantages	Regional expression High levels of expression achievable (high MOI) Requires small volumes of virus Reduced off-target effects	CNS or PNS-wide transduction Quick, noninvasive Does not require surgical expertise Lower more uniform expression Sparse labeling is possible	IT injection can be used to target spinal motor neurons and dorsal root ganglia Neonatal ICV injections can provide widespread gene delivery to the CNS May allow CNS expression in the presence of neutralizing antibodies (Gray et al., 2013)
Disadvantages	Requires invasive surgery Damage to the targeted area Challenging in certain deep brain structures Transduction gradient from injection site	Higher dose and volume of virus required Greater risk of immune response Off-target effects may confound experiment	Expression is not confined to the CNS Requires moderately large volumes of virus Expression is not as uniform as it is after systemic delivery
Expression considerations	High levels of expression may be important for opsin expression High level expression makes cell-type specific transgene expression using regulatory elements more challenging	Moderate expression provided by IV AAV-PHP.B/eB may be preferable for GCaMP6 expression (no nuclear expression observed, see (Hillier et al., 2017))	Expression is higher around CSF spaces and the brain/SC surface
Capsids	AAV2 – confined spread, mostly neuronal AAV-DJ – Confined spread, higher expression (vs AAV2) AAV1, 5 and 8 – widespread, moderate expression, neurons and glia AAV2-Retro – widespread distribution, enhanced axonal uptake and retrograde expression AAV1 – paired with Cre exhibits trans-synaptic (anterograde) transduction	AAV9 and rh.10 – efficient neonatal CNS transduction AAV-BR1 – brain endothelial cell specific AAV-PHP.B – enhanced neuron and glial transduction after adult IV injection in mice AAV-PHP.eB – further evolved AAV-PHP.B variant with improved neuronal transduction AAV-PHP.S – evolved capsid with improved transduction of peripheral nerves and heart	AAV7, AAV9 and Rh.10 are the most widely tested serotypes for delivery into the CSF (Borel et al., 2016; Federici et al., 2012; Gurda et al., 2016; Samaranch et al., 2013) AAV4 enables transduction of ependymal cells (Liu et al., 2005) AAV SCH9 and AAV4.18 enable SVZ progenitor cell transduction (Murlidharan et al., 2015; Ojala et al., 2018)

Table 2: Ubiquitous enhancers and promoters

Promoter	Characteristics	Length (bp)	Notes	References
CMV, Cytomegalovirus early enhancer and promoter	Ubiquitous	590-800	Robust, rapid, long term expression in all cell types. Prone to silencing in some tissues, specifically the hippocampus, striatum, and substantia nigra. Silenced by 10 weeks in the spinal cord. Only modest expression in glial cells in rat. Minimal expression in rAAV2-retro helper- packaged AAV.	(Gray et al., 2011; Klein et al., 1998; McCown et al., 1996; Paterna et al., 2000; Tenenbaum et al., 2004; Thomsen et al., 1984; Yaguchi et al., 2013)
CAG, CMV enhancer, CBA promoter, globin intron	Ubiquitous	1700		(Miyazaki et al., 1989)
CAGGS, CMV immediate-early enhancer, CBA promoter, CBA intron1/exon1	Ubiquitous, strong in neurons	1600	Ubiquitous and long term expression in the brain.	(Klein et al., 1998; Niwa et al., 1991)
CBh, CBA hybrid intron: CMV early enhancer, CBA promoter, CBA/MVM intron	Ubiquitous, strong in neurons	800	Stronger expression than the CBA promoter	(Gray et al., 2011)
EF1a, Elongation Factor 1a	Ubiquitous, strong in neurons	1200, 2500	Moderate, lower expression in glia compared with CMV/CAG	(Gill et al., 2001; Kim et al., 1990)
EFS, EF1a short version	Ubiquitous	250		(Montiel-Equihua et al., 2012)
UBC, Ubiquitin C	Ubiquitous, weak	400, 1200		(Seita et al., 2019)
PGK, phosphoglycerate kinase	Ubiquitous	425	weak	(Qin et al., 2010)

Table 3: Cell type-specific promoters

Promoter	Characteristics	Length (bp)	Notes	References
hSyn1, human Synapsin1	Neuronal, broad	485	Broadly neuronal in mice, low-level expression in Purkinje cells. Excitatory neuron-specific expression in monkey and rat.	(Dittgen et al., 2004; Hoesche et al., 1993; Kügler et al., 2003; Yaguchi et al., 2013)
MeCP2, mMeCP2 promoter	Mostly neuronal, broad, weak expression	229	Expresses in neurons and in spinal cord motor neurons	(Gray et al., 2011)
NSE, Neuron specific enolase	Neuronal, broad	1300, 1800	Provides strong and long-term expression.	(Xu et al., 2001)
BM88, Neuron-specific protein	Preferentially neuronal	88		(Pignataro et al., 2017)
CaMKII, Ca2+/Calmodulin-dependent kinase II	Neuronal, glutamatergic (cortical)	400, 1200, 2300	Excitatory neuron-specific expression in monkey and rat.	(Dittgen et al., 2004; Hioki et al., 2007; Yaguchi et al., 2013)
mDLX, mouse DLX5/6 enhancer, minimal promoter and chimeric intron	Forebrain GABAergic neurons	850		(Dimidschstein et al., 2016)
mTH/rTH, mouse/rat Tyrosine Hydroxylase	Catecholamine neurons	2500		(Chan et al., 2017; Oh et al., 2009)
DBH, Dopamine β hydroxylase	Adrenergic and noradrenergic neurons	1150		(Hwang et al., 2001)
PRSx8, DBH synthetic	Adrenergic and noradrenergic neurons	NR	Evaluated in noradrenergic neurons in the LC	(Hwang et al., 2001)
PCP2, Purkinje Cell Protein 2 (Ple155)	Purkinje neurons	1650		(Chan et al., 2017; de Leeuw et al., 2016)
FEV, ETS transcription factor (Ple67)	Serotonergic neurons	2000	Serotonergic neurons	(Chan et al., 2017; de Leeuw et al., 2016)
MCH, Melanin-concentrating hormone	Subpopulation, dorsal lateral hypothalamus	830		(van den Pol et al., 2004)
SLC6A4, Serotonin Transporter (Ple198)		3050	Expression is strongest in the thalamus	(de Leeuw et al., 2016)
NR2E1 (Ple264)	Müller glia	2030		(de Leeuw et al., 2016)

GfABC1D, truncated GFAP	Astrocytes	680		(Lee et al., 2008)
Aldh1l1	Astrocytes	1300		(Koh et al., 2017)
mMBP, mouse myelin basic protein	Oligodendrocytes	1900		(Gow et al., 1992)
MAG, Myelin-Associated Glycoprotein	Oligodendrocytes	300, 1500, 2200	All provide expression in Ols, 1500 and 2200 bp versions are more specific	(von Jonquieres et al., 2016)
ICAM-2, Intracellular Adhesion Molecule 2	Endothelial	330		(Cowan et al., 1998)
CLDN5, Claudin5 (Ple261)	Endothelial	2960		(de Leeuw et al., 2016)
Tie-2, TEK Receptor Tyrosine Kinase	Endothelial	730		(Leung et al., 2009)
vWF, von Willebrand Factor	Endothelial	730		(Jahroudi and Lynch, 1994)
FLT1, Endothelial Growth Factor Receptor	Endothelial	1030		(Morishita et al., 1995)
TRE, rtTA-tTA responsive element	Inducible	320-400, (1400 w/tTA)		(Chan et al., 2017; Chenuaud et al., 2004)
c-FOS	Activity dependent	760		(Ye et al., 2016)
eSARE	Activity dependent	980		(Kawashima et al., 2014)

Table 4: General expression considerations for specific transgenes and applications

Transgene	Application	Optimal expression level
Opsins	Optogenetics	High
DREADDs	Chemogenetics	Low for optimal specificity
Ca^{2+} sensors	Activity monitoring	Moderate
Voltage sensors	Activity monitoring	Moderate-high
dLight1	Dopamine indicator	Not yet known
Fluorescent reporters (XFPs)	Expression reporters, protein tagging	Moderate
Luciferase/AkaLuc	Expression reporter	Low-High
Cre/FlpO/Dre/KD/B3 Recombinases	Intersectional expression/ Circuit studies	Low
CRISPR-Cas9	Gene editing	Moderate-High
TVA and rabies G	Circuit studies/retrograde tracing/TRIO/cTRIO	TVA (low) Rabies G (Moderate)

Table 5: Bicistronic Expression Options

	Bicistronic Expression Elements	
	2A	IRES
Advantages	Requires minimal sequence space Results in similar expression of both proteins Can be used to express >2 proteins	Protein products are unmodified
Disadvantages	May reduce expression of both proteins Adds peptide sequence to the C-terminus of the first protein and a proline to the N-terminus of the second protein	May not provide equivalent expression of both transgenes IRES sequences are >500 bp

Table 6: Methods of delivering Cre for cell-type targeting, labeling, and manipulation.

	Method of Cre delivery		
	Targeted knock-in Cre mouse line	**Transgenic Cre mouse line (not generated by homologous recombination)**	**Cre via AAV**
Advantages	• Specific and reliable by genetic targeting to locus of interest (higher certainty that driver activity will reflect the endogenous expression of the gene of interest) • Comprehensive with Cre mouse lines • Sparse if using CreER by adjusting tamoxifen dose • Can combine with viral strategies to achieve spatial control or very strong expression • Lower Cre expression than AAV	• Cheap and easy to produce mouse lines • Lower Cre expression than AAV	• Stable over time • Spatial control: can restrict delivery to a particular region • Can be delivered broadly by systemic (e.g., tail vein or retro-orbital) injection
Disadvantages	• Time-consuming and costly to produce and maintain mouse lines	• Does not necessarily recapitulate the endogenous gene's expression • Genetic silencing in mouse lines can affect Cre expression • Sexual dimorphism can arise that does not reflect the gene's native expression profile • Transgenic animals can lose specificity over time	• Expression gradients from injection site(s) AAV vectors increase interleukin levels Cre toxicity if delivered at high dose •AAV expression may affect the cell's DNA and RNA

References

Borel F, Gernoux G, Cardozo B, Metterville JP, Toro Cabrera GC, Song L, Su Q, Gao GP, Elmallah MK, Brown RH Jr, Mueller C. 2016. Therapeutic rAAVrh10 Mediated SOD1 Silencing in Adult SOD1(G93A) Mice and Nonhuman Primates. *Hum Gene Ther* **27**:19–31. doi:10.1089/hum.2015.122

Chan KY, Jang MJ, Yoo BB, Greenbaum A, Ravi N, Wu W-L, Sánchez-Guardado L, Lois C, Mazmanian SK, Deverman BE, Gradinaru V. 2017. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat Neurosci* **20**:1172–1179. doi:10.1038/nn.4593

Chenuaud P, Larcher T, Rabinowitz JE, Provost N, Joussemet B, Bujard H, Samulski RJS, Favre D, Moullier P. 2004. Optimal design of a single recombinant adeno-associated virus derived from serotypes 1 and 2 to achieve more tightly regulated transgene expression from nonhuman primate muscle. *Mol Ther* **9**:410–418. doi:10.1016/j.ymthe.2003.12.015

Cowan PJ, Tsang D, Pedic CM, Abbott LR, Shinkel TA, d'Apice AJ, Pearse MJ. 1998. The human ICAM-2 promoter is endothelial cell-specific in vitro and in vivo and contains critical Sp1 and GATA binding sites. *J Biol Chem* **273**:11737–11744.

Davidsson M, Wang G, Aldrin-Kirk P, Cardoso T, Nolbrant S, Hartnor M, Parmar M, Bjorklund T. n.d. Barcoded Rational AAV Vector Evolution enables systematic in vivo mapping of peptide binding motifs. doi:10.1101/335372

de Leeuw CN, Korecki AJ, Berry GE, Hickmott JW, Lam SL, Lengyell TC, Bonaguro RJ, Borretta LJ, Chopra V, Chou AY, D'Souza CA, Kaspieva O, Laprise S, McInerny SC, Portales-Casamar E, Swanson-Newman MI, Wong K, Yang GS, Zhou M, Jones SJM, Holt RA, Asokan A, Goldowitz D, Wasserman WW, Simpson EM. 2016. rAAV-compatible MiniPromoters for restricted expression in the brain and eye. *Mol Brain* **9**:52. doi:10.1186/s13041-016-0232-4

Deverman BE, Pravdo PL, Simpson BP, Kumar SR, Chan KY, Banerjee A, Wu W-L, Yang B, Huber N, Pasca SP, Gradinaru V. 2016. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat Biotechnol* **34**:204–209. doi:10.1038/nbt.3440

Dimidschstein J, Chen Q, Tremblay R, Rogers SL, Saldi G-A, Guo L, Xu Q, Liu R, Lu C, Chu J, Grimley JS, Krostag A-R, Kaykas A, Avery MC, Rashid MS, Baek M, Jacob AL, Smith GB, Wilson DE, Kosche G, Kruglikov I, Rusielewicz T, Kotak VC, Mowery TM, Anderson SA, Callaway EM, Dasen JS, Fitzpatrick D, Fossati V, Long MA, Noggle S, Reynolds JH, Sanes DH, Rudy B, Feng G, Fishell G. 2016. A viral strategy for targeting and manipulating interneurons across vertebrate species. *Nat Neurosci* **19**:1743–1749. doi:10.1038/nn.4430

Dittgen T, Nimmerjahn A, Komai S, Licznerski P, Waters J, Margrie TW, Helmchen F, Denk W, Brecht M, Osten P. 2004. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. *Proceedings of the National Academy of Sciences* **101**:18206–18211. doi:10.1073/pnas.0407976101

Drouin LM, Agbandje-McKenna M. 2013. Adeno-associated virus structural biology as a tool in vector development. *Future Virol* **8**:1183–1199. doi:10.2217/fvl.13.112

Dufour BD, Smith CA, Clark RL, Walker TR, McBride JL. 2014. Intrajugular Vein Delivery of AAV9-RNAi Prevents Neuropathological Changes and Weight Loss in Huntington's Disease Mice. *Mol Ther* **22**:797–810. doi:10.1038/mt.2013.289

Federici T, Taub JS, Baum GR, Gray SJ, Grieger JC, Matthews KA, Handy CR, Passini MA, Samulski RJ, Boulis NM. 2012. Robust spinal motor neuron transduction following intrathecal delivery of AAV9 in pigs. *Gene Ther* **19**:852–859. doi:10.1038/gt.2011.130

Feil S, Valtcheva N, Feil R. 2009. Inducible Cre MiceMethods in Molecular Biology. pp. 343–363. doi:10.1007/978-1-59745-471-1_18

Fenno LE, Mattis J, Ramakrishnan C, Deisseroth K. 2017. A Guide to Creating and Testing New

INTRSECT Constructs. *Curr Protoc Neurosci* **80**:4.39.1–4.39.24. doi:10.1002/cpns.30

Fenno LE, Mattis J, Ramakrishnan C, Hyun M, Lee SY, He M, Tucciarone J, Selimbeyoglu A, Berndt A, Grosenick L, Zalocusky KA, Bernstein H, Swanson H, Perry C, Diester I, Boyce FM, Bass CE, Neve R, Huang ZJ, Deisseroth K. 2014. Targeting cells with single vectors using multiple-feature Boolean logic. *Nat Methods* **11**:763–772. doi:10.1038/nmeth.2996

Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. 2009. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* **27**:59–65. doi:10.1038/nbt.1515

Foust KD, Salazar DL, Likhite S, Ferraiuolo L, Ditsworth D, Ilieva H, Meyer K, Schmelzer L, Braun L, Cleveland DW, Kaspar BK. 2013. Therapeutic AAV9-mediated suppression of mutant SOD1 slows disease progression and extends survival in models of inherited ALS. *Mol Ther* **21**:2148–2159. doi:10.1038/mt.2013.211

Gill DR, Smyth SE, Goddard CA, Pringle IA, Higgins CF, Colledge WH, Hyde SC. 2001. Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor1α promoter. *Gene Therapy*. doi:10.1038/sj.gt.3301561

Gow A, Friedrich VL Jr, Lazzarini RA. 1992. Myelin basic protein gene contains separate enhancers for oligodendrocyte and Schwann cell expression. *J Cell Biol* **119**:605–616.

Gray SJ, Foti SB, Schwartz JW, Bachaboina L, Taylor-Blake B, Coleman J, Ehlers MD, Zylka MJ, McCown TJ, Jude Samulski R. 2011. 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**:1143–1153. doi:10.1089/hum.2010.245

Gray SJ, Nagabhushan Kalburgi S, McCown TJ, Jude Samulski R. 2013. Global CNS gene delivery and evasion of anti-AAV-neutralizing antibodies by intrathecal AAV administration in non-human primates. *Gene Ther* **20**:450–459. doi:10.1038/gt.2012.101

Gurda BL, De Guilhem De Lataillade A, Bell P, Zhu Y, Yu H, Wang P, Bagel J, Vite CH, Sikora T, Hinderer C, Calcedo R, Yox AD, Steet RA, Ruane T, O'Donnell P, Gao G, Wilson JM, Casal M, Ponder KP, Haskins ME. 2016. Evaluation of AAV-mediated Gene Therapy for Central Nervous System Disease in Canine Mucopolysaccharidosis VII. *Mol Ther* **24**:206–216. doi:10.1038/mt.2015.189

Hillier D, Fiscella M, Drinnenberg A, Trenholm S, Rompani SB, Raics Z, Katona G, Juettner J, Hierlemann A, Rozsa B, Roska B. 2017. Causal evidence for retina-dependent and -independent visual motion computations in mouse cortex. *Nat Neurosci* **20**:960–968. doi:10.1038/nn.4566

Hioki H, Kameda H, Nakamura H, Okunomiya T, Ohira K, Nakamura K, Kuroda M, Furuta T, Kaneko T. 2007. Efficient gene transduction of neurons by lentivirus with enhanced neuron-specific promoters. *Gene Ther* **14**:872–882. doi:10.1038/sj.gt.3302924

Hoesche C, Sauerwald A, Veh RW, Krippl B, Kilimann MW. 1993. The 5'-flanking region of the rat synapsin I gene directs neuron-specific and developmentally regulated reporter gene expression in transgenic mice. *J Biol Chem* **268**:26494–26502.

Huang Q, Chan KY, Tobey IG, Chan YA, Poterba T, Boutros CL, Balazs AB, Daneman R, Bloom JM, Seed C, Deverman BE. n.d. Delivering genes across the blood-brain barrier: LY6A, a novel cellular receptor for AAV-PHP.B capsids. doi:10.1101/538421

Huang ZJ, Taniguchi H, He M, Kuhlman S. 2014. Cre-dependent adeno-associated virus preparation and delivery for labeling neurons in the mouse brain. *Cold Spring Harb Protoc* **2014**:190–194. doi:10.1101/pdb.prot080382

Hwang DY, Carlezon WA Jr, Isacson O, Kim KS. 2001. A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther* **12**:1731–1740. doi:10.1089/104303401750476230

Jahn HM, Kasakow CV, Helfer A, Michely J, Verkhratsky A, Maurer HH, Scheller A, Kirchhoff F. 2018. Refined protocols of tamoxifen injection for inducible DNA recombination in mouse astroglia. *Sci Rep* **8**:5913. doi:10.1038/s41598-018-24085-9

Jahroudi N, Lynch DC. 1994. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol* **14**:999–1008.

Kawashima T, Okuno H, Bito H. 2014. A new era for functional labeling of neurons: activity-dependent promoters have come of age. *Front Neural Circuits* **8**:37. doi:10.3389/fncir.2014.00037

Keaveney MK, Tseng H-A, Ta TL, Gritton HJ, Man H-Y, Han X. 2018. A MicroRNA-Based Gene-Targeting Tool for Virally Labeling Interneurons in the Rodent Cortex. *Cell Rep* **24**:294–303. doi:10.1016/j.celrep.2018.06.049

Kim DW, Uetsuki T, Kaziro Y, Yamaguchi N, Sugano S. 1990. Use of the human elongation factor 1α promoter as a versatile and efficient expression system. *Gene* **91**:217–223. doi:10.1016/0378-1119(90)90091-5

Klein RL, Meyer EM, Peel AL, Zolotukhin S, Meyers C, Muzyczka N, King MA. 1998. Neuron-specific transduction in the rat septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors. *Exp Neurol* **150**:183–194. doi:10.1006/exnr.1997.6736

Koh W, Park YM, Lee SE, Justin Lee C. 2017. AAV-Mediated Astrocyte-Specific Gene Expression under Human ALDH1L1 Promoter in Mouse Thalamus. *Experimental Neurobiology*. doi:10.5607/en.2017.26.6.350

Kügler S, Kilic E, Bähr M. 2003. 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. doi:10.1038/sj.gt.3301905

Lee Y, Messing A, Su M, Brenner M. 2008. GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia* **56**:481–493. doi:10.1002/glia.20622

Leung JWC, Chung SSM, Chung SK. 2009. Endothelial endothelin-1 over-expression using receptor tyrosine kinase tie-1 promoter leads to more severe vascular permeability and blood brain barrier breakdown after transient middle cerebral artery occlusion. *Brain Research*. doi:10.1016/j.brainres.2009.01.070

Liu G, Martins IH, Chiorini JA, Davidson BL. 2005. Adeno-associated virus type 4 (AAV4) targets ependyma and astrocytes in the subventricular zone and RMS. *Gene Ther* **12**:1503–1508. doi:10.1038/sj.gt.3302554

McCown TJ, Xiao X, Li J, Breese GR, Samulski RJ. 1996. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res* **713**:99–107.

Miyanohara A, Kamizato K, Juhas S, Juhasova J, Navarro M, Marsala S, Lukacova N, Hruska-Plochan M, Curtis E, Gabel B, Ciacci J, Ahrens ET, Kaspar BK, Cleveland D, Marsala M. 2016. Potent spinal parenchymal AAV9-mediated gene delivery by subpial injection in adult rats and pigs. *Mol Ther Methods Clin Dev* **3**:16046. doi:10.1038/mtm.2016.46

Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* **79**:269–277.

Montiel-Equihua CA, Zhang L, Knight S, Saadeh H, Scholz S, Carmo M, Alonso-Ferrero ME, Blundell MP, Monkeviciute A, Schulz R, Collins M, Takeuchi Y, Schmidt M, Fairbanks L, Antoniou M, Thrasher AJ, Gaspar HB. 2012. The β-globin locus control region in combination with the EF1α short promoter allows enhanced lentiviral vector-mediated erythroid gene expression with conserved multilineage activity. *Mol Ther* **20**:1400–1409. doi:10.1038/mt.2012.50

Morishita K, Johnson DE, Williams LT. 1995. A novel promoter for vascular endothelial growth factor receptor (flt-1) that confers endothelial-specific gene expression. *J Biol Chem* **270**:27948–27953.

Murlidharan G, Corriher T, Ghashghaei HT, Asokan A. 2015. Unique glycan signatures regulate adeno-associated virus tropism in the developing brain. *J Virol* **89**:3976–3987. doi:10.1128/JVI.02951-14

Nassi JJ, Cepko CL, Born RT, Beier KT. 2015. Neuroanatomy goes viral! *Front Neuroanat* **9**:80. doi:10.3389/fnana.2015.00080

Nathanson JL. 2009. Short promoters in viral vectors drive selective expression in mammalian inhibitory neurons, but do not restrict activity to specific inhibitory cell-types. *Front Neural Circuits* **3**. doi:10.3389/neuro.04.019.2009

Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193–199.

Oh MS, Hong SJ, Huh Y, Kim K-S. 2009. Expression of transgenes in midbrain dopamine neurons using the tyrosine hydroxylase promoter. *Gene Ther* **16**:437–440. doi:10.1038/gt.2008.148

Ojala DS, Sun S, Santiago-Ortiz JL, Shapiro MG, Romero PA, Schaffer DV. 2018. In Vivo Selection of a Computationally Designed SCHEMA AAV Library Yields a Novel Variant for Infection of Adult Neural Stem Cells in the SVZ. *Mol Ther* **26**:304–319. doi:10.1016/j.ymthe.2017.09.006

Paterna JC, Moccetti T, Mura A, Feldon J, Büeler H. 2000. Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. *Gene Ther* **7**:1304–1311. doi:10.1038/sj.gt.3301221

Pignataro D, Sucunza D, Vanrell L, Lopez-Franco E, Dopeso-Reyes IG, Vales A, Hommel M, Rico AJ, Lanciego JL, Gonzalez-Aseguinolaza G. 2017. Adeno-Associated Viral Vectors Serotype 8 for Cell-Specific Delivery of Therapeutic Genes in the Central Nervous System. *Front Neuroanat* **11**:2. doi:10.3389/fnana.2017.00002

Qin JY, Zhang L, Clift KL, Hulur I, Xiang AP, Ren B-Z, Lahn BT. 2010. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One* **5**:e10611. doi:10.1371/journal.pone.0010611

Samaranch L, Salegio EA, Sebastian WS, Kells AP, Bringas JR, Forsayeth J, Bankiewicz KS. 2013. Strong Cortical and Spinal Cord Transduction After AAV7 and AAV9 Delivery into the Cerebrospinal Fluid of Nonhuman Primates. *Hum Gene Ther* **24**:526–532. doi:10.1089/hum.2013.005

Schultz BR, Chamberlain JS. 2008. Recombinant Adeno-associated Virus Transduction and Integration. *Mol Ther* **16**:1189–1199. doi:10.1038/mt.2008.103

Schuster DJ, Dykstra JA, Riedl MS, Kitto KF, Belur LR, McIvor RS, Elde RP, Fairbanks CA, Vulchanova L. 2014. Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Front Neuroanat* **8**:42. doi:10.3389/fnana.2014.00042

Seita Y, Tsukiyama T, Azami T, Kobayashi K, Iwatani C, Tsuchiya H, Nakaya M, Tanabe H, Hitoshi S, Miyoshi H, Nakamura S, Kawauchi A, Ema M. 2019. Comprehensive evaluation of ubiquitous promoters suitable for the generation of transgenic cynomolgus monkeys†. *Biol Reprod*. doi:10.1093/biolre/ioz040

Taniguchi H. 2014. Genetic dissection of GABAergic neural circuits in mouse neocortex. *Front Cell Neurosci* **8**:8. doi:10.3389/fncel.2014.00008

Tenenbaum L, Chtarto A, Lehtonen E, Velu T, Brotchi J, Levivier M. 2004. Recombinant AAV-mediated gene delivery to the central nervous system. *J Gene Med* **6 Suppl 1**:S212–22. doi:10.1002/jgm.506

Tervo DGR, Hwang B-Y, Viswanathan S, Gaj T, Lavzin M, Ritola KD, Lindo S, Michael S, Kuleshova E, Ojala D, Huang C-C, Gerfen CR, Schiller J, Dudman JT, Hantman AW, Looger LL, Schaffer DV, Karpova AY. 2016. A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. *Neuron* **92**:372–382. doi:10.1016/j.neuron.2016.09.021

Thomsen DR, Stenberg RM, Goins WF, Stinski MF. 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc Natl Acad Sci U S A* **81**:659–663.

van den Pol AN, Acuna-Goycolea C, Clark KR, Ghosh PK. 2004. Physiological properties of hypothalamic MCH neurons identified with selective expression of reporter gene after

recombinant virus infection. *Neuron* **42**:635–652.

Vaughan A, Vaughan A, Brumwell A, Chapman H. 2015. Tamoxifen Administration for Lineage Tracing Using CreERT2 mice. *Protocol Exchange*. doi:10.1038/protex.2015.018
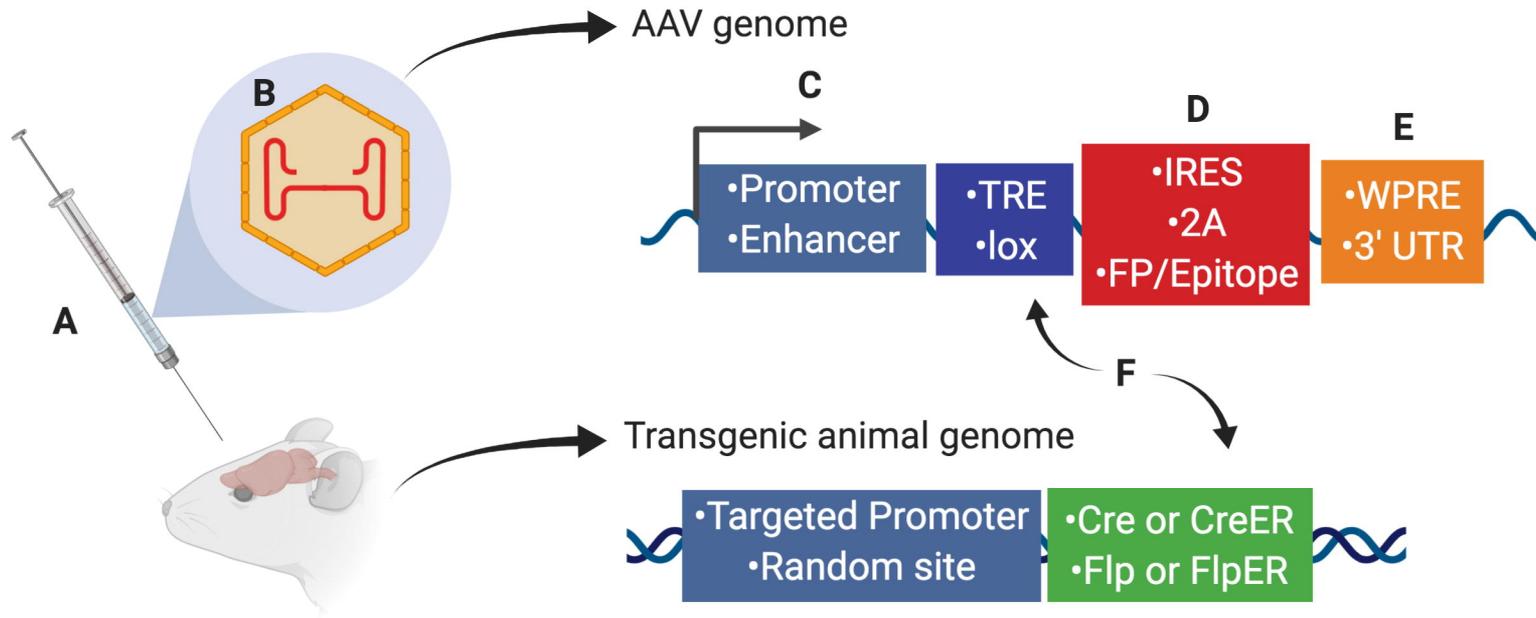
von Jonquieres G, Fröhlich D, Klugmann CB, Wen X, Harasta AE, Ramkumar R, Spencer ZHT, Housley GD, Klugmann M. 2016. Recombinant Human Myelin-Associated Glycoprotein Promoter Drives Selective AAV-Mediated Transgene Expression in Oligodendrocytes. *Front Mol Neurosci* **9**:13. doi:10.3389/fnmol.2016.00013

Xie J, Xie Q, Zhang H, Ameres SL, Hung J-H, Su Q, He R, Mu X, Seher Ahmed S, Park S, Kato H, Li C, Mueller C, Mello CC, Weng Z, Flotte TR, Zamore PD, Gao G. 2011. MicroRNA-regulated, systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. *Mol Ther* **19**:526–535. doi:10.1038/mt.2010.279

Xu R, Janson CG, Mastakov M, Lawlor P, Young D, Mouravlev A, Fitzsimons H, Choi KL, Ma H, Dragunow M, Leone P, Chen Q, Dicker B, During MJ. 2001. Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. *Gene Ther* **8**:1323–1332. doi:10.1038/sj.gt.3301529

Yaguchi M, Ohashi Y, Tsubota T, Sato A, Koyano KW, Wang N, Miyashita Y. 2013. Characterization of the properties of seven promoters in the motor cortex of rats and monkeys after lentiviral vector-mediated gene transfer. *Hum Gene Ther Methods* **24**:333–344. doi:10.1089/hgtb.2012.238

Ye L, Allen WE, Thompson KR, Tian Q, Hsueh B, Ramakrishnan C, Wang A-C, Jennings JH, Adhikari A, Halpern CH, Witten IB, Barth AL, Luo L, McNab JA, Deisseroth K. 2016. Wiring and Molecular Features of Prefrontal Ensembles Representing Distinct Experiences. *Cell* **165**:1776–1788. doi:10.1016/j.cell.2016.05.010

AAV genome

B
C
• Promoter
• Enhancer
• TRE
• lox
D
• IRES
• 2A
• FP/Epitope
E
• WPRE
• 3' UTR

A

F

Transgenic animal genome

• Targeted Promoter
• Random site
• Cre or CreER
• Flp or FlpER