Cell-Specific Targeting of Genetically Encoded Tools for Neuroscience

Lucas Sjulson,[1,2] Daniela Cassataro,[2]
Shamik DasGupta,[3,4] and Gero Miesenböck[3]

[1]Department of Psychiatry, New York University School of Medicine, New York, NY 10016;
email: lukesjulson@gmail.com

[2]Department of Neuroscience and Physiology, Smilow Neuroscience Program, and New York
University Neuroscience Institute, New York, NY 10016

[3]Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, OX1 3SR,
United Kingdom; email: gero.miesenboeck@cncb.ox.ac.uk

[4]Present address: Tata Institute of Fundamental Research, Mumbai, 400005, India

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Abstract

Genetically encoded tools for visualizing and manipulating neurons in vivo
have led to significant advances in neuroscience, in large part because of
the ability to target expression to specific cell populations of interest. Cur-
rent methods enable targeting based on marker gene expression, develop-
ment, anatomical projection pattern, synaptic connectivity, and recent activ-
ity as well as combinations of these factors. Here, we review these methods,
focusing on issues of practical implementation as well as areas for future
improvement.

INTRODUCTION

Over the past 20 years, genetically encoded tools have revolutionized neuroscience. The first major advance was the development of green fluorescent protein (GFP) and other encodable fluorophores (21, 61), which enabled the visualization of gene expression and protein localization in neurons. Soon thereafter came genetically encoded fluorescent indicators for visualizing calcium signals, vesicle fusion, membrane potential, and other physiological variables (109, 111, 114, 148). More recently, we have seen the development of optogenetic (8, 17, 89, 90, 181, 182), chemogenetic (2, 86, 101, 150, 182), and thermogenetic (23, 56, 74, 78, 156, 157, 171) technologies that allow electrical activity to be evoked or suppressed in specific neuronal populations, analogous to the use of activating or interfering mutations in genetics. One primary advantage of each of these technologies over their respective predecessors is their cellular specificity. Encoding a sensor or actuator molecule in DNA permits selective targeting of transgene expression based on endogenous gene expression, anatomy, connectivity, development, or neural activity. Although the tools themselves have been discussed and reviewed at length (42, 107, 108, 110, 134, 149, 164), comparatively less attention has been paid to the equally important technologies for targeted transgene expression to specific neuronal populations (96, 108). Here, we review the most common of these methods, focusing on practical issues of implementation and areas for future improvement.

ONE SIZE DOES NOT FIT ALL: OPTIMAL TRANSGENE EXPRESSION STRATEGIES DIFFER FOR DIFFERENT TRANSGENES

When choosing a strategy to target transgene expression to a given neuronal population, it is easy to overlook the fact that different transgenes require different expression levels to exert their desired effects (**Table 1**). This is important because many expression systems exhibit off-target leaky expression in the "wrong" cells and an upper bound on expression level in the "right" cells; the expression strategy should therefore be chosen with the desired expression levels in mind. For example, light-activated ion pumps and channels such as channelrhodopsin-2 (ChR2) generate small unitary currents (24, 41) and thus require extremely high expression levels. As a result, early successes expressing ChR2 in cultured neurons were obtained under conditions favoring high expression levels, using viral vectors that can introduce multiple transgene copies per cell (17, 89). In contrast, early ChR2 knock-in mice, which contain one or two transgene copies per cell, yielded expression levels that were adequate for some neurons but inadequate for others (69) and required subsequent optimization (99, 100). On the positive side, modest expression levels in these knock-in animals prevented the accumulation of ChR2 in axons, dendrites, and synaptic terminals—a useful property for distinguishing synaptic connections that originate locally from those that project into the illuminated volume (69). What constitutes the right expression level thus also depends on the intended use of a tool.

Multiple factors affect transgene expression level, such as copy number, choice of promoter or genomic insertion site (127), codon usage (72), presence or absence of introns (124), and whether the protein is soluble or membrane-bound. In order to choose the optimal targeting strategy, it is helpful first to know what expression level is desired (**Table 1**). After implementing a particular targeting strategy, it is then critical to assess whether or not the desired transgene expression profile was actually attained. For fluorescent proteins or indicators that can be visualized directly, this is relatively straightforward. However, for optogenetic, chemogenetic, or thermogenetic actuators, direct verification requires electrophysiological measurements to demonstrate that the cells of interest are being modulated appropriately. In practice, most investigators assess

Table 1 Genetically encoded tools for neuroscience

Class of tool	Examples	Mechanism of action	Expression level required
Fluorescent proteins	GFP, mCherry, GCaMP, synapto-pHluorin, ArcLight, ASAP	Cyclization reaction between side chains generates at most one fluorophore per protein	Medium to high. Fluorescence may be insufficiently sensitive to report transcriptional leak detectable by other methods
Ion channels with low unitary conductance	Channelrhodopsin and other cation-conducting microbial opsins	Modulates membrane potential. Absorption of a single photon leads to translocation of multiple ions	Very high. Off-target transcriptional leak can nonetheless be relevant
Ion channels with high unitary conductance	PSAM, Kir2.1, TRPV1, P2X2, TrpA1, GtACR2 opsin	Modulates membrane potential. Ligand, light, or heat leads to the flow of a large number of ions across plasma membrane	Variable: low to high
Ion pumps	Halorhodopsin, archaerhodopsin, Jaws	Modulates membrane potential by ion transport across plasma membrane. Absorption of each photon results in translocation of at most a single ion	Very high. Off-target transcriptional leak unlikely to be relevant
GPCRs	DREADDs, animal opsins	Activates endogenous G-protein signaling pathways	Not well-studied in vivo; likely to be cell dependent
Dominant-negative subunits	Dynamin (*shibire*[ts])	Forms stoichiometric complex with endogenous counterpart	Variable: low to high, depending on the expression level of the endogenous counterpart
Transcriptional activators	Tet-on, tet-off systems; GAL4-UAS; LexA-lexAop; Q-QUAS	Binds to exogenous promoter sequence, activating transcription of another transgene	Low to medium
DNA recombinases	Cre, Flp	Recombines double-stranded DNA, often leading to activation or inactivation of a gene	Variable: very low to high, depending on the recombination target. Transcriptional leak known to be functionally relevant
High efficiency enzymes	Tetanus toxin light chain, diphtheria toxin, Reaper, Dam methylase	Single protein molecule affects many substrate molecules	Very low, potentially below detectable levels. Off-target expression may be a significant concern

Abbreviations: DREADDs, designer receptors exclusively activated by designer drugs; GFP, green fluorescent protein; GPCRs, G-protein coupled receptors.

transgene expression by visualizing a coexpressed fluorescent protein, and in many cases this approach is sufficient. This method is used ideally under circumstances in which transgene expression is binary (e.g., Cre-Lox mediated) and the required level of expression is high, as in the case of microbial opsins. Because fluorescent proteins require high levels of expression to be visible, a more problematic case involves coexpression with a transgene that is active at low levels, such as Cre recombinase or tetanus toxin (**Table 1**). Under these circumstances, the absence of fluorescence does not conclusively rule out functional expression of the transgene. When using transgenes such as designer receptors exclusively activated by designer drugs (DREADDs) (2), for

which functionally relevant expression levels are less well established, the specificity of targeting should be verified physiologically if off-target expression is a possibility.

TARGETING "CELL TYPES" BASED ON EXPRESSION OF ENDOGENOUS GENES

The most common approach for targeting subpopulations of neurons is based on marker genes, the expression of which is used to delineate neuronal "cell types." The relationship between gene expression and neuronal identity is complex and the topic of much debate (34, 44, 96, 123). In our view, a major contributor to the confusion surrounding this topic is the problematic notion of "cell type" itself. Studies of the generation of neuronal diversity during development suggest that rather than being organized into discrete, objectively definable cell types, neuronal identity instead has a hierarchical organization (**Figure 1***a*) (44). As a result, the number of cell types in the brain can be correctly asserted to be arbitrarily large or small, depending on which level of the hierarchy one counts. Semantic issues aside, there is a consensus that substantial electrophysiological and morphological heterogeneity exists within the populations of neurons that express most of the common marker genes currently used for targeting transgene expression (167, 179). The recent development of single-cell RNA-seq in neurons promises to resolve some of these controversies, as well as to identify candidate marker genes that can be used to label more homogenous populations of neurons (**Figure 1***b*) (167, 179).

One common method to target transgene expression based on endogenous marker gene expression is site-specific integration to create a knock-in line (20, 136) (**Figure 2***a*). This method is most popular in mice but is increasingly applied to other species based on CRISPR-Cas9 technology (27, 67). Several strategies are used to create knock-in animals, leading to important differences in properties of the resulting lines. In some cases, endogenous exons are replaced with the transgene, meaning that the knock-in allele does not express the endogenous gene and the animal may have an abnormal phenotype, especially if both copies are affected. In other cases, the transgene is inserted into the last exon of the endogenous gene, preceded by a viral internal ribosome entry site (IRES) (22, 122) or 2A self-cleavage sequence (139) that permits simultaneous expression of both the endogenous gene and the transgene (73, 168). Self-cleavage sequences are generally preferable, as they lead to translation of the desired transgene product at a 1:1 stoichiometric ratio to the endogenous protein with little disruption of endogenous gene expression. IRES sequences, in contrast, produce lower, less predictable levels of transgene expression and can also reduce mRNA stability (147), decreasing expression of the endogenous gene (7) and thereby possibly producing phenotypes of their own.

Bacterial artificial chromosome (BAC) transgenic lines (62, 170, 178) are a cheaper and faster alternative to knock-in lines that also achieve selective transgene expression based on an endogenous marker gene. The process of generating knock-in lines requires the painstaking step of inserting the transgene into a specific location in the full animal genome (20, 136). BAC transgenics circumvent this by instead incorporating the transgene into a BAC carrying a fragment of the animal genome that contains the desired insertion site (**Figure 2***b*). The entire BAC is then integrated into the full animal genome at a random location (178). The large size of the BAC (∼150–350 kb) helps mitigate alterations of the transgene expression pattern imposed by chromosomal location and is likely to capture more of the regulatory context provided by distal enhancers (62, 170), but multiple insertions must still be screened to select one exhibiting the same expression pattern as the endogenous gene or a knock-in line. Of note, BAC transgenic lines faithfully reproducing endogenous expression patterns in one brain region often fail to do so in other brain regions, and thorough examination of expression patterns can reveal subtle differences that were not
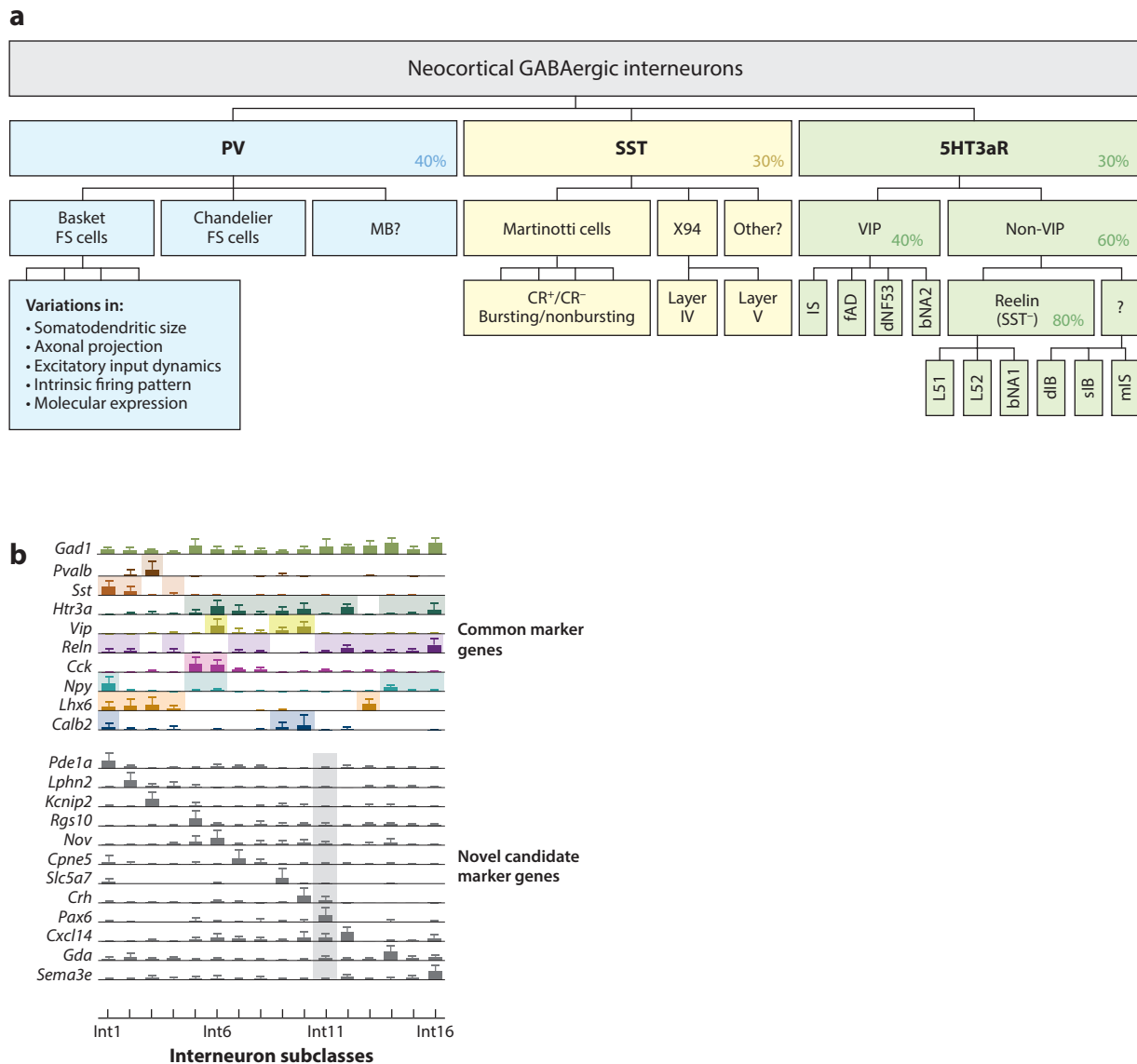
a

Neocortical GABAergic interneurons

PV 40%

SST 30%

5HT3aR 30%

Basket FS cells

Chandelier FS cells

MB?

Martinotti cells

X94

Other?

VIP 40%

Non-VIP 60%

Variations in:
• Somatodendritic size
• Axonal projection
• Excitatory input dynamics
• Intrinsic firing pattern
• Molecular expression

CR⁺/CR⁻ Bursting/nonbursting

Layer IV

Layer V

IS fAD dNF53 bNA2

Reelin (SST⁻) 80%   ?

L51 L52 bNA1 dlB slB mlS

b

Gad1
Pvalb
Sst
Htr3a
Vip
Reln
Cck
Npy
Lhx6
Calb2

Common marker genes

Pde1a
Lphn2
Kcnip2
Rgs10
Nov
Cpne5
Slc5a7
Crh
Pax6
Cxcl14
Gda
Sema3e

Novel candidate marker genes

Int1   Int6   Int11   Int16

Interneuron subclasses

Figure 1

The relationship between cell type and gene expression is complex. (*a*) Neocortical GABAergic interneurons illustrate the hierarchical organization of neuronal identity. Owing to this organization, the number of cell types can be correctly asserted to be arbitrarily large or small, highlighting the heuristic nature of the very concept of cell type. Figure adapted from Reference 138. (*b*) Single-cell RNA-seq reveals that common marker genes are expressed across multiple subclasses of interneurons. The results suggest novel candidate marker genes that may allow more precise targeting of diverse neuronal subpopulations. Figure adapted from Reference 179.

immediately apparent (84, 163). Investigators working with these lines should therefore verify expression profiles with careful histology. Additionally, BAC insertion can disrupt endogenous genes, and some BAC lines are constructed such that additional copies of endogenous genes carried in the BAC are overexpressed. Both of these effects can alter the animal's phenotype (30, 76, 77), and investigators should be circumspect to control for these appropriately, for example by
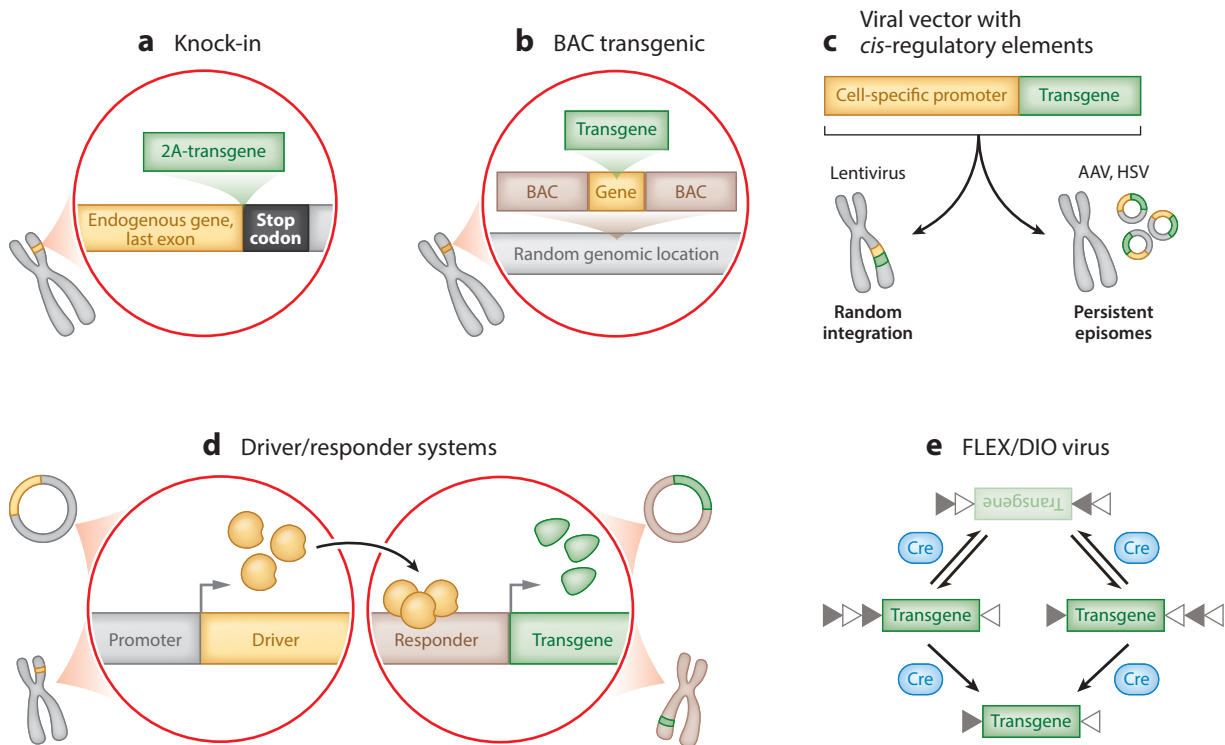
a Knock-in

2A-transgene

Endogenous gene, last exon | Stop codon

b BAC transgenic

Transgene

BAC | Gene | BAC

Random genomic location

c Viral vector with *cis*-regulatory elements

Cell-specific promoter | Transgene

Lentivirus | AAV, HSV

Random integration | **Persistent episomes**

d Driver/responder systems

Promoter | Driver | Responder | Transgene

e FLEX/DIO virus

Transgene

Cre | Cre

Transgene | Transgene

Cre | Cre

Transgene

Figure 2

Targeting based on expression of endogenous genes. (*a*) Knock-in lines use site-specific integration to insert the transgene of interest directly into the marker gene locus, causing the transgene to be expressed wherever the marker gene is expressed. Use of a self-cleaving 2A sequence reduces alterations in expression of the endogenous gene. (*b*) Bacterial artificial chromosome (BAC) transgenic lines insert the transgene of interest into the marker gene in a large genomic fragment maintained in a BAC. The BAC is then inserted into a random genomic location. (*c*) Viral vectors can be targeted using short promoter sequences that replicate the endogenous gene expression pattern. (*d*) Two-component driver/responder systems allow versatility through combinatorial use of a selectively expressed driver, which activates transgene expression, and a responder, which contains the transgene of interest. Prototypical examples include the GAL4-UAS system, the Cre-Lox system, and the tTA-TRE/tetO system. Both the driver and responder can be either genomic or encoded in a viral vector. (*e*) One of the most popular responder viral vector strategies is a Cre-activated FLEX or DIO switch. The transgene of interest is initially inactive in an inverted orientation and flanked by two pairs of incompatible Cre target sites, such as LoxP and Lox511. The presence of Cre irreversibly flips the transgene into the correct orientation via two recombination steps: The first, reversible step inverts the orientation of the transgene, and the second step locks the transgene in the correct orientation by excising one copy of either Cre target site, thus preventing further recombination. This strategy has been used with great success in adeno-associated viruses (AAVs) but is less effective in other systems, possibly because efficient inversion of the transgene may rely on intermolecular recombination between multiple copies of the viral genome.

comparing a BAC line to the same BAC line in a negative control condition rather than comparing transgenic to wild-type animals. An advantage of BAC transgenics is that in knock-in lines transgenes are expressed at levels similar to that of the targeted endogenous marker gene, which is often insufficient for opsins or genetically encoded activity indicators. With BAC transgenics, one can attain higher expression levels by screening for lines with strong expression, which typically correspond to insertion of many copies of the BAC.

Another method to target transgene expression based on endogenous marker gene expression is the use of viral vectors (115, 116, 172) containing regulatory DNA sequences taken from the marker gene of interest (**Figure 2*c***). Viral vectors have the benefits of reduced cost and time

relative to transgenic animals as well as the feature of anatomically restricted expression when a specific brain region is injected. This is also the most straightforward approach to implement in human subjects for translational studies involving therapeutic transgene expression. However, this approach has met with limited success for several reasons. First, most viral vectors have packaging capacities that are too small to hold entire genomic regulatory sequences (38, 81), which are difficult to map and may span megabases (16, 104). Viral vectors with ostensibly cell-specific promoter sequences can also exhibit insufficient expression levels, as well as nonspecific leaky expression, especially when multiple copies are present in a single cell. Combining viral promoter sequences with viral serotypes that preferentially infect a particular neuronal population has been shown to improve targeting in some circumstances (117). Despite these shortcomings, viruses carrying cell-specific promoters have proven relatively successful in several cases, including the CaMKIIa promoter for targeting excitatory cells in the cortex (36), the Dlx1/2 promoter for GABAergic neurons (87), the tyrosine hydroxylase promoter for dopaminergic cells (119), and the substance P and enkephalin promoters for targeting the direct and indirect pathway cells of the striatum (63, 177). It is worth noting that the definition of success depends on the transgene because strong promoters capable of driving expression of microbial opsins or fluorescent proteins in specific populations can exhibit leaky expression elsewhere. This low-level leak may be virtually undetectable as light responsiveness or fluorescence but can be a serious issue when expressing Cre recombinase. Likewise, weak promoters can be insufficient to express functional levels of sensors or actuators but adequate to express transcriptional activators like tTA (63), which drives strong expression at the TRE promoter in the absence of doxycycline.

Many of the limitations of these systems are addressed in what is probably the most popular and versatile approach, using a two-component system for transgene expression (**Figure 2d**). The prototypical two-component driver/responder system is the GAL4-UAS system used in flies (18) and zebrafish (145). In this system, a transgenic driver line expressing the yeast transcription factor GAL4 in a specific population of cells is crossed with a responder line containing the transgene of interest preceded by UAS sequences. The progeny then exhibit strong responder transgene expression specifically in the cells containing GAL4. That said, there is a possibility of leaky expression of the undriven responder transgene, which should be controlled for by testing animals carrying the responder cassette but lacking the GAL4 driver.

In *Drosophila*, endogenous expression patterns are tapped either by shotgun or directed approaches; both have produced libraries of many thousands of GAL4 driver lines (13, 49, 59, 65, 102, 126, 133). Shotgun techniques use mobile genetic elements (P elements) to insert GAL4 at random genomic locations (12, 14, 174). Because insertions in fact favor the 5′ untranslated regions of genes (11), they tend to "trap" (i.e., reproduce) the expression profiles of endogenous enhancers. Shotgun approaches have several drawbacks: Many expression patterns are unacceptably broad; P elements have insertional hot spots (153), resulting in uneven genome coverage; and it is often impossible to identify and reuse the regulatory sequences responsible for a specific expression pattern. If the relevant enhancer elements can be isolated experimentally (28, 118) or by sequence analysis (see, for example, 26, 33, 46, 126), they can be coupled to a synthetic core promoter (126) and inserted by site-specific ΦC31 recombination into an intergenic landing site (15), overcoming many of these drawbacks. The LexA-lexAop and Q systems (using components derived from bacteria and fungi, respectively) are orthogonal two-component systems for concurrent use with GAL4-UAS (82, 128).

The GAL4-UAS system never gained popularity in rodents, but analogous systems have been developed using Cre recombinase driver lines and Cre-dependent responder lines (83, 142), as well as tetracycline-dependent tTA driver lines and TRE responder lines (47, 50). (Rodent responder lines are often referred to as "reporter" lines, even if they do not express a reporter protein.

We prefer the generic term responder.) The primary advantage of two-component systems is versatility, in that each driver or responder can be used in many different combinations, reducing the number of transgenic lines that must be created. Additionally, responder lines can be optimized for stronger transgene expression, thus amplifying weak cell-specific expression, and expression levels can be titrated (within limits) by using inducible or repressible drivers.

In other versions of this technique, the driver, the responder, or both can be supplied through a viral vector. The most common example is adeno-associated virus (AAV) vectors that contain a strong nonspecific promoter and a transgene that is silent until activated by Cre-mediated recombination (80). Similar to Cre-dependent responder lines (83), the earliest version of this approach (80) used a floxed stop cassette, which led to some leaky expression in Cre-negative cells. Improved stop cassettes that are small enough to fit within the viral payload have been developed (55), but the most common approach to avoid leaky expression is called FLEX (flip excision) (4) or DIO (double-floxed inverted open reading frame) (151) (**Figure 2e**). In this approach, the transgene starts in the inverted orientation and is thus inactive. When Cre is present, recombination flips the orientation of the transgene, activating transcription. When many copies of the transgene are present, as with AAV infection, the FLEX/DIO approach works well, but floxed stop cassettes appear to be more efficient in larger viral vectors or genomic locations. Other versions of Cre-dependent AAV exist in which Cre can inactivate transcription of a transgene, or even switch from expressing one transgene to another (143). Cre-dependent viral vectors have some complexities stemming primarily from the fact that they create DNA fragments that are capable of recombining with compatible genomic Lox sites present in some transgenic mice. For example, we and others have found that the presence of a Cre-dependent AAV often suppresses expression of a Cre-dependent genomic transgene if the Lox sites are compatible (143). An important difference between Cre-dependent AAV and Cre-dependent genomic loci is that Cre-dependent AAV expresses the transgene in all infected cells expressing Cre since the time of AAV injection, but Cre-dependent genomic loci are transcriptionally active in cells that expressed Cre at any point throughout development. Many Cre lines express Cre more broadly during early development than adulthood (166), which can lead to transgene expression in undesired cell populations (135).

TARGETING BASED ON DEVELOPMENT

Another strategy for targeting specific neuronal populations is to label neurons that are generated at a specific developmental time point. This can be accomplished through several means. In rodents, a common technique is in utero electroporation, in which naked plasmid DNA is injected into the brain of mouse pups in utero, followed by transcranial electrical stimulation to cause plasmid uptake (**Figure 3a**) (165). Because plasmids are lost in cells undergoing mitotic division, neurons born on the day of electroporation preferentially carry the plasmid in the adult (85). This technique has the advantages of being fast and inexpensive, as well as the fact that the plasmid can be much larger than the payload of most viral vectors. However, not all anatomical areas are amenable to this technique, and in many circumstances a relatively low and variable proportion of neurons take up the plasmid DNA.

A technique common in rodents is the use of Cre recombinase lines that are inducible at specific developmental time points (**Figure 3b**). The most common form of inducible Cre is CreER^{T2}, which contains a modified estrogen receptor binding domain that prevents Cre from entering the nucleus in the absence of a ligand (40, 106). Tamoxifen is administered on a specific day of pre- or postnatal development, which allows low levels of Cre to enter the nucleus, causing preferential recombination in the cells that express high levels of CreER^{T2} on that day, with an apparent bias toward actively dividing cells. This approach has been used to label various cell

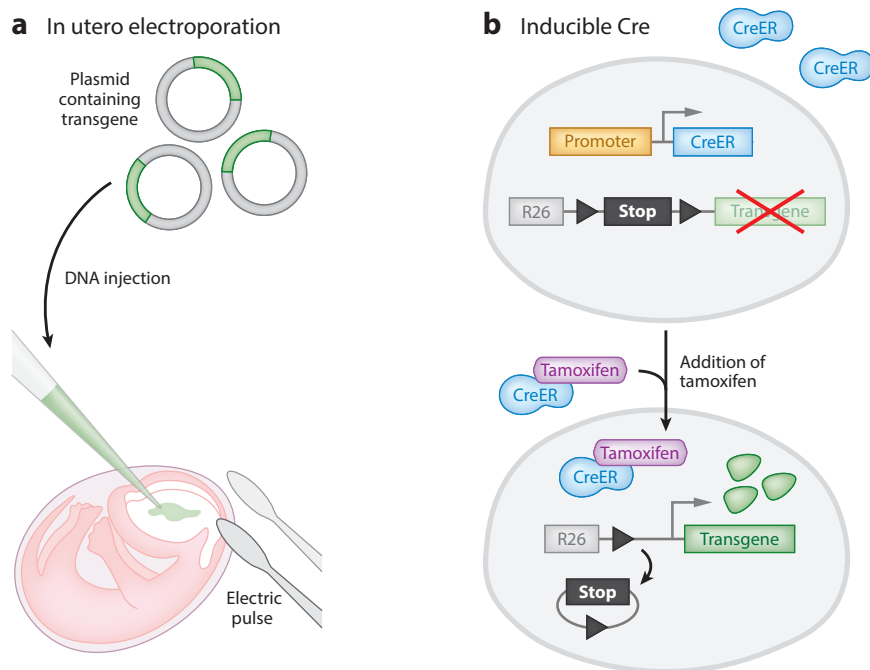**a** In utero electroporation

b Inducible Cre

Figure 3

Targeting based on development. (*a*) In utero electroporation. Naked plasmid DNA is injected into the brain of a mouse pup at a specific day of embryonic development, and a series of electrical pulses is applied to facilitate plasmid uptake. Because the plasmid is not retained in actively dividing cells, this method targets transgene expression to neurons born on the day of electroporation. (*b*) Another method to target cells at specific developmental time points is through the use of mouse lines expressing CreER^{T2} under the control of developmentally regulated genes. Because CreER^{T2} is inactive until induced with a pulse of tamoxifen, this method targets neurons expressing maximal levels of CreER^{T2} at the time of tamoxifen induction, with a possible bias for actively dividing cells.

populations in multiple brain regions (32, 69, 98, 183, 184). Despite its popularity, CreER^{T2} has several drawbacks: in the absence of tamoxifen, it exhibits some activity, and in the presence of tamoxifen it is substantially less efficient than unmodified Cre. Newer inducible recombinases have been developed to remedy some of these issues, including Cre*PR (176), an inducible Cre that is activated by mifepristone, a progesterone receptor antagonist. Cre*PR exhibits a substantially lower leak in vitro than CreER^{T2}, in part because of the removal of a cryptic splice site near the 3′ end of the Cre coding sequence that can lead to alternatively spliced isoforms lacking the regulatory PR domain (176). To our knowledge, this splice site has never been removed from CreER^{T2}, despite the likely advantage it would impart. One complicating factor with Cre*PR is that mifepristone terminates pregnancy unless administered with a compensatory dose of progesterone (137). Another inducible Cre, known as DD-Cre, is based on Cre fused to a degradation domain (DD) that is inhibited by trimethoprim, a commonly used antibiotic (141). This inducible Cre has two advantages: First, the level of Cre activity is titratable over a large range of trimethoprim doses; second, at high doses DD-Cre is more efficient than CreER^{T2}. Unfortunately, some mouse lines expressing DD-Cre exhibit leaky trimethoprim-independent recombination that may be greater than what is observed with CreER^{T2} (141). The ideal inducible recombinase therefore still does not exist, although a recombinase regulated by two simultaneous methods (e.g., the PR domain

and the DD), or possibly by light-sensitive domains (57, 71, 107), may rectify some of these current deficiencies.

In *Drosophila*, the most commonly used method is MARCM (mosaic analysis with a repressible cell marker) (88). In this method, heat shock at a specific developmental time point expresses Flp recombinase, which selectively activates transgene expression in a subset of actively dividing cells. Although MARCM and other techniques for developmental targeting (185) have provided valuable anatomical insights, their inherent stochasticity and sparsity of labeling have limited their utility for functional studies. The cellular composition of MARCM clones typically differs between individuals and brain hemispheres, requiring laborious post hoc verification of each expression pattern and complicating behavioral analyses. Where functional conclusions have been drawn, they are based on phenotypic similarities or differences, rather than direct inferences, among animals harboring partially overlapping MARCM clones (31, 75).

TARGETING BASED ON ANATOMY

A third strategy is to target specific neuronal populations based on anatomical projections and connectivity. The simplest such case is to target the population of neurons in area A that project to area B. For optogenetic stimulation, one approach is to express the transgene nonspecifically in region A, then illuminate the axon terminals in region B (**Figure 4a**), thus selectively activating connections from region A that project to region B. This approach was first used in acute slices (125) and has since been extended to behavioral experiments (162) and the identification of neurons with specific projection patterns in extracellular recordings (66). One under-recognized caveat of this technique in behavioral studies is the fact that optical stimulation of axon terminals causes antidromic propagation of action potentials to the soma, evoking transmission from axon terminals outside the illuminated region. In some cases, a behavioral phenotype attributed to vesicle release from directly illuminated axon terminals could in fact be due to activity in a different brain region. Unfortunately, this is not a trivial effect to control for, as simple measures such as injecting lidocaine into the region harboring the somata of the photostimulated cells (162) may independently alter the behavioral phenotype. The recent development of InSynC (92), a method to inactivate vesicle release optically without affecting antidromic action potentials, may provide a solution to this problem.

Analogous strategies have been used to inhibit vesicle release in neurons projecting from A to B, either by focal illumination of axon terminals expressing a hyperpolarizing opsin (152) or by focal injection of clozapine-N-oxide near the axon terminals of cells expressing the inhibitory DREADD hM4Di (154).

An alternative approach is to inject a retrograde viral vector encoding the transgene of interest in area B and illuminate cell bodies in area A (**Figure 4b**) or, more generally, a Cre-dependent viral vector in area A and a retrograde viral vector encoding Cre in area B. Examples of such retrograde viral vectors that infect axon terminals include canine adenovirus 2 (79), pseudorabies virus (121), herpesvirus (155), and AAV (140). One noteworthy observation is that many AAV serotypes that appear to cause minimal retrograde infection when expressing fluorescent proteins can in fact show significant retrograde infectivity when expressing Cre recombinase (Jordane Dimidschstein, personal communication). This is an example of the principle that weak expression of some transgenes may be undetectable and/or functionally irrelevant, whereas weak or even transient expression of others can have a significant effect.

A refinement of this approach uses replication-competent viruses that travel transsynaptically, enabling targeting based on synaptic connectivity. For retrograde transsynaptic targeting, the most common technique is to use a rabies virus vector with three modifications: (*a*) Its genome
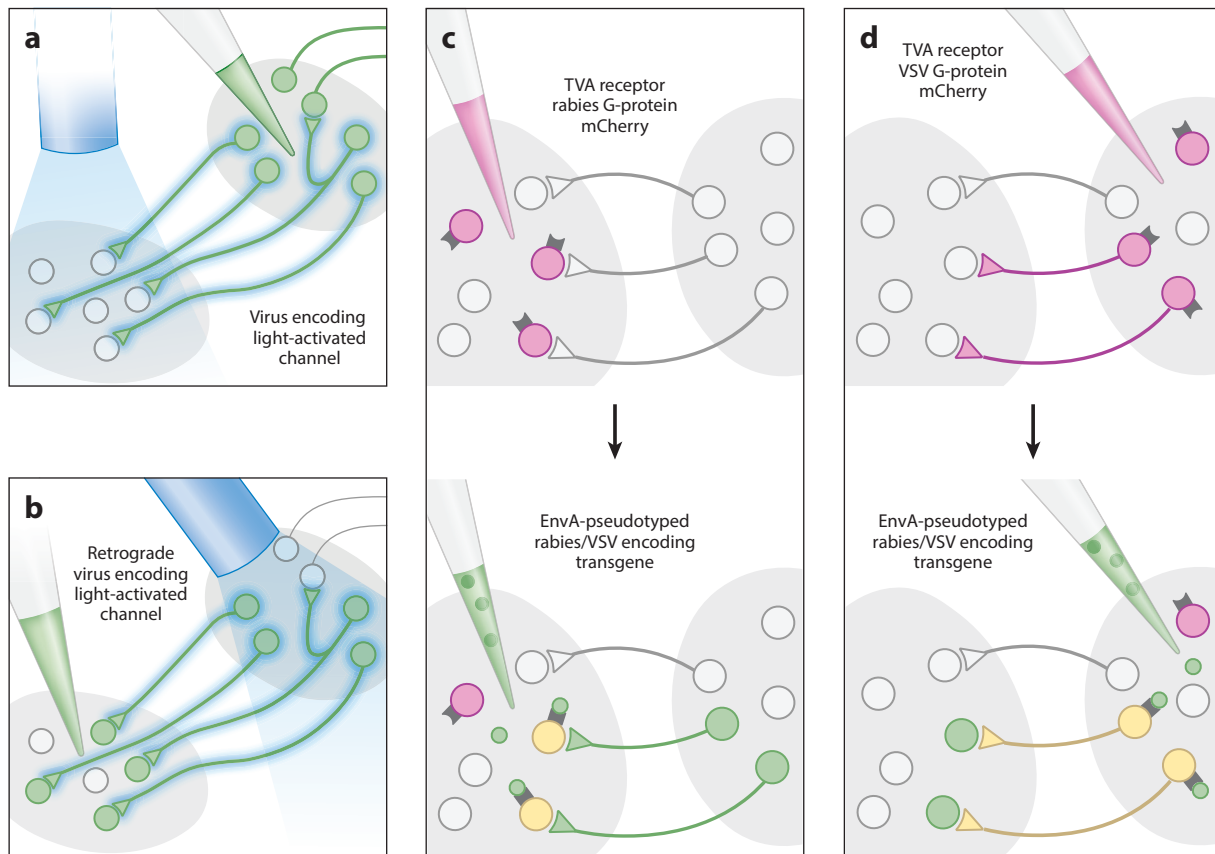
Figure 4

Targeting based on anatomy. (*a*) A common method for targeting optogenetic activation to a population of cells projecting from one area to another. A viral vector encoding a light-activated channel is injected into the first area, and an optical fiber is implanted above the second area. Illumination of the axon terminals in the second area evokes action potentials selectively in the cells projecting from the first area. (*b*) Another method to achieve the same goal is to inject a retrograde viral vector encoding a light-activated channel into the second area and implant an optical fiber into the first area. A common variation of this approach uses a retrograde viral vector encoding Cre recombinase. (*c*) An approach for monosynaptic retrograde targeting involves first injecting adeno-associated viruses (AAVs) or plasmids encoding the TVA receptor, rabies glycoprotein G (G-protein), and mCherry. These infect a population of starter cells (*pink*). Later, EnvA-pseudotyped rabies or vesicular stomatitis virus (VSV) vector encoding green fluorescent protein (GFP) (*green*) is injected; the protein infects TVA-positive starter cells and travels retrogradely across one synapse. (*d*) For monosynaptic anterograde targeting, AAVs encoding the TVA receptor, VSV glycoprotein G, and mCherry are injected. The area is later infected with EnvA-pseudotyped rabies/VSV encoding GFP (*green*), which infects TVA-positive starter cells and travels anterogradely across one synapse.

carries the transgene of interest and a GFP marker, (*b*) the injected viral particles are pseudo-typed with the EnvA coat protein and can only infect neurons expressing the avian TVA receptor (39), and (*c*) glycoprotein G is deleted from the genome; thus, the virus cannot replicate or travel transsynaptically (173). A typical experiment involves targeting a defined population of starter cells to express TVA, glycoprotein G, and a red fluorescent protein; this is done using AAV, a transgenic mouse line, single-cell electroporation (103), or transfection of plasmid DNA through the patch pipette (130) (**Figure 4*c***). The modified rabies virus is then injected, infects the starter cells through the TVA receptor, and is able to replicate because of the presence of gly-coprotein G. The virus then spreads retrogradely across a single synapse and infects presynaptic

neurons but is unable to spread further because of the absence of glycoprotein G. This strategy can also be used with G-deleted vesicular stomatitis virus (VSV), which travels retrogradely when paired with the rabies G-protein (10) or anterogradely when used with the native VSV G-protein (**Figure 4d**) (9).

Herpes simplex virus (HSV) strain H129 (37) travels in an exclusively anterograde direction in the central nervous system (CNS) (180). A variant exists in which only Cre-positive cells act as starter cells (94), but unlike the G-deleted rabies or VSV systems, the HSV will continue traveling anterogradely across multiple synapses (169). In theory, a monosynaptically restricted version could be made by removing an essential gene from the HSV genome and supplying it in *trans* with a second viral vector, analogous to G-deleted rabies virus and VSV. Another practical drawback to the HSV H129 strain is that it contains a full viral genome more than 150 kb in size, meaning that inserting transgenes of interest requires tedious recombineering and selection in vitro rather than routine plasmid cloning. A significant disadvantage of all of these transsynaptic targeting strategies is that they require viral replication in vivo, which leads to toxicity after a period of several days (9, 94, 173). This restricts the time window in which a behavioral or neurophysiological experiment can be performed, but newer viral strains are becoming available that increase the length of this window to several weeks (131).

Nontoxic methods for transsynaptic targeting have been tested, but none of these have achieved widespread popularity. For instance, replication-incompetent AAV particles can be transferred anterogradely across synapses for stable long-term labeling (64), but in practice the efficiency of this process is too low for most experimental applications. Transsynaptic retrograde targeting has also been achieved in vivo by using AAV to express a wheat germ agglutinin-Cre (WGA-Cre) fusion protein that travels retrogradely across synapses to activate Cre-dependent transgene expression in presynaptic cells (51). One caveat of this method is that most AAV serotypes can infect axon terminals with low efficiency, which could produce artifactual labeling in cells that do not synapse directly on the WGA-Cre expressing neurons.

TARGETING BASED ON ACTIVITY PATTERN

It is often desirable to target populations of neurons with specific firing properties, e.g., neurons firing in one context but not another, or responding to one sensory stimulus but not another. For purposes of genetically encoded neuromodulation, this is typically achieved by harnessing immediate early genes (IEGs) such as c-*fos* or *arc*, which are rapidly upregulated following increases in neuronal firing (52, 112). Most of the work along these lines has been performed using mouse lines in which an inducible driver such as tTA or CreER^{T2} is expressed under the control of a promoter from an IEG such as c-*fos* or *arc* (54, 132) (**Figure 5a**). These driver lines are crossed with a responder line or injected with a Cre/tTA-dependent viral vector, and a specific behavioral condition is paired with the appropriate inducer, e.g., 4-OH-tamoxifen for CreER^{T2} or doxycycline withdrawal for tTA. This enables expression of genetically encoded actuators such as DREADDs or opsins preferentially in the subpopulation of neurons that were most active during the corresponding behavioral condition (29, 48, 93, 129) (**Figure 5b**). Additionally, there now exist viral promoters containing regulatory sequences from the *arc* gene that confer activity-dependent expression in wild-type animals (60, 70), in principle including nonrodent species.

Optimal use of these methods is complex in part because the quantitative relationship between IEG expression and firing rate (or changes in firing rate) is not well understood. One general guideline is to choose a method that activates transgene expression in approximately the proportion of cells expected to show the firing patterns of interest. For example, the dentate gyrus (DG) and CA1 regions of the hippocampus are both known to encode spatial location. A study using a
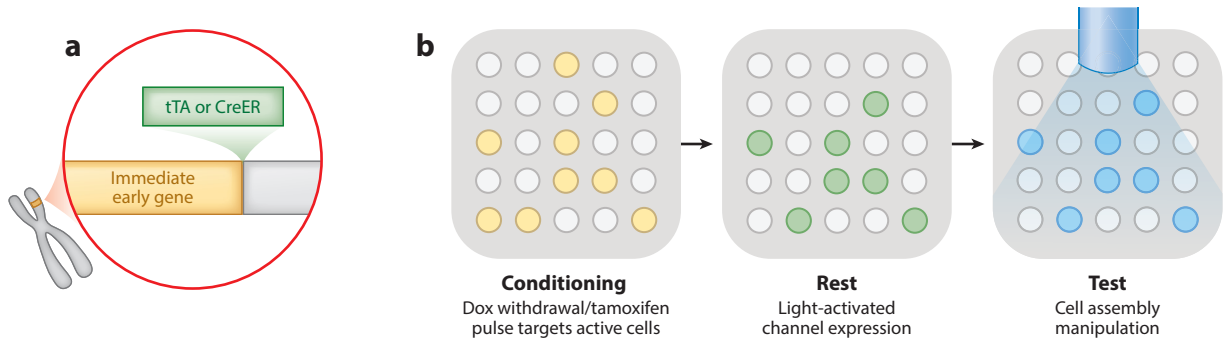
a

tTA or CreER

Immediate
early gene

b

Conditioning
Dox withdrawal/tamoxifen
pulse targets active cells

Rest
Light-activated
channel expression

Test
Cell assembly
manipulation

Figure 5

Targeting based on recent activity. (*a*) Activity-dependent transgene expression relies on inserting a chemically inducible driver transgene such as tTA or CreER^{T2} into the genomic location of an immediate early gene, which is rapidly upregulated upon increased neuronal firing. (*b*) In a typical experiment, the animal is exposed to a context or stimulus paired with doxycycline (Dox) withdrawal or a tamoxifen pulse. This causes the expression of light-activated channels preferentially in the cells most active during conditioning. After a sufficient number of light-activated channels have been produced, illumination enables activation of the ensemble of neurons that were active during conditioning.

c-*fos*-tTA line to target ChR2 expression to cells active in a specific spatial location found that 2–6% of cells were labeled in DG (129), which is approximately the percentage expected to fire based on physiological recordings (68). However, 40–70% of cells were labeled in CA1, which is larger than the percentage of CA1 neurons expected to encode a specific location (120). This suggests that cells encoding multiple locations were targeted, which was confirmed experimentally in the same study (129) and may be the reason why optically stimulating the targeted cells in DG, but not in CA1, produced the expected behavioral phenotype. Targeting of brain regions such as CA1 may benefit from other IEG-based mouse lines that label CA1 more sparsely (54) or from improved lines in which the proportion of cells labeled is titratable over a larger dynamic range.

COMBINATORIAL TARGETING

In practice, the most productive cell-targeting strategies have often been those that use a combination of the techniques described above. The most straightforward examples of this involve targeting populations of cells that simultaneously express Flp recombinase and either Cre or GAL4, thus making transgene expression contingent upon simultaneous expression of two marker genes (a logical AND) (5, 158). This can be achieved using either responder lines (91, 99, 158) or AAV (43). An alternative in flies, deployed recently to target the complete collection of mushroom body output neurons (3), is to encode the DNA binding and transcription activation domains of GAL4 as separate entities and reconstitute functional transcription factors in neurons that coexpress the split-GAL4 domains (95). GAL80, a repressor of GAL4 (97), is widely used in *Drosophila* to subtract one expression domain from another (a logical NOT) (88). A temperature-sensitive version of GAL80 offers temperature-inducible transgene expression at specific time points or developmental stages (105). Analogous NOT operations can be performed in mammals by flanking the transgene with Lox or FRT sites such that the presence of Cre or Flp inactivates, rather than activates, the transgene (53).

Cells are also commonly targeted based on the intersection of gene expression and an anatomical projection pattern. Several groups have targeted specific projections of midbrain dopaminergic neurons, for example by injecting a Cre-dependent AAV vector that encodes ChR2 into the

ventral tegmental area of a TH-Cre animal and placing an optical fiber in the nucleus accumbens to stimulate dopaminergic terminals (1, 175) or by injecting a retrograde Cre-dependent Flp virus into the lateral habenula of TH-Cre mice (155). It is also possible to target cell populations based on the combination of endogenous gene expression and synaptic connectivity. This is best illustrated in the use of a pseudotyped rabies virus that infects only cells expressing the TVA receptor (173). Of the many possible combinations, only a small number have been implemented.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The role of genetically encoded tools in neuroscience continues to grow, underscoring the importance of technologies to target transgene expression to neuronal populations of interest. Optimal use of these technologies requires assessment of the desired expression level, knowledge of the potential pitfalls, and careful post hoc verification. Much has been accomplished with existing cell-targeting technologies, but there are several areas in which continuing advances will lead to substantial improvements. The most straightforward advance is that single-cell RNA-seq is starting to provide candidate marker genes that promise to target populations more precisely. In addition to this, the main areas for improvement fall into four categories: designer promoters; long-term, nontoxic targeting based on synaptic connectivity; improvement of activity-dependent targeting; and eliminating dependence on germline genome editing.

Designer Promoters

Control of transgene expression is often achieved by fusing fragments of natural *cis*-regulatory modules to heterologous transcription factors or recombinases (26, 33, 46, 126). Opportunities for refining the resulting expression patterns are limited to intersections of just two expression domains (e.g., Cre plus Flp, split-GAL4, GAL4 plus GAL80). Artificial *cis*-regulatory modules containing binding sites for potentially large numbers of endogenous—activating and repressing—transcription factors could potentially overcome this limitation.

Long-Term, Nontoxic Targeting Based on Synaptic Connectivity

Restricting viral infections to specific starter populations and limiting their spread to single synapses have been major advances (173). However, transsynaptic spread requires infectious viral particles to be generated in vivo, which typically kills the infected neurons after several days to weeks, precluding use in many settings. Advances in this area will likely involve a mechanism to restrict expression of the viral genes required for replication to a brief period in time, after which the cells can recover normal function, or nonviral alternatives for activating transgene expression via synaptic contacts. For example, an expression cassette lying dormant in the genome might become active when a contact receptor at the pre- or postsynaptic cell surface releases a transcriptional transactivator or recombinase. Biological precedents for this type of mechanism exist: The transmembrane receptors Lin-12 in *C. elegans* and Notch in *Drosophila* undergo proteolytic cleavage in response to ligand; the liberated intracellular domains subsequently shuttle to the nucleus, where they activate gene expression (146, 160, 161).

Improving Activity-Dependent Targeting

The current systems based on IEG expression have opened new avenues of research (29, 48, 93, 129), but they suffer from leaky expression and a narrow dynamic range. These limitations

will likely be addressed through improvements in inducible recombinases, such as simultaneous regulation by two exogenous factors, e.g., 4-OH-tamoxifen and trimethoprim.

Many applications require higher temporal resolution than IEG-based methods can provide, for example restricting transgene expression to neurons firing at a specific phase of a complex task. The recent development of CaMPARI, a calcium-sensitive fluorescent protein that preferentially photoconverts from green to red in neurons that are firing at the time of illumination (45), suggests how finer temporal resolution might be achieved. The mechanism of photoconversion is the light-dependent cleavage of a peptide bond (58), which could in principle be used to activate a recombinase and thus permanent transgene expression. A fundamental difficulty of this approach is the fact that peptide bond cleavage requires high energy photons at near-UV wavelengths, which have poor tissue penetration. As a result, illumination of a behaviorally relevant volume of brain tissue is heterogeneous, likely producing false-positive targeting of inactive cells near the illumination site and insufficient expression farther away. Nevertheless, this is an area requiring greater investigation.

Eliminating Dependence on Germline Genome Editing

Many strategies involve germline genome editing to create transgenic animals that are subsequently bred for experiments. The cost, labor, and delays associated with generating, breeding, and maintaining transgenic lines are substantial, and the burden increases with larger or less common model organisms. Primates, in particular, are difficult because of small litter sizes and long generation times, with humans being the most challenging of all. The ability to safely target expression of therapeutic transgenes to specific neuronal populations in the human CNS would revolutionize clinical neuropsychiatry, just as the corresponding methods in laboratory animals have revolutionized basic neuroscience. There are therefore several compelling reasons to eliminate dependence on germline genome modification for neuronal targeting. We envision technological developments along several lines.

First, cell-specific targeting has in a small number of cases been achieved with viral vectors carrying short regulatory sequences (36, 63, 87, 177). Successful generalization to arbitrary genes of interest will likely require rigorous bioinformatics analyses of genomic regulatory regions combined with high-throughput screening methods to validate candidate promoter sequences. MicroRNA binding sites can enhance or suppress transgene expression based on the presence or absence of endogenous microRNAs. This strategy has been implemented successfully by adding microRNA binding sites to transgenes in AAV to target GABAergic neurons in cortex (144), and it could likely be generalized.

Second, viral capsids are important determinants of the efficiency and cellular specificity of infection. Improvements in these parameters will likely be achieved by screens in which libraries of viral particles carrying mutagenized capsid sequences are injected into the brain, and capsid sequences with increased efficiency or specificity are isolated from infected neurons (35, 113).

Third, a caveat of viral vectors is the possibility that animals can develop a cytotoxic T-cell–mediated response against the transgene product that kills neurons expressing it. That this happens infrequently reflects the fact that the brain is an immune-privileged site, and expression of transgenes in neurons generally induces immune tolerance. However, viral serotypes and promoters that drive transgene expression in microglia can trigger catastrophic immune responses (25). Avoiding microglial transgene expression using Cre lines, neuron-specific promoters, and appropriate microRNA binding sites (19) should reduce the likelihood of this occurring. Nevertheless, finding an effective general solution to this problem is a high priority, particularly for translational applications.

Fourth, another method to render transgene expression conditional on endogenous gene expression is to use modified versions of a group I self-splicing ribozyme to *trans*-splice mRNAs encoding a nonfunctional transgene onto endogenous mRNAs, activating transgene expression (6). Although this technique has not yet been applied broadly, it has the potential to enable rapid targeting of viral vectors based on arbitrary marker genes without requiring any mechanistic knowledge of how these genes are regulated.

Fifth, virally encoded CRISPR-Cas9 has already been used for targeted gene deletion in vivo (159), and with further development this could possibly be used to regulate transgene expression through direct integration into a specific genomic locus.

CONCLUSION

A great strength of genetically encoded tools is their exquisite molecular specificity. Achieving comparable specificity in targeting these tools—selectively, comprehensively, at the right time, and in the right amounts—to the cells of interest is key to using them successfully. This is true even for optogenetic devices, whose ability to respond to optical signals that can be patterned in space and time provides an extra layer of spatiotemporal control on top of the genetic resolution of cells.

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Contents

Errata

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