Topic Introduction

Genetic Labeling of Neurons in Mouse Brain

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Mammalian central nervous systems consist of highly diverse types of neurons, which are the functional units of neural circuits. To understand the organization, assembly, and function of neural circuits, it is necessary to develop and to improve technologies that allow efficient and robust visualization of neurons in their native environment *in vivo*. Here we discuss various genetic strategies for achieving specific and robust neuron labeling in mice.

INTRODUCTION

The challenges in labeling and visualizing neurons in mammalian brains include (1) highly heterogeneous neuron types that are often intermingled extensively, (2) complex geometry and trajectory of axonal and dendritic arbors, and (3) minute and densely packed structures such as axons and synapses. Traditional extracellular or intracellular dye injection methods and neurochemical staining have very limited capacity to meet these challenges. A genetic approach in mice is ideal to achieve specific, efficient, and robust neuron labeling (Luo et al. 2008). A genetic strategy that engages the intrinsic gene regulatory mechanisms generating and maintaining cell identity and diversity is often the best, if not the only, method to label a specific neuron type. In addition, genetically encoded fluorescent proteins (FPs) (such as green FP [GFP] and its variants, XFPs) and the continuous invention and improvement of their variants have revolutionized neuronal labeling and biological imaging (Giepmans et al. 2006). Most genetic labeling strategies involve the expression of FPs in defined neuron types or populations. Therefore, successful genetic labeling methods should fulfill two technical requirements that appear deceptively simple: cell-type specificity and high-level FP expression. Here we focus on various strategies in mice to achieve these two technical goals. We also discuss some of the unique challenges in mice in comparison with other neurogenetic systems such as *Drosophila* and zebrafish.

HOW TO ACHIEVE SPECIFICITY AND RELIABILITY OF CELL LABELING

A dream for many neuroscientists is to be able to generate or to have easy access to genetically engineered mouse lines in which specific neuron types or populations of interest are labeled reliably, but with thousands of neuron types in the mouse brain, this dream is far from being realized. The difficulty is not just the number of cell types. There are also deep conceptual and technical roots to the problem. The first issue has to do with the definition of neuron type; however, an in-depth discussion of this complex and contentious subject is beyond the scope of this introduction. In addition, the relationship between neuron types and gene expression, the basis of genetic labeling, is complex. Although there is evidence that gene expression profiles may determine cell phenotypes and, thus,

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cell identity (Arlotta et al. 2005; Nelson et al. 2006; Sugino et al. 2006), there is often no simple relationship between the expression of one gene and a morphologically and functionally defined cell type. With these considerations in mind, we will discuss neuron types in a largely practical sense based on their expression of one or two genes that are suitable for current genetic strategies. In this context, a major issue in achieving specificity of neuron labeling comes down to harnessing strategies to recapitulate the expression pattern of an endogenous gene with that of exogenous marker genes (e.g., FPs).

An alternative strategy is to use genetic elements and techniques to randomly or semiselectively label subsets of neurons in a high-throughput and large-scale scheme. Indeed, this approach has been applied to *Drosophila* (Pfeiffer et al. 2008), zebrafish (Scott et al. 2007), and mice (Gong et al. 2003). An important issue here is whether such a strategy can parse neurons into functionally meaningful and specific subsets (vs. mixed or largely random cell populations). Because the genetic identities of labeled neurons are often unclear, a major challenge associated with this strategy is the extensive effort necessary to discover the identity and to characterize the property of labeled neurons.

We discuss both strategies here, but will focus on methods aimed at recapitulating endogenous gene expression that can be put into routine practice by most academic laboratories. In particular, we will compare the pros and cons of transgenic (Tg) and gene-targeting approaches.

Transgenic and Bacterial Artificial Chromosome Transgenic Approaches

Transgenesis is the most common method for genetic labeling. In these experiments, the starting point is often the identification of a gene X, which is expressed in neurons of interest. The goal then is to express FPs in these neurons, or a specific subset of them, using the promoter elements of gene X. In a conventional Tg approach, a DNA fragment containing several to tens of kilobases (kb) of the 5' promoter region of gene X is linked to the GFP-coding sequence by routine molecular cloning. Through pronuclear injection, this fusion gene is integrated into the mouse genome at random locations (Brinster et al. 1982) (Fig. 1). The expression of GFP is, thus, determined by the promoter fragment of gene X contained in the transgene as well as by the gene regulatory elements near the genomic loci of Tg integration.

In the bacterial artificial chromosome (BAC) Tg approach, a much larger DNA fragment of gene X (up to ~200 kb) is used to control GFP expression (Yang et al. 1997). The GFP-coding sequence is inserted into a BAC clone containing the coding region and certain regulatory regions of gene X (depending on the size of the gene), often at the translation initiation site (Fig. 1B). This can be

FIGURE 1. Comparison of transgenesis and gene targeting. (Rectangles) coding exons; (arrows) transcription promoter; (thick lines) chromosomal DNA; (thin lines) transgenic (Tg) DNA; (ovals, triangle, and diamond) enhancer or repressor elements. (A) Genomic organization of an endogenous gene. (B) Schematic of a Tg or a bacterial artificial chromosome (BAC) Tg construct (top) and two configurations of Tg alleles at different genomic integration sites. Green fluorescent (GFP) cDNA is inserted in at the ATG site of the coding exon in the Tg BAC clone. Different colors and shapes represent different genomic loci and different enhancer elements. (C) The knockin allele is identical to the endogenous allele, with all the regulatory elements intact, except for the insertion of the GFP cDNA in a coding exon.

achieved by several recombineering procedures that are now well established (Copeland et al. 2001; Muylers et al. 2001; Gong et al. 2002). The BAC transgene is also integrated into the mouse genome through random insertion.

The advantages of the Tg approach include (1) easy and rapid construction of transgenes, (2) potentially high levels of Tg expression caused by multiple copies often arranged in a tandem array at the genomic integration site, and (3) relatively low cost. The main caveat of the Tg approach is that Tg expression patterns often do not recapitulate those of the endogenous gene. This is not surprising given our increasing knowledge of the mouse genome and gene regulation. First, the *cis*-regulatory elements (enhancers, repressors, and insulators) are often very distant from the transcription start site (Kapranov et al. 2007). Therefore, conventional and even most BAC Tg constructs do not contain the full complements of regulatory elements of the gene of interest. Second, because *cis*-regulatory elements can act at a very long distance, enhancers and repressors near the Tg integration site (but unrelated to the promoter elements in the transgene) will influence Tg transcription, leading to ectopic expression. In addition, different Tg lines will have different expression patterns because of differential enhancer/repressor influences at different genomic integration sites. Third, in addition to promoters and enhancers, gene expression is strongly influenced by chromatin architecture determined by histone modification and DNA methylation patterns; transgenes inserted into a foreign chromatin environment can be silenced or altered epigenetically in unpredictable ways.

These are serious issues because cell specificity is the basis for successful genetic labeling and manipulation. Here, it seems worthwhile to make a comparison with Tg labeling in *Drosophila*. The vast majority of genetic manipulations in flies is achieved through conventional Tg approaches with relatively small promoter regions. The success of this approach is obvious. Transgenes in the *Drosophila* genome should presumably face the same issues raised above, but several factors may have contributed to better success for the Tg approach in flies. First, the size of *Drosophila* genes is smaller than that of the mouse; thus, a conventional Tg construct is more likely to contain the main regulatory elements. Indeed, BAC Tgs are rarely used in flies. Second, there appear to be significant differences in chromatin architectures between *Drosophila* and mammals. Although much of the mouse genome is tagged by methylation of DNA cytosine throughout the mouse life span, and this epigenetic modification likely plays important roles in gene regulation, DNA methylation is sparse and is largely restricted to early embryonic development in *Drosophila* (Gowher et al. 2000; Lyko et al. 2000; Suzuki and Bird 2008). The impact of this profound difference in global DNA methylation patterns on Tg expression is not yet clear, but one should no longer be surprised that a genetic strategy that works well in flies may not work equally well in mice. Third, transgenes in flies clearly often do not fully recapitulate the endogenous patterns of gene expression, but the ease and low cost of generating and screening many Tg lines often allow researchers to identify lines that are useful for their experiments.

Of course, these issues and discussions should by no means discourage the use of Tg mice in neuron labeling. Rate of success, which is unpredictable in mice, should be kept in mind when planning the experiments. Also, one should have realistic expectations. There have been numerous examples in which the Tg approach has yielded useful and sometimes spectacular results. In most of these cases, the success resulted from generating many Tg lines and screening for those that suited the research goal. In some cases, the unexpected or unintended Tg expression patterns, in fact, defined new research projects.

Gene Targeting Approach

Gene targeting is achieved by homologous recombination in embryonic stem cells (Capecchi 1989). It allows highly precise engineering of the mouse genome (e.g., by deletion, insertion, or point mutations) at defined genetic loci with single-base accuracy (Bradley et al. 1992). In genetic labeling, once a gene of interest is identified, cDNAs coding for FPs can be inserted at any desired location of the endogenous gene X through gene targeting. Because the FP cDNA is embedded in the native chromatin environment with its regulatory elements largely intact (Fig. 1C), FP expression often precisely and reliably recapitulates the expression of gene X (Mombaerts et al. 1996; Tamamaki et al. 2003;

Bozza et al. 2004). This is the most important advantage of gene targeting. Indeed, in most cases, the only method to recapitulate endogenous gene expression is through gene targeting. Therefore, gene targeting allows the rational design of precise and reliable genetic manipulations that are essential for specific neuron labeling.

There are several disadvantages of the gene targeting strategy. First, the construction of the gene targeting vector is more demanding, although recombineering technology now greatly simplifies this task. Second, the procedure of homologous recombination in embryonic stem cells, although now routine and reliable, is still labor intensive and costly. Third, and most important for neuron labeling, the expression level of the FP is determined by the expression level of endogenous gene Xs, which varies greatly among different genes. In most cases, these levels are simply not strong enough to yield useful labeling for axons and dendrites. This is a fatal problem, for if FP expression is invisible, specificity is irrelevant. Fortunately, this problem can be solved by binary gene expression strategies that dissociate specificity and expression levels. Gene targeting strategy applied in a binary expression system is probably the most versatile and powerful approach for neuron labeling (see below).

GENE TARGETING AND BINARY EXPRESSION SYSTEMS

In binary systems (Fig. 2), gene expression is controlled by two Tg alleles that are bred together from two independent mouse lines. Most schemes include a driver allele, which is engineered to define the pattern and the specificity of expression, and a reporter allele, which is activated by the driver and directs the expression of a marker gene (e.g., FP) with a strong promoter.

In the recombination-based binary system (Dymecki and Kim 2007) (Fig. 2), a DNA fragment encoding a sequence-specific recombinase (e.g., Cre, Flp, or Dre) is inserted into an endogenous gene X locus through gene targeting (i.e., by knockin) to generate a driver mouse line, thus, establishing a genetic handle for neurons defined by the expression of gene X. In a separate reporter line, FP expression is driven by a strong and often ubiquitous promoter but is conditional on Cre-mediated recombination. This conditional reporter allele is often built with a transcription–translation STOP cassette flanked by *loxP* sites, and this *loxP*–STOP–*loxP* cassette is inserted just before the FP-coding sequence (Fig. 2). An example of Cre/*loxP*-mediated neuron labeling is shown in Figure 3. In a transcription activation-based binary system (Fig. 2), potent transcriptional activators such as tTA (Sprengel and Hasan 2007) or Gal4 (Ornitz et al. 1991) can be used in place of Cre to create driver lines. In the corresponding reporter lines, a strong promoter, tet operator (tetO) or upstream activating sequence (UAS), respectively, are used to drive FP expression.

FIGURE 2. Binary systems for genetic control of gene expression. (Arrows) Transcription promoter; (ovals) *cis*-regulatory elements. See text for further description.

FIGURE 3. *Cre/loxP*-mediated labeling of GABAergic neurons. The driver is an inducible *Gad2-CreER* knockin mouse line (H. Taniguchi and Z.J. Huang, unpubl.); the reporter is the mouse line (Sousa et al. 2009). (A) A single dose of tamoxifen was given at embryonic day 16 to the pregnant female, and the progeny was perfused at P25. This sagittal section was enhanced with GFP immunofluorescence and was imaged with a NanoZoomer 2.0-HT (Hamamatsu). (B,C) Same sample as in A, showing the diverse types of GABAergic neurons in the neocortex. Neuronal soma (arrow) and axons (arrowhead) are indicated. (D,E) Neuronal labeling in the cerebellum in a P40 mouse after a single dose of tamoxifen induction at P25. (D) A Purkinje cell (arrow), stellate and basket cells in the molecular layer (double arrow), and Golgi cells (arrowhead) are indicated. (E) A basket cell (arrow) and basket cell axon terminals (arrowhead) are indicated. Scale bars, 200 μ m in B, 50 μ m in C, 400 μ m in D, 50 μ m in E.

The binary system distributes the two requirements for genetic labeling (i.e., specificity and high-level expression) to two independent genetic modifications. This scheme potentially can overcome the problems of low and variable expression levels intrinsic to the gene targeting strategy if high-level expression reporter alleles can be constructed (see the next section). Binary systems also confer combinatorial power and flexibility because different drivers and reporters can be combined to label different neuron types with different FPs. Furthermore, a binary system can convert transient gene expression during development into stable marker expression, thus, allowing fate mapping and lineage tracing.

HOW TO ACHIEVE ROBUST AND SPECIFIC NEURON LABELING

Cre/loxP System

The requirement for robust and strong FP expression seems obvious—axons and synapses need to be brightly labeled for visualization by various imaging methods. What was not appreciated until recently was the level of FP expression necessary to achieve bright labeling of these minute structures in large mammalian neurons. FP expression needs to be extremely high to fill axons and their terminals with enough fluorescence for high-resolution imaging, especially *in vivo*. This demand for high-level expression is typically not able to be met by most endogenous promoters targeted by the gene knockin approach, with rare exceptions such as the olfactory receptor genes (Potter et al. 2001).

The *Cre/loxP* binary system allows enhancement of FP expression through the reporter allele. An ideal *Cre*-activated reporter allele should have the following properties. The first is high-level expression: Reporter alleles are often driven with artificial promoters consisting of strong viral promoter and enhancer elements, complemented with an RNA stabilization sequence. The second is the potential

for ubiquitous expression in all cells, or at least, in all neurons after Cre/*loxP* recombination. Third, the reporter allele can only contain a single expression cassette. Because the reporter allele contains *loxP* sites, multiple copies in tandem arrays will be reduced to a single copy on Cre-mediated recombination. This property is specific to a recombination-activated reporter allele and eliminates the opportunity for boosting the expression level by increasing Tg copy numbers. These three properties are not as easy to achieve as they may seem. Currently, there are only two to three well-characterized genomic loci (e.g., *Rosa26*, *Col1a*) (Zambrowicz et al. 1997; Soriano 1999) that confer ubiquitous expression to an exogenous gene; however, all of these loci have rather weak expression levels as a single copy.

To date, there are only a handful of Cre-activated reporter lines that are useful for neuron labeling to various extents. The RCE reporter is built at the *Rosa26* locus driven by the CAGG promoter (Sousa et al. 2009). RCE does allow visualization of neuron somata in brain slices for targeted electrophysiology recording, fluorescence-activated cell sorting, and labeling of axons with GFP immunofluorescence (Fig. 3). Z/EG is a single-copy Tg reporter at an undefined genomic locus (Novak et al. 2000). GFP expression from Z/EG is comparable or slightly stronger than RCE, but it is not ubiquitous. The best reporter alleles include Ai9 and Ai14, which express Tdtomato driven by the CAGG promoter from the *Rosa26* locus supplemented with the WPRE (woodchuck posttranscriptional regulatory element) RNA stabilization sequence (Madisen et al. 2010). As a single copy, Ai9 allows imaging of axon terminals in vivo. In addition to Ai9, the Ai6 GFP reporter also allows intense labeling of neuronal soma without the use of antibodies (Madisen et al. 2010). These recent advances show that a strong, ubiquitous, and single-copy reporter allele at the *Rosa26* locus is possible. It is reasonable to expect that further improvement and modifications at the *Rosa26* locus will yield additional useful reporter alleles. In addition, efforts are underway to identify other strong and ubiquitous loci in the mouse genome based on the knowledge of gene expression in the mouse brain gained from the Allen brain atlas <http://www.brain-map.org/>.

Transcription Activation System

The Gal4–UAS system (Fig. 2) is widely used in several species, especially *Drosophila* and zebrafish. In mice, the tetracycline trans-activator (tTA) combined with a tetO-driven reporter (Fig. 2) have proven to be a robust expression system (Krestel et al. 2001; Sprengel and Hasan 2007). Although the tTA–tetO system was originally designed for inducible gene expression, this system achieves high levels of gene expression in the mouse brain in the absence of tetracycline. Robust tetO reporter alleles have been established and have been characterized (Krestel et al. 2001; Sprengel and Hasan 2007). However, cell-type-specific tTA driver lines are currently quite limited and more need to be generated, especially with a gene targeting approach. In addition to the tTA–tetO system, the Gal4–UAS system should be better explored in mice.

Viruses as Reporter Components in Binary Systems

Virus-mediated gene delivery represents an alternative and powerful strategy for labeling and manipulating neurons in mammalian brains. Because of their multicopy transfection of a single neuron and the use of strong and ubiquitous promoters, virus-mediated gene delivery often achieves high-level expression and, thus, bright labeling of fine structures such as synapses. In addition, viral transfection can be targeted to specific brain regions by stereotactic injection and at defined developmental stages. Furthermore, neurotrophic viruses suitable for longitudinal studies have been well characterized and can now be efficiently engineered at low cost. However, a major drawback of virus-mediated gene delivery has been the lack of cell-type specificity.

An important recent advancement is the invention of conditional viral vectors as the reporter component of a binary system. For example, we and others have described methods that combine Cre knockin mice and Cre-activated adeno-associated viruses (AAVs) to achieve high-level, stable, and cell-type-specific gene expression (Atasoy et al. 2008; Kuhlman and Huang 2008). This method is simple, highly efficient, and allows chronic live imaging of defined classes of synapses in the mouse

brain (Fig. 4). With the increasing number of Cre driver lines, conditional viral vectors represent a general and particularly powerful strategy for visualizing and manipulating specific neuron types in vivo. In addition to the Cre/loxP strategy, transcription activation-based viral vectors are also being developed. In addition to AAV, conditional lentiviral vectors and other retroviral vectors are being explored. A protocol for the design and delivery of Cre-dependent AAVs is presented in **Cre-Dependent Adeno-Associated Virus Preparation and Delivery for Labeling Neurons in the Mouse Brain** (Huang et al. 2014).

Tg Overexpression

Numerous Tg lines have proven to be useful for neuron labeling, and among these, the Thy1–XFP lines are probably the best examples (Feng et al. 2000). Because of their strong promoter and multi-copy Tgs, the Thy1–XFP lines are among the few genetically engineered mouse lines that allow direct live imaging of synapses in vivo (Grutzendler et al. 2002; Trachtenberg et al. 2002). Another key factor for their success was the generation and the screening of many different Tg lines. Although none of these lines recapitulated endogenous Thy1 expression, different lines (Thy1–XFP) with different labeling frequencies and patterns serve different experimental goals. A number of BAC Tg lines have been useful in labeling subsets of neurons for targeted physiological recordings and for morphological studies (Ango et al. 2004; Di Cristo et al. 2004; Inta et al. 2008; Kim et al. 2008). Again, the key is to screen through multiple lines and to identify those that are useful.

FIGURE 4. Neuron labeling by GFP expression using Cre-activated AAV vectors. Two strategies have been used to render GFP expression conditional on Cre-mediated recombination. (A) A loxP-STOP-loxP cassette is inserted between the promoter and the GFP gene. (B) A GFP gene flanked by a pair of incompatible antiparallel loxP sites is cloned in the opposite orientation to an upstream cytomegalovirus (CMV) promoter; Cre recombination results in an inversion of this flip-excision (FLEX) switch and, thus, expression of the GFP gene. (C) Injection of the AAV-lox-STOP-lox-GFP virus into the neocortex of a Pv-cre mouse specifically labeled a subclass of GABAergic neurons (S. Kuhlman and Z.J. Huang, unpubl.). (D–F) In vivo two-photon imaging of GFP-labeled cortical basket interneurons in Pv-Cre mice. (D) z-Projections of an image stack 85–90 μm below the pia mater. Note the smooth, aspiny dendrites (blue arrow) and the dense cluster of boutons of varying size (yellow arrowheads). (E) Projection of a z-series 60–170 μm below the pia mater from a sparsely labeled area, showing an isolated basket interneuron. Dendrites (blue arrows) could be traced back to the soma. Axonal boutons (yellow arrowheads) appear as a cloudy signal at this magnification. (F) Examples of a well-isolated basket cell axon (yellow arrow) with distinct boutons (yellow arrowheads). Scale bars, 100 μm in C, 20 μm in E, 5 μm in D and F. (D–F, Adapted from Kuhlman and Huang 2008.)

Technical Tips on Driver Alleles and Mice

To recapitulate the expression of an endogenous gene X, driver cDNAs (Cre, CreER, rTA, and Gal4) can be targeted to the translation initiation codon of gene X. This will inactivate one allele of gene X but may or may not influence the experimental results or interpretation, depending on the particular gene or experiment. Alternatively, driver cDNAs can be inserted after the terminal codon of gene X using a bicistronic strategy. The bicistronic cassette will contain either an internal ribosomal entry site or a DNA fragment coding for the self-cleaving peptide T2A, followed by the driver cDNA. In this case, the expression of gene X should be largely intact.

Although Cre is generally quite efficient in mediating recombination at the *loxP* sites, the efficiency of the inducible CreER driver is quite variable. This variability may result from (1) expression levels, (2) cell-type difference in concentration, localization, or nuclear transport of CreER, (3) methods and dose of tamoxifen administration, or (4) accessibility of the cells of interest to tamoxifen. In addition, genomic locations of the reporter or target alleles (i.e., *loxP* sequences) may have different accessibilities by Cre or CreER and, thus, may have different recombination efficiencies.

OTHER RELATED GENETIC STRATEGIES

Increased Specificity Through Intersectional Recombination

As discussed earlier, the correlation between the expression of a single gene and a specific cell type is often less than perfect. On the other hand, overlapping the expression patterns of two genes can define quite exquisite cell types or populations. For example, neurogliaform cells (NGFCs) are a highly unique class of neocortical GABAergic interneurons (i.e., transmit or secrete γ -aminobutyric acid) that extend extremely dense axon arbors and mediate volume instead of synaptic transmission (Olah et al. 2009). The expression of α -actinin2 or the GABA_A receptor δ distinguishes NGFCs from other cortical GABAergic neurons, but both genes are also expressed in a subset of cortical glutamatergic neurons (Olah et al. 2009). On the other hand, the overlap of a pan-GABA marker and α -actinin2 or GABA_AR δ would specifically define NGFCs. Such genetic labeling can be achieved by an intersectional method using two site-specific recombination systems (Awatramani et al. 2003; Dymecki and Kim 2007) (Fig. 5). In this scheme, two driver alleles are generated, one expressing Cre and the other expressing Flp (or Dre). The reporter allele is designed to be activated only when both Cre and Flp are present. Although this strategy requires the combination of three alleles, and more dual reporters need to be generated, it significantly improves labeling specificity and is increasingly used in neuron labeling and manipulation.

Mosaic Analysis with Double Markers

Mosaic analysis with double markers (MADM) allows simultaneous labeling and gene knockout in clones of somatic cells or isolated single cells in vivo (Zong et al. 2005). Two reciprocally chimeric genes, each containing the amino terminus of one marker and the carboxyl terminus of the other marker interrupted by a *loxP*-containing intron, are knocked in at identical locations on homologous chromosomes. Functional expression of markers requires Cre-mediated interchromosomal recombination, which can be induced in both mitotic and postmitotic neurons in vivo. MADM has been used to label cells, to determine cell lineage, to trace neuronal connections, and to create conditional knockouts in small populations of cells (Espinosa and Luo 2008; Espinosa et al. 2009).

Brainbow

Brainbow is a method to genetically label neurons with multiple distinct colors (Livet et al. 2007; Tian et al. 2011). Brainbow transgenes are reporter alleles designed to create a stochastic choice of expression between three or more XFPs on inducible Cre-mediated recombination. Integration of tandem Brainbow transgenes yields combinatorial XFP expression and, thus, many colors, thereby providing a

FIGURE 5. Intersectional strategy. Different designs of intersectional reporter alleles (*left*) can give rise to different labeling patterns (*right*) following the intersection of Cre and Flp driver alleles. (*Top*) Sequential arrangement of *loxP*- and *frt*-flanked stop cassettes result in GFP expression in cells defined by the overlap of the Flp (A) and Cre (B) drivers. (*Middle*) This reporter allele includes both red FP and GFP cassettes in tandem and results in both intersectional (green, A/B) and subtractive (red, B not A) labeling. (*Bottom*) This reporter allele contains *loxP* and *frt* cassettes in a different order and results in intersectional (green, A/B) and a different subtractive (red, A not B) labeling.

way to distinguish adjacent neurons. For example, hundreds of neighboring axons and multiple synaptic contacts that show approximately 90 labeled colors in a small volume of the cerebellum have been reconstructed. The ability of the Brainbow system to uniquely label many individual cells within a population may facilitate the analysis of neuronal circuitry on a large scale.

Single-Neuron Labeling with Inducible Cre-Mediated Knockout

Single-neuron labeling with inducible Cre-mediated knockout (SLICK) is achieved by coexpressing a drug-inducible form of Cre and an FP in a small subset of neurons, thus, combining genetic manipulation with fluorescent labeling of single neurons (Young et al. 2008). Using the Thy1 promoter, inducible genetic manipulation of several types of neurons has been shown using SLICK. For example, Thy1-SLICK was used to inactivate the choline acetyltransferase gene, thus eliminating synaptic transmission in a small subset of neuromuscular junctions.

RELATIVE MERITS OF DIFFERENT METHODS AND FUTURE OUTLOOK

Binary System Versus Direct Labeling

The binary system has several major advantages: (1) conversion of weak and transient expression of a driver allele to strong and stable expression from the reporter allele, (2) combinatorial power for both neuron labeling and functional manipulation, and (3) it can be used for fate mapping and lineage tracing. One caveat of the Cre/*loxP* system is that reporter expression in mature brain results from integrated Cre activity throughout development (Fig. 6). This may blunt the specificity of labeling based on adult expression of a gene X but could be circumvented by using an inducible form of Cre or by using a Cre-dependent virus injected to the adult brain or at a desired developmental stage (Figs. 4 and 6).

Specificity: Gene Targeting Versus Transgenesis

Gene targeting through Cre (or Flp) knockin is clearly superior to transgenesis in recapitulating endogenous gene expression patterns. With improved Cre-dependent reporter alleles and viral

FIGURE 6. The pattern of neuron labeling achieved by Cre recombination may differ from the gene expression pattern driving Cre activity. (Large open ovals) Brain tissue; (green circle and oval) different cell populations throughout development. (*Top row*) Gene X is transiently expressed in a cell during the embryonic stage, then in b cells from the neonatal stage onward, and in c cells in the adult brain. (*Second row*) In a Tg mouse that contains the gene X–Cre driver allele and the Cre-dependent reporter, transient Cre activity in the embryo is permanently registered by the reporter allele. The recombination pattern (thus, neuron labeling) is an integration of Cre activity throughout development. (*Third row*) In a Tg mouse that contains the gene X–Cre driver allele, targeted injection of a Cre-dependent AAV in the adult brain can selectively label c (or b) cells. (*Bottom row*) In a Tg mouse that contains an inducible gene X–CreER driver allele and a Cre-dependent reporter, selective labeling of b cells can be achieved by administering tamoxifen at neonatal ages.

vectors, the Cre-knockin binary system will be the method of choice for many future experiments. On the other hand, characterization of various Cre Tg mice will yield useful lines. Also, by chance and not by design (thus, unpredictable), Tg lines can occasionally generate surprisingly specific patterns not yet represented by known patterns of gene expression.

Expression Levels

The requirement for expression levels of markers used for neuron labeling depends on the nature of the experiments. For cell labeling in fixed tissues, amplification of FP signals by immunofluorescence may well serve the purpose. For identifying cell somata in electrophysiology and calcium imaging experiments in living tissues, the current Cre-dependent reporters are mostly sufficient. For imaging dendrites and axon terminals in living tissues or deep into the tissue, the most demanding task for neuron labeling, the Ai9 reporter is useful; and better reporters will likely be developed. In addition, Cre-dependent viruses certainly will serve this task well.

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