Challenges in Retinal Circuit Regeneration: Linking Neuronal Connectivity to Circuit Function

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Tremendous progress has been made in retinal regeneration, as exemplified by successful transplantation of retinal pigment epithelia and photoreceptor cells in the adult retina, as well as by generation of retinal tissue from embryonic stem cells and induced pluripotent cells. However, it remains unknown how new photoreceptors integrate within retinal circuits and contribute to vision restoration. There is a large gap in our understanding, at both the cellular and behavioral levels, of the functional roles of new neurons in the adult retina. This gap largely arises from the lack of appropriate methods for analyzing the organization and function of new neurons at the circuit level. To bridge this gap and understand the functional roles of new neurons in living animals, it will be necessary to identify newly formed connections, correlate them with function, manipulate their activity, and assess the behavioral outcome of these manipulations. Recombinant viral vectors are powerful tools not only for controlling gene expression and reprogramming cells, but also for tracing cell fates and neuronal connectivity, monitoring biological functions, and manipulating the physiological state of a specific cell population. These virus-based approaches, combined with electrophysiology and optical imaging, will provide circuit-level insight into neural regeneration and facilitate new strategies for achieving vision restoration in the adult retina. Herein, we discuss challenges and future directions in retinal regeneration research.

Key words stem cell; neural circuit; retina; rabies virus; transsynaptic tracing; visual system

1. INTRODUCTION

The mammalian retina, a highly layered structure a few hundred micrometers thick, consists of six cell classes: photoreceptors, horizontal cells, bipolar cells, amacrine cells, retinal ganglion cells (RGCs), and Müller glia.1,2) The processing of visual information is initiated in rod and cone photoreceptors when photoreceptors capture photons, convert them into electrical signals, and stimulate multiple neural circuits in the retina. This initial signal encoded by photoreceptors is transformed into an adequate representation of the entire visual scene. Incoming visual signals are processed by at least 80 anatomically and physiologically distinct neural cell populations and at least 20 separate circuits in the retina. RGCs produce the sole output of the retina, and each RGC type sends a discrete representation of visual features to various targets in the brain via the optic nerve. The lateral geniculate nucleus (LGN) is the primary relay center for visual information received from the retina. Neurons of the LGN send their axons to the primary visual cortex V1, which extracts the relevant information from this reduced signal and further elaborates and integrates the information into a unified and coherent perceptual experience.

We rely on vision as a primary sense for evaluating our surroundings and guiding our behavior. Therefore, loss of vision dramatically reduces our quality of life. The leading cause of blindness in developed countries is retinal degeneration. Many degenerative retinopathies culminate in the same final consequences: loss of photoreceptors and vision. Once retinal neurons are lost, they are unable to regenerate, and radical treatments for retinal degeneration have not been developed.3,4) Hence, new therapies for vision loss are urgently required.

Because first-order neurons are selectively affected in retinitis pigmentosa and age-related macular degeneration (AMD), the neural circuitry mediating higher-order visual processing is maintained during the early phase of degeneration. Therefore, replenishment of retinal cells may make it possible to reconstitute retinal circuits and recover visual function.5–8) Due to the proliferation and differentiation potential of stem cells, stem cell therapy represents a promising future approach to replenishing retinal cells.9–12) To achieve vision restoration using stem cells, two strategies have been proposed: drug therapy and transplantation therapy.13) Although these strategies are different, they have a common goal: making new retinal neurons that can integrate and function in pre-existing neural circuits.

2. RETINAL REGENERATION: PAST, PRESENT, AND FUTURE

Müller glia, which generate various types of retinal neurons in response to injury in the adult, are the primary targets of drug therapy for retinal regeneration.14,15) However, the number of regenerated retinal neurons is very low. Therefore, we need to increase the number of new functional neurons using drugs. There are three main steps involved in retinal repair:
proliferation, differentiation, and synapse formation. Each step involves multiple genes and molecular cascades, any of which represent potential targets for drug development. Extrinsic and intrinsic factors cooperate to regulate the neurogenic potential of Müller glia. Proliferation is promoted by Wnt, Notch, sonic hedgehog (Shh), and fibroblast growth factors (FGF).\textsuperscript{16–38} Differentiation is promoted by retinoic acid and valproic acid, in addition to basic helix-loop-helix transcription factors and homeodomain transcription factors.\textsuperscript{69} Moreover, the pre-synaptic marker synaptophysin has been expressed in newly generated photoreceptors in an N-methyl-N-nitrosourea-induced injury model of rats.\textsuperscript{19} S334ter rats are a model of rod degeneration; when these animals receive Wnt2b and Jag1 followed by Shh and DAPT (N-(S)-phenyl-glycine-t-butyl ester; a γ-secretase inhibitor that blocks Notch signaling), they exhibit functional recovery based on an optokinetic response (a behavioral test for head–neck tracking of visual stimuli).\textsuperscript{20} It is difficult to determine whether this functional recovery can be attributed to regeneration or neuroprotection. To date, there is no direct evidence that regenerated neurons derived from Müller glia form functional synapses, integrate into pre-existing retinal circuits, or contribute to functional recovery.

Cell transplantation therapy confers two effects on the host retina: supplementation of trophic factors released from transplanted cells and replacement of lost cells with transplanted cells. Among many cell types in the retina, transplantation of retinal pigmented epithelia (RPE) is the most fully developed technology.\textsuperscript{5,21,22} Functional recovery has been achieved by transplantation of human embryonic stem (ES) cell-derived RPE or human induced pluripotent stem (iPS) cell-derived RPE.\textsuperscript{5,23,24} A clinical trial of RPE transplantation using human iPS cells was launched in Japan in 2014. This trial aims to assess the safety and feasibility of autologous transplantation of iPS cell-derived RPE sheets in patients with wet AMD, which is characterized by progressive damage to the RPE due to leakage caused by neovascularization. Among the various types of retinal neurons, transplantation of photoreceptors is the most promising technology at present. Although several attempts have been made to transplant RGCs to treat RGC degeneration syndromes such as glaucoma, regeneration of distant RGC projections (the optic nerve) has not been achieved. Transplantation of other retinal neurons has not yet been attempted.

In proof-of-concept experiments for rod and cone transplantation, photoreceptor cells derived from mouse neonates were used to verify feasibility and effectiveness of cell transplantation in the adult retina.\textsuperscript{7,25–28} Photoreceptor precursors at specific ontogenic stages (P4–P6) integrated into recipient adult retinas.\textsuperscript{27} We theorized that if ES cells could differentiate into photoreceptors that correspond to the P4–P6 stages, those cells might integrate into the host retina and restore visual function.\textsuperscript{29} We have successfully generated rod and cone photoreceptor cells from mouse, monkey, and human ES and iPS cells in vitro\textsuperscript{29–35} (Fig. 1). Other groups have also reported differentiation of photoreceptors from human ES and iPS cells.\textsuperscript{8,36,37}

Several attempts at retinal transplantation have been made using ES and iPS cells. Transplanted cells that differentiated from human ES cells expressed photoreceptor markers in the immunosuppressed mouse retina and restored some visual function in a photoreceptor degeneration model using Cx-342 Vol. 38, No. 3 (2015)deficient mice\textsuperscript{38}; however, in that study, a mixed population of differentiated cells was transplanted without selection of photoreceptors. For clinical applications, retinal cells must be purified in order to avoid tumor formation after transplantation, as well as to enhance photoreceptor integration in the host retina.\textsuperscript{34,38} Lamba et al. showed that human iPS cell-derived photoreceptors survived in the host mouse retina following transplantation.\textsuperscript{39} However, fluorescence activated cell sorting (FACS)-purified, iPS cell-derived photoreceptors that express GFP under the photoreceptor-specific, interphotoreceptor retinoid-binding protein (IRBP) promoter did not survive as well as unsorted cells, and the number of integrated cells available for functional restoration was far lower.\textsuperscript{39} We have also attempted purification of Nrl+ photoreceptor cells from differentiated ES cells for use in transplantation. To this end, we generated mouse ES cells with double-fluorescent Rx-GFP/Nrl-RFP reporters; however, it was difficult to obtain sufficient numbers of Nrl-RFP+ donor cells from mouse ES cells, even with an improved differentiation protocol.\textsuperscript{34} Although several different protocols for retinal differentiation are available, a common issue with these protocols is that differentiated photoreceptors fail to form the outer segment in two dimensional (2D) culture. However, recent studies have shown that mouse and human ES cells can spontaneously form the 3D structure of the optic cup, the retinal primordia.\textsuperscript{40–42} Gonzalez-Cordero et al. showed that mouse ES cell-derived photoreceptors from 3D culture can integrate within degenerating mouse retina and mature into outer segment-bearing photoreceptors.\textsuperscript{43} However, functional recovery using stem cell-derived photoreceptors has not yet been reported. Presumably, for functional recovery to occur, at least 150000 rod photoreceptors would need to integrate into the degenerating retina. It should be noted that the acceptability of transplanted cells depends on the conditions of the host retina. Grafted cells survive best when transplanted within a defined period after massive cell death, but before host gliosis progresses. We recently reported successful transplantation of 3D retinal sheets derived from mouse ES and iPS cells into an advanced retinal degeneration model that completely lacked the outer nuclear layer.\textsuperscript{43} Functional assays will be required to determine whether photoreceptors from 3D retinal sheets can reconstruct retinal circuit and restore visual function.

Retinal regeneration strategies are still developing. Emerging evidence has indicated that transplantation of photoreceptors derived from developing mice can improve some visual function in mouse retinal degeneration models,\textsuperscript{26} and that transplanted photoreceptors that differentiate from mouse ES cells can connect to host bipolar cells and horizontal cells in mice. However, the extent of visual restoration after transplantation is too small for this method to be considered as a strategy for photoreceptor transplantation therapy. Along with cell transplantation, drug treatments that promote regeneration processes will be required in order to achieve visual restoration. Currently, however, we do not know how to enhance synapse formation by new retinal neurons, nor do we know how to enhance functional recovery of the degenerated retina. This is largely because we still lack a mechanistic understanding of how new neurons make synapses with correct partners, rewire neural circuits, and contribute to visual function. Despite advances in culture systems for ES and iPS cells, as well as transplantation strategies, there has been much less prog-
In our understanding of circuit formation by transplanted retinal neurons or Müller glia-derived retinal neurons. In essence, the roles of newly formed circuits are totally unknown. A circuit-level understanding on regeneration should help us develop novel strategies to enhance neural circuit reconstruction and functional recovery.

The biggest challenge in retinal regeneration is the formation of appropriate synapses between pre-existing neurons and newly generated neurons or transplanted neurons in the degenerating retina, in order to generate functional neural circuits. Currently, there is a large gap in our understanding of the functional roles of new neurons in the adult retina, at both the cellular and behavioral levels. It has been difficult to experimentally investigate how new neurons form neural circuits and participate in visual function in living animals. A central challenge is the identification of structural and functional links of new neurons, which remains difficult due to the lack of appropriate methods for relating new synaptic connections to circuit function in the normal and diseased central nervous system (CNS). To bridge this gap and decipher the functional roles of newly generated connections at the circuit level, it will be necessary to identify newly formed connections, correlate them with function, manipulate their activity, and assess the behavioral outcome of these manipulations. In the past decade, innovations in molecular biology, bioengineering, microscopy, imaging, and computer science have provided an unprecedented opportunity to elucidate the organization and function of neural circuits. These new methods and tools allow fine-scale mapping, quantitation, and perturbation of individual connections, as well as experimental tests of the role of particular cell types, such as newly generated neurons or transplanted neurons, within a functioning neural network. Here, we discuss genetic and viral tools to study the link between neuronal connectivity and circuit function in the normal and diseased CNS.
3. CELL-TYPE-SPECIFIC GENETIC MANIPULATION

The brain and retina consist of heterogeneous populations of cells. Underlying the function and dysfunction of the CNS are complex neuronal circuits assembled from thousands of neuronal cell types that differ in their chemical content, electrophysiological characteristics, and connectivity. In addition, glial cells, including astrocytes and microglia, play important roles in regulating inflammation processes and forming the blood–brain barrier, as well as in modulating synaptic properties and circuit functions.

Endogenous neural stem cells and their derivatives, another diverse population in the adult CNS, are considered to contribute to memory, learning, and mood. When we transplant cells in the brain or retina, those transplanted cells become a new population to be investigated. Our understanding of the functional roles of particular cell types in the adult CNS is still far from complete.

To understand the roles of particular cell types, we need to dissect many different populations and selectively manipulate individual cell types in gain-of-function and loss-of-function analyses. In many CNS regions, a detailed description of cell morphologies and their correlation with immunohistochemical markers has been obtained based on studies performed in fixed tissues. However, the functions of cells identified in vivo are not fully understood. Circuit-level approaches will help us to understand the biological or physiological roles of particular cells in vivo. For example, to determine the role of new neurons in neural circuits, it is necessary to establish causal relationships between newly generated connections and behaviors. Cell type-specific targeting by genetic manipulation will enable analyses of the functional roles of endogenous stem cells, newborn neurons, and transplanted cells in the intact brain and retina of living animals. In addition, genetic manipulation is also necessary in order to produce iPSC cells or other reprogrammed cell types. Uncovering the molecular and cellular mechanisms underlying nuclear reprogramming also requires precise control of gene expression with high temporal resolution in a specific population of living cells. Powerful tools for these purposes include induction of reporter genes (e.g., XFPs, lacZ), genetic modulators (e.g., Cre, FLP, toolbox transgenes), and gene silencing (e.g., shRNA) in targeted cell types. In particular, optogenetics and circuit tracing will be crucial to our efforts to correlate neuronal circuits with function.

ChR2 is a light-sensitive cation-conducting channel that depolarizes neurons when illuminated with blue light at 470 nm. NpHR is a chloride pump that hyperpolarizes neurons when illuminated with blue light at 540 nm. ChR2 is a chloride pump that hyperpolarizes neurons when illuminated with blue light at 540 nm. In addition, glial cells, including astrocytes and microglia, play important roles in regulating inflammation processes and forming the blood–brain barrier, as well as in modulating synaptic properties and circuit functions. NpHR is a chloride pump that hyperpolarizes neurons when illuminated with blue light at 540 nm. In addition, glial cells, including astrocytes and microglia, play important roles in regulating inflammation processes and forming the blood–brain barrier, as well as in modulating synaptic properties and circuit functions. Endogenous neural stem cells and their derivatives, another diverse population in the adult CNS, are considered to contribute to memory, learning, and mood. When we transplant cells in the brain or retina, those transplanted cells become a new population to be investigated. Our understanding of the functional roles of particular cell types in the adult CNS is still far from complete.

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ChR2 is a light-sensitive cation-conducting channel that depolarizes neurons when illuminated with blue light at 470 nm. NpHR is a chloride pump that hyperpolarizes neurons upon illumination with yellow light. Many ChR2 variants have been developed. The most commonly used variant is ChR2 (H134R), and mouse lines expressing this opsin in a Cre-dependent manner (Ai27 or Ai32) are a powerful tool for selectively stimulating genetically targeted populations. ChR2 (E123T: ChETA) has fast channel closure kinetics and permits high-frequency stimulation at ca. 40 Hz. ChR2 (C128S/D156A), which opens under blue light illumination and closes under green light illumination, can maintain an activated state for 20–30 min. Because near-infrared light scatters less through biological tissue and is absorbed less by blood than the blue to green wavelengths that are required by other ChR variants, the red-shifted ChR variant ReaChR can be activated through the intact skull. Some of these variants have superior membrane localization and lower aggregation as misfolded proteins in the Golgi, endoplasmic reticulum, and other intracellular compartments. Because each ChR variant has different performance, including peak photocurrent, kinetics, activation wavelength spectrum, and light sensitivity, it is important to select the opsin best suited to the experimental goals. However, because the retina itself is photosensitive, it may be impractical to manipulate neural activity in a specific population in the retina using optogenetics. Therefore, chemogenetics (chemical ligand-operated systems) and magnetogenetics (magnetic field-operated systems) might be more suitable for retinal research.

In addition to neuronal excitability, cellular functions such as signaling pathways, protein localization, transcription, and post-translational modification can be controlled by light. For example, engineered synthetic rhodopsins (Opto-XR) have been designed to control intracellular biochemical signaling using light. The intracellular loops of rhodopsin have been replaced with the intracellular loops of G protein-coupled receptors (GPCRs) to yield light-activated chimeric GPCRs. Upon green light illumination, Opto-XR activates downstream Gq and Gi signaling pathways. Strikingly, gene expression also can be controlled by light. For this end, Wang et al. have established the LightOn system, which is based on a light-sensitive transcription factor named GAVPO consisting of VVD protein fused to the LOV domain (Light-Oxygen-Voltage (LOV) domain), which changes conformation in response to blue light, the GAL4 DNA-binding domain, and the p65 transcription activation domain. In this system, the gene of interest is expressed under the control of the UAS element, where the GAVPO binds. Konermann et al. have described the light-inducible transcriptional effector (LITE) system, which is based on a fusion of the blue-light-sensitive CRY-CIB1 and the TALE DNA binding domain. TALEs can be customized to specifically target a wide range of genomic loci and other DNA-binding domains. Notably, epigenetic states can be modified with TALE-mediated targeting of histone effectors (epiTALEs). For in vivo applications of light-induced gene-expression systems, it will be necessary to improve expression levels, response timescale, and signal-to-noise ratio, as well as develop new variants with different spectral properties.

Another powerful strategy is knockdown of a specific gene. Selective gene silencing by RNAi can be achieved by two methods: cytoplasmic delivery of short double-stranded interfering RNA oligonucleotides (siRNA), in which the gene silencing effect is only transient in nature and may not be suitable for all applications; or nuclear delivery of gene-expression cassettes that express short hairpin RNA (shRNA), which are processed like endogenous interfering RNAs and lead to stable gene downregulation.

In order to identify cells of interest such as transplanted cells and newborn cells, it is also important to express transgenes in combination with reporter genes such as green fluorescent protein (GFP), β-galactosidase, or alkaline phosphatase. An internal ribosome entry site (IRES), a self-cleavage 2A-element, a second promoter, and fusion of an open reading frame (ORF) with a reporter are all well suited for...
this purpose. The production of IRES-containing bicistronic transcripts driven by a single promoter allows independent translation of a second protein from a single transcript, resulting in stable co-expression of two proteins. However, expression levels often differ significantly for the ORFs before and after the IRES. Although the 2A system (e.g., F2A, E2A, T2A, and P2A) results in equal expression of more than two transgenes via a translation-skip mechanism, cleavage efficiency varies. Expression of two genes from two different promoters also works, but can lead to competition between the promoters and eventual repression of one of the promoter elements. To overcome this issue, an insulator can be used to eliminate interactions between the promoters. Alternatively, bidirectional constructs allow for simultaneous expression of the gene of interest and a reporter gene. Production of fusion proteins with reporters such as GFP also enables visualization of the subcellular localization of the expressed protein (e.g., Synaptophysin-GFP and ChR2-GFP), but this strategy potentially interferes with the original protein structure and its interaction with other proteins. Thus, although all of the approaches described above are useful, their efficiency and results will likely vary depending on the design of the constructs.

Recent advances in the development of molecular, genetic, and viral-based tools enable structural and functional investigation in the CNS, as well as monitoring and manipulation of neural activity and gene expression with high spatiotemporal resolution at the level of specific cell types or even single cells. The creation of interfaces between genetically engineered animals and viral vectors via electrophysiology and optical imaging will make it possible to address circuit-level questions that have not been readily addressed thus far. Here, we present an overview of the genetic manipulation techniques that will be useful for studying circuit regeneration and pathophysiology of the CNS.

4. GENETICALLY ENGINEERED ANIMALS

Mouse genetics is one of the most powerful strategies in modern biology. In particular, recombinase-based systems such as Cre/loxP and FLP/frt allow for conditional mutagenesis, fate-mapping, transgenesis, and gain-of-function and loss-of-function analyses in a cell-type specific fashion (Figs. 2A, B). In addition to Cre and FLP, other site-specific recombination systems have been developed: for example, PhiC31o and codon-improved Dre recognize different target sequences. More select populations can be captured with an intersectional or subtractive approach using a combination

Fig. 2. Site-Specific Recombination-Mediated Targeting

(A) Cre-dependent gene targeting. A plasmid or virus carries reverse-complemented gene sequences flanked by two mutually exclusive sets of lox sites, termed FLEX or DIO. Cre acts on either pair of lox sites, lox2272 (black) or loxP (white), but cannot mediate recombination between different types of lox sites. Through Cre-mediated inversion of antiparallel lox sites, followed by Cre-mediated deletion of identical parallel lox sites, this double-floxed system stabilizes the Cre-dependent cassette in the proper orientation, allowing for gene expression. (B) Cre- and FLP-dependent intersectional approach. The Creon-FLPon construct expresses transgenes in cells expressing both Cre and FLP. Cre recognizes two sets of lox sites (red and pink), whereas FLP recombines two sets of frt sites (blue and sky blue). After recombination with both Cre and FLP, Exons 1, 2, and 3 are oriented in the forward direction to generate pre-messenger RNA (and, following splicing, mRNA), resulting in expression of the gene of interest only in the Cre+ and FLP+ population. The combined use of both Cre and FLP allows genetic manipulation in restricted cell populations, e.g., the Cre+/FLP+, Cre+/FLP−, or Cre+/FLP+ population.
of multiple recombination systems\textsuperscript{79–83} (Fig. 2B).

Knock-in and knockout strategies are extremely versatile techniques for altering the germ line. Using this approach, the target gene is inserted \textit{via} homologous recombination in mouse ES cells at the endogenous locus of the gene.\textsuperscript{82,83} Knock-in lines generally exhibit less variegation and more faithful patterns of reporter-gene expression than transgenic lines.\textsuperscript{74} In contrast to the line-to-line variability in expression in transgenic lines, which is caused by differences in transgene location and copy number, variability between knock-in lines is very low. On the other hand, transgenic techniques involve the random insertion of relatively small constructs, in which promoters control transgene expression. To generate transgenic animals, transgene constructs are injected into the pronuclei of fertilized eggs, and the resultant founders are screened by polymerase chain reaction (PCR) and Southern blotting to detect the transgene. These founders transmit the stably integrated transgene through the germ line to their offspring, which can then be analyzed to determine the pattern of transgene expression. In general, only a fraction of the resultant lines express the injected transgene, and the expression pattern may vary from line to line. Indeed, transgenes are expressed in only a subset of the expected cells. These variegated expression patterns are generally ascribed to the chromatin structure at the site of integration of the transgene.

Bacterial artificial chromosome (BAC) transgenesis represents a variant strategy in which a large chromosomal fragment containing a gene of interest is engineered to direct the expression of the transgene.\textsuperscript{84} A large fragment (100–200kb), often containing a relatively complete set of \textit{cis}-regulatory elements, will more reliably reproduce the endogenous expression pattern than a small promoter fragment, and will consequently direct reporter expression more precisely to that cell population. The Gene Expression Nervous System Atlas (GENSAT) project generated hundreds of mouse lines that express GFP or Cre driven by different BACs.\textsuperscript{76,84} However, many lines of evidence have indicated that BAC-mediated transgenesis cannot completely recapitulate endogenous gene expression, probably due to random integration and the incompleteness of \textit{cis}-regulatory elements. Nonetheless, in some cases, we can unexpectedly obtain BAC transgenic lines that mark specific cell populations, even if their expression patterns are different from those of the endogenous gene.\textsuperscript{76}

Rats have important advantages over mice as an experimental system for physiological and pharmacological investigations. For example, rats are able to learn complicated tasks, allowing behavior studies. However, due to the lack of germline-competent rat ES cells, rat genetics has not been developed to the same extent as mouse genetics. Intriguingly, recently improved culture techniques and an understanding of the underpinnings of pluripotency are making it possible to apply the knock-in and knockout strategies into many species, including rats.\textsuperscript{85} Established rat ES cell lines are not germline-competent, and instead possess so-called “primed pluripotency”\textsuperscript{86–90} Although pluripotent EpiSCs (post-implantation epiblast-derived stem cells) have been derived from rat embryos at 7.5 d postcoitus,\textsuperscript{93} these EpiSCs do not contribute to chimeras. The lack of germline-competent rat ES cells with “naïve pluripotency” has significantly restricted the availability of genetically engineered models in this species.

Derivation and maintenance of the undifferentiated state of mouse ES cells originally relied on co-culture with feeder cells, usually mitotically inactivated mouse embryonic fibroblasts, and the presence of serum. Later, it was shown that leukemia inhibitory factor (LIF) is the key cytokine secreted by feeders to support mouse ES cell self-renewal.\textsuperscript{92,93} Emerging evidence indicates ground state of the pluripotency by showing that the application of SU5402 (an FGF receptor inhibitor), PD184352 (a MEK inhibitor), and CHIR99021 (a glycogen synthase kinase-3 inhibitor) in serum-free and LIF-free media (3i method) can maintain the pluripotency of mouse ES cells.\textsuperscript{94} The 3i method can also maintain the naïve pluripotency of rat ES cells; cells in this state yield high rates of chimerism when reintroduced into early stage embryos and can be transmitted through the germline.\textsuperscript{95,96} Establishment of germline-competent rat ES cells enables generation of knock-in and knockout rats, opening up applications in drug discovery and neuroscience research.

It is not clear whether findings from studies using rodent models will translate across species. Larger animals that are more closely related to humans, such as monkeys, therefore indispensable for preclinical studies. The common marmoset (\textit{Callithrix jacchus}), a New World monkey, represents a versatile model of human disease, drug development, and physiology studies because of its small size, ease of handling, and potential for genetic manipulation. Marmosets have a relatively short gestation period (about 144 d) and reach sexual maturity at 12–18 months. Marmoset females usually have twins, and produce a total of 40–80 offspring during their life. In the context of vision research, marmosets have high visual acuity, a well-segregated layered LGN, and ocular dominance columns in the primary visual cortex VI\textsuperscript{77–100}. In addition, they can perform a variety of visual discrimination and cognitive tasks.\textsuperscript{101,102} Strikingly, Sasaki \textit{et al.} have generated transgenic marmosets (\textit{Callithrix jacchus}) that exhibit germline transmission.\textsuperscript{103} Many recent technologies will facilitate marmoset genetics. Genome editing technology such as zinc finger nuclease, TALEN, and CRISPR/Cas9 make it possible to generate knock-in and knockout marmoset lines.\textsuperscript{104,105} In addition, the tetraploid complementation method should accelerate the process of transgenic marmosets: electrofusión of a two-cell stage embryo (4N) followed by culture to the blastocyst stage and injection of fully pluripotent ES/iPS cells (2N) generates 2N pups completely derived from ES/iPS cells, while the tetraploid host blastocyst contributes to the extraembryonic lineages but not to the embryo.\textsuperscript{106,107} In vitro differentiation of sperm and eggs from ES/iPS cells is also a promising approach for gametogenesis and genetic manipulation.\textsuperscript{108,109} The Brain Mapping by Integrated Neurotechnologies for Disease Studies project (Brain/MINDS) in Japan will facilitate the development of marmoset genetics, as well as whole-brain circuit mapping.\textsuperscript{110}

5. RECOMBINANT VIRAL VECTORS

Viral vectors can efficiently deliver genes to neurons, glia, and stem cells both \textit{in vitro} and \textit{in vivo}.\textsuperscript{45,111–119} In particular, they are very useful in species in which typical transgenic technologies are impractical, such as ferrets, cats, and monkeys. Transgenes can be expressed in cells and regions of interest by injection of viruses into particular locations. In particular, targeting of viruses to different types of neurons
in the nervous system is based on the genetic identity of the cells, their specific connectivity, or a combination of these two attributes (Figs. 2, 3). In general, results using viral vectors are more variable than those obtained by mouse genetics, largely due to variations in virus preparation, injection, and surgery. Moreover, virus injection is much more invasive than crossing mice of two different lines. Quality control of virus preparations, by quantitation of titer, is important for minimizing variation between batches of viruses. Larger volumes of virus injection deliver more viral particles but can cause tissue damage. Slow-speed pressure injection can distribute viruses better, whereas iontophoresis of adeno-associated viruses (AAVs) can restrict viral infection to a small location. However, early withdrawal of injection pipettes can cause distribution of the virus along the needle track before the viral particles diffuse into the tissue.

More than ten types of viral vectors are now in use. The available vectors differ in their suitability for different applications. Each vector has limits, including the size of the transgene that can be accommodated, viral tropism, duration of gene expression, and route of delivery. Viral vectors enable us to express or knock down genes, monitor biological functions, and change the physiological state of specific cell populations. For example, recombinant AAV and lentivirus are promising vectors for gene therapy because they can infect post-mitotic neurons and mediate stable expression with minimal toxicity. Retroviruses are ideal for targeting endogenous stem/progenitor cells in the adult brain because they infect only dividing cells. In addition, rabies virus makes it possible to probe the structure and function of neural circuits because it spreads transsynaptically, exclusively in the retrograde direction and only between connected neurons. These viral vectors are extremely powerful tools for understanding reprogramming, neural stem cells, and neural circuits. However, it is very important to choose viral vectors that are appropriate for the experimental design and purpose. To take advantage of viral approaches, we will outline four major types of viral vectors used for stem cell biology and neuroscience.

6. MOMLV RETROVIRUSES

Mooney murine leukemia virus (MoMLV) is a (+)-strand-ed RNA retrovirus that is characterized by its ability to generate double-stranded DNA (dsDNA) from its RNA genome through reverse transcription. Upon entry into host cells through a specific interaction between viral surface glycoprotein and host cell membrane receptor, the retroviral RNA genome is transcribed into dsDNA in the cytoplasm of the host cell. The newly synthesized dsDNA enters the nucleus, where it integrates into the chromosomal DNA of the host cell. It should be noted that the preintegration complex containing the viral dsDNA does not cross an intact nuclear membrane; however, it enters the nucleus in dividing cells because the nuclear membrane is disassembled during the prophase–prometaphase transition at the onset of mitosis.

The retrovirus genome has three genes, gag, pol, and env, which are flanked by long term repeats (LTR). The gag gene codes for structural proteins such as capsid, matrix, and nucleocapsid. The pol gene codes for viral protease, reverse transcriptase, and integrase. The rev gene codes for viral glycoproteins. Virus infection is mediated by an interaction between envelope proteins and cell surface receptors. The tropism of retroviral vectors is determined by the type of envelope protein used for viral production; the use of heterologous Env proteins is called pseudotyping. The most commonly used Env protein is the vesicular stomatitis virus glycoprotein (VSV-G), which gives the virus a broad tropism because VSV-G binds to ubiquitous phospholipid components of the
plasma membrane. Another advantage of VSV-G is that it can be easily concentrated to high titers by ultracentrifugation.

MoMLV vectors infect dividing cells, whereas vectors derived from human immunodeficiency virus (HIV) infect both dividing and non-dividing cells. The inability of MoMLV retroviral vectors to deliver genes to non-dividing cells makes it an ideal tool for selectively targeting proliferative stem/progenitor cells in the adult CNS.118,121,122 Historically, BrDU labeling of dividing cells has been used to detect endogenous stem cells in the adult brain. However, BrDU is not only incorporated into dividing cells, but is also picked up during cell death or DNA repair.124 Thus, BrDU-based results need to be confirmed by independent methods. Furthermore, neurogenesis is prevented by anti-mitotic drugs and irradiation, because the proliferative nature of neural progenitors makes them more sensitive to these treatments than differentiated neurons. However, these treatments disrupt cell-cycle propagation in other cell types in the body and cause side effects such as general health deterioration and inflammation. Cell type-specific targeting with the MoMLV retrovirus allows for ablation of neural progenitors in the adult CNS. Induction of the mitosis blocker thymidine kinase, the pro-apoptotic factor Bax, or the toxin 2-diphtheria toxin fragment in the MoML retrovirus can kill neural progenitors in the adult CNS. The use of a neuron-specific promoter to drive thymidine kinase, Bax, or the 2-diphtheria toxin fragment in the MoML retrovirus permits ablation of newborn neurons after differentiation in the adult CNS. In addition, combination of the tet-inducible system (rtTA/tetO system) or tamoxifen-inducible Cre-dependent recombination system (CreER/lox system) makes it possible to kill cells at a specific time. By taking advantage of the ability of the MoMLV retrovirus to infect only dividing cells in the adult CNS, MoMLV retrovirus-mediated gene transfer will help us to label and kill new neurons, trace cell fates, analyze physiological properties of newly generated neurons and synapses, and understand the molecular mechanisms that regulate self-renewal, differentiation, maturation, and integration of new neurons. A combination of cell type or time-specific promoters is useful for precise manipulation. In particular, in the adult retina, the use of retinal neuron-specific promoters in the MoMLV retrovirus makes it possible to visualize newly generated retinal neurons that differentiate from endogenous Müller glia.

Another feature of retroviruses is that retroviral transcripts are often silenced in host cells such as ES cells, embryonic carcinoma cells, and hematopoietic stem cells. Moreover, retroviruses have been used to establish iPSCs: gene transfer of Oct3/4, Sox2, Klf4, and c-Myc using retroviruses resulted in reprogramming of somatic cells into pluripotent stem cells.113,125 It should be noted that generation of iPSCs requires transgenes to be silenced after reprogramming events are complete, because subsequent differentiation of iPSCs requires silencing of exogenous genes.126 The retroviral transcripts of the four transcription factors are eventually silenced, and the endogenous expression of pluripotent genes is turned on. Although lentiviruses permit the transduction of even non-dividing cell types with high efficiencies, transgenes introduced by lentiviruses are poorly silenced in the pluripotent state.

7. HIV LENTIVIRUSES

HIV-derived lentiviruses infect both dividing and non-dividing cells, including neurons. Lentiviruses have more complex genomes that contain gag, pol, env, as well as additional regulatory and accessory genes. The most studied lentivirus is HIV-1, which infects CD4-positive T lymphocytes and macrophages (the CD4 antigen acts as the primary surface receptor for HIV-1) and causes chronic immune depletion known as acquired immune deficiency syndrome (AIDS). The HIV-1 genome is 9.2 kb, and it contains two additional regulatory genes, tat and rev, as well as four accessory genes, vpr, vpu, vif, and nef. The Tat protein enhances transcriptional activity from the promoter region in the 5’LTR of an integrated provirus, the rev protein enhances transport of HIV unspliced mRNA from the nucleus, and the accessory proteins mainly enhance virulence against the host organism. The lentivirus-specific proteins (vif, vpr, vpu, vif, nef, tat, and rev) also play various roles in the life cycle of the virus that are not essential for gene transfer or integration of the provirus. Therefore, most of these proteins are deleted in recently developed third-generation vector systems.127,128 Because only the rev gene is required to produce lentivirus, the rev gene is supplied from the packaging cell lines, and the rev-responsive element is included in the vector genome. For lentivirus production, the gag-pol and env genes are expressed in a packaging cell line or by transient expression. Lentiviral env can be specific for particular cell types: for example, HIV env binds CD4, resulting in specificity for a particular T-cell subset. The tropism of lentiviral vectors can be altered by the type of envelope protein that is used for viral production. Pseudotyping with VSV-G is the most popular method because the titers of VSV-G lentivirus can be easily increased by ultracentrifugation, and the virus can then infect a broad range of cell types in various species. Rabies glycoprotein-pseudotyped lentivirus can infect neurons through their axon terminals, providing a very powerful method of targeting projection neurons based on their connectivity.129,130 One advantage of lentiviral vectors is their larger packaging capacity (about 9 kb) relative to AAV (about 5 kb).

One clear advantage of lentiviral vectors over other recombinant vector systems is their applicability to efficient generation of transgenic animals. In contrast to other retroviral vectors, such as MoMLV vectors, expression from integrated lentiviral vectors is not silenced during development. Single-cell embryos can be injected with lentivirus into the perivitelline space and subsequently implanted into the uterus of pseudopregnant females.131 Expression from cell-specific promoters is restricted to relevant tissues in the resultant transgenic animals, e.g., muscle-specific expression from the myogenin promoter or thymus-specific expression from the T lymphocyte lck promoter in mice.131 Other transgenic species generated using lentiviral vectors include cats, chickens, pigs, and monkeys. Interestingly, Sasaki et al. have used lentivirus vectors to produce transgenic marmosets (Callithrix jacchus) that exhibit germline transmission.103 Among the commonly used model organisms, monkeys most resemble humans, and disease models in non-human primates will be very useful for the study of human disease and drug development.
8. ADENO-ASSOCIATED VIRUSES

AAV is single-stranded DNA viruses that belong to the Parvoviridae family. The virion is a non-enveloped capsid 18–26 nm in diameter, and the DNA genome size is 4.7 kb. The AAV genome consists of two genes, rep and cap, flanked by 145-nucleotide palindromic sequences, called inverted terminal repeats (ITRs). The ITRs contain all cis-acting sequences critical for virus packaging, replication, and integration. The rep gene contains two promoters, and each transcript is regulated by internal splicing, resulting in the production of four nonstructural Rep proteins, Rep78, Rep68, Rep52, and Rep40. The Reps regulate replication, viral transcription, packaging of the AAV genome, and site-specific integration. The cap gene encodes three structural proteins (VP1, VP2, and VP3), which form the capsid of the virus. To generate a recombinant AAV vector, these rep and cap genes are replaced by a promoter and transgene cassette. Only the ITRs are kept to serve as the essential replication/packaging signal.

Serotypes determine the selectivity of infection. Viral tropism and transduction efficiency can be controlled by capsids, which mediate initial receptor attachment, cellular entry, and trafficking mechanisms. Specific glycan motifs have been identified as primary receptors for some AAV serotypes: AAV2 uses heparin sulfate for cell recognition, AAV5 binds to sialic acid, and AAV8 and AAV9 bind to the laminin receptor. AAV serotype 2 preferentially infects neurons, and AAV5 binds to the laminin receptor. AAV8 and AAV9 bind to the laminin receptor. AAV2 uses heparin sulfate for cell recognition, AAV5 binds to sialic acid, and AAV8 and AAV9 bind to the laminin receptor.

AAV vectors can mediate safe, long-term gene expression. In addition, AAVs have the distinct advantage that they infect larger areas (or more cells), relative to lentivirus, following direct injection into various tissues. This is largely due to the small size of these viruses: the AAV virion is 20 nm, whereas the lentivirus virion is 100 nm. On the other hand, one problem with recombinant AAV vectors in certain tissue types is that onset of transgene expression is delayed, and does not reach peak levels for up to 4 weeks. This delay can be attributed to the need for efficient synthesis of the complementary strand of the single-stranded vector genome prior to transgene expression.

Interface AAVs with mouse genetics, in particular the Cre/lox and/or FLP/frt recombination system, is extremely powerful. Crossing a Cre-expressing driver mouse with a floxed mouse make it possible to knock out or introduce a particular gene in molecularly defined cell types. In addition, injection of Cre-dependent FLEX/DIO AAV into particular regions of Cre-expressing driver mice enables targeting of gene expression to spatially restricted subsets of defined neurons (Fig. 2A). The FLEX/DIO switch, which contains two pairs of different lox sites, is a very powerful tool for use in this strategy.

Both loxP and lox2272 sites are recognized by Cre recombinases; however, lox2272 sites recombine efficiently with themselves but not with loxP sites, and vice versa. The FLEX/DIO switch uses two pairs of heterotypic, antiparallel loxP-type recombination sites, which first undergo an inversion of the coding sequence, followed by excision of two sites; as a result, one of each orthogonal recombination site is oriented in the opposite direction, and therefore incapable of further recombination.

Devoting additional resources to developing Cre- or FLP-expressing mouse lines to target particular cell types (q.v. initiatives by GENSAT and the Allen Institute for Brain Science) will provide tremendous opportunities for targeting expression of transgenes to specific cell types. In particular, intersectional strategies using Cre and FLP can target smaller subpopulations of genetically defined cells. Cre-dependent and FLP-dependent FLEX/DIO AAV vectors will expand the application of the interventional strategies (Fig. 2B).

Self-complementary AAV (scaAV) vectors contain a double-stranded vector genome generated by deletion of the terminal resolution site (TR) from one rAAV TR, preventing the initiation of replication at the mutated end. These constructs generate single-stranded, inverted-repeat genomes, with a wild-type TR at each end and a mutated TR in the middle. Compared with conventional single-strand AAV vectors, scaAV vectors improve transduction efficiency in various tissues, including the brain. ScaAV vectors confer rapid onset and higher ultimate levels of transgene expression. One disadvantage of these vectors is the size of the scaAV expression cassette, which is only 2.2 kb. The potential applications of scaAV will depend on trade-offs between the ability to package the transgene and the gains in transduction efficiency.

9. RABIES VIRUSES

The rabies virus is a (−) strand RNA virus belonging to the Rhadoviridae family. The virion structure is a bullet-shaped particle approximately 250 nm long and 70 nm in diameter. The RNA genome of the rabies virus encodes five genes over a length of 11–12 kb. These genes code for nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral RNA polymerase (L).

The mammalian CNS consists of billions of neurons, including thousands of cell types, connected into circuits by trillions of synapses. When it is not possible to target cells based on genetic identity using specific promoters or Cre/FLP mouse lines, specific circuit connectivity can be used to selectively target cells in a specific region. Rabies virus infects neurons through axon terminals and spreads transsynaptically, exclusively in the retrograde direction, and only between connected neurons in the CNS (Fig. 4A). To delve into complex neural circuits in the CNS, the rabies virus is a very powerful tool for tracing synaptic connections and linking circuit structure to function. Conventional anterograde and retrograde neuronal tracers have allowed the collection of an extensive amount of information about connectivity in various
Monosynaptically Restricted Circuit Mapping with G-Deleted RABV

(A) Polysynaptic labeling of connected neurons with G-intact RABV (brown with red envelope). G-intact RABV infects neurons through axon terminals and keeps spreading between neurons (green) in a retrograde direction. (B) G-deleted RABV (RABVΔG: white with red envelope) infects neurons through axon terminals, but does not spread beyond the initially infected neurons (green). (C) Gene delivery to genetically targeted neurons via the EnvA/TVA system. Infection with virus pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA can be restricted to neurons that express TVA, an avian receptor protein that is absent from mammalian cells unless provided through gene-delivery vectors. (D) EnvA-pseudotyped RABVΔG (white with blue envelope) can be used to selectively infect neurons (green) that have been targeted for expression of the EnvA receptor, TVA. Although RABVΔG is capable of infecting cells and replicating to produce large quantities of transgenes such as GFP, GCaMP and ChR, they are not able to spread out of those cells without the help of another DNA expression vector that provides rabies virus glycoprotein. (E) Because the G gene was deleted from the rabies genome, complementation is required to allow spread of the virus from infected cells. This can be accomplished by targeted expression of RABV-G in the same cells that express TVA. (F) Following infection with EnvA-pseudotyped RABVΔG (white with blue envelope) and RABV-G complementation in the initially infected neurons (green), rabies virus (white with red envelope) spreads retrogradely to directly “presynaptic” neurons (green). However, the virus cannot spread beyond these secondarily infected neurons because they do not express RABV-G.
species. These data reveal areas that are directly connected, the location of the cells that provide the connections, and the laminar zones of termination of afferent axons. This information has been used to guide studies in which likely functional interactions between areas are inferred and tested using lesions or electrical stimulation. However, conventional tracers do not have sufficient resolution to elucidate connectivity or guide functional studies at higher levels of complexity. For example, they do not reveal whether axons that terminate at a particular location make synaptic contacts onto particular cell types or instead onto cells that in turn connect to other cells. Therefore, we require methods that can reveal multisympathetic pathways and identify connections to and from particular cell types. Ideally, such methods should be able to be integrated with functional studies.

Rabies virus can only infect neurons via receptors for the rabies envelope glycoprotein, which appear to be restricted to presynaptic nerve terminals. In particular, a genetically modified rabies virus that is missing the glycoprotein gene required for spread across synapses (RABVΔG) is a powerful retrograde tracer for use in the CNS, because it is unable to be transported transsynaptically; thus, it differs from normal rabies virus, which travels retrogradely across multiple synapses in a time-dependent fashion (Fig. 4B). One advantage of RABVΔG is that it can introduce transgenes at very high levels. For example, RABVΔG encoding the fluorescent proteins GFP, mCherry, DsRed, or BFP allows clear visualization of detailed neuronal morphology. However, rabies viruses have several disadvantages. Long exposure to rabies viruses induces cytotoxicity in infected neurons; therefore, successful use of this virus will require a reduction in its cytotoxicity. Furthermore, because the rabies virus is a RNA virus whose life cycle occurs in the host cytoplasm, that rabies viruses cannot confer promoter-specific expression of transgenes. To overcome this disadvantage, target transduction of TVA in specific cell types or single cells, followed by infection with EnvA-pseudotyped RABVΔG, allows specific targeting (Figs. 4C, D). In particular, Cre-, FLP-, or tTA-expressing transgenic or knock-in mice under the control of a cell type-specific promoter are extremely powerful tools for introducing TVA in a particular cell type (Figs. 2A, B). Bridge proteins, i.e., fusion proteins of TVB with a ligand for a receptor that is specifically expressed in a targeted cell type, are also useful for cell type-specific transduction (Fig. 3). Co-injection of EnvB-pseudotyped RABVΔG with the neuregulin-TVb bridge protein can preferentially target Erb4 inhibitory neurons in the somatosensory cortex. Moreover, electroporation and targeted patching of single cells of interest in vivo to introduce TVA, followed by infection of the TVA-expressing cells with EnvA-pseudotyped rabies viruses, allows analysis of single neuronal networks with single-cell resolution. The most striking feature of RABVΔG is that complementation of G in trans enables transsynaptic spreading of RABVΔG to directly-connected, presynaptic neurons (Figs. 4E, F). Targeted expression of TVA or TVB in particular cell types or single neurons permits specific infection by rabies viruses pseudotyped with EnvA or EnvB, respectively, and subsequent labeling of the monosynaptically restricted inputs to the population by G complementation. Several anterograde tracing strategies also have been used to identify post-synaptic neurons. To understand how neural circuits function, we have developed new rabies virus variants that have the power to combine rabies viral labeling of connectionally defined neuronal populations with functional assays that monitor or manipulate neuronal function. In living animals, the use of RABVΔG expressing transgenes, such as genetically expressed activity sensors or manipulators, can reveal direct correlations between circuit structure and function and perturbations of connectionally defined neural populations. Interfacing rabies viruses that express genes that can control gene expression, such as Cre recombinase, FLP recombinase, or the Tet transactivator, with mouse lines that express transgenes of interest under the control of these proteins will also permit selective manipulation in defined circuits. These approaches facilitate the tests of the causal relationships between connectivity and function within defined neural circuits. These rabies virus tools will pave the way for novel approaches in which neurons targeted based on their connectivity can be functionally assayed or subjected to manipulation of activity or gene expression.

10. CONCLUDING REMARKS

The last decade has witnessed tremendous progress in the field of retinal regeneration, fueled by the successful transplantation of RPE and photoreceptor cells in the retina and the directed differentiation of RPE, photoreceptors, and retinal tissue from ES and iPS cells. Accumulating evidence suggests that organization of neural networks is more flexible and plastic than previously thought. For example, newborn neurons integrate into neural circuits in the adult hippocampus and olfactory bulb, and activity-dependent synaptic plasticity participates in reconstruction of neural networks. These findings have opened up the possibility of novel therapeutic strategies for reacquisition of functions based on the replenishment of diseased or injured CNS with new neurons. However, many questions remain to be answered before retinal regeneration can be applied in the clinic. One challenge involves determining how stem cell-derived photoreceptors function within retinal circuits in the degenerating retina, and how stem cell-derived photoreceptors contribute to vision restoration; these questions will drive important research over the coming years. Due to the emergence of powerful new tools, as described here, we have an unprecedented opportunity to tackle questions that were difficult or impossible to address using previous tools. A growing arsenal of technology and methodology that allow direct investigation of the relationships between the structure and function of neural circuits will enable us to achieve new insights into circuit reconstruction in the adult retina.

Recombinant viral vectors have proven to be useful in gain-of-function and loss-of-function analyses, fate mapping, transsynaptic circuit tracing, and nuclear reprogramming, but restriction of viral vector-mediated expression in a single cell type remains challenging. Although interfacing viral vectors with mouse genetics such as Cre-, FLP-, or tTA-driver mice will permit highly selective transduction, targeting a particular population in monkeys is very difficult. Marmoset genetics might pave the way for cell type-specific manipulation in non-human primates. In this regard, one challenge is limited viral packaging capacity. Many promoter sequences that en-
able cell type-specific gene expression have been reported, but most of them are quite long. To accommodate a cell type-specific promoter in viral vectors, it is necessary to identify short promoters that target gene expression to particular cell types. In addition to the viral vectors that we discussed here, other powerful biotechnology methods have also been developed. The GFP reconstitution across synaptic partners (GRASP) technique enables mapping of synaptic connectivity in mammalian neurons. Two-photon imaging can optically record live neural activity of cells in a relatively deep location. Genetically encoded sensors for intracellular calcium, membrane voltage transients, or glutamate release have made it possible to monitor activities from particular cell types and even at axons, dendrites, and spines in vivo, using two-photon microscopy. A miniaturized fiber-optic epifluorescence microscope also allows real-time imaging of brain function with high temporal and spatial resolution in freely moving mice. Moreover, because longer wavelengths (>900 nm) do not activate endogenous opsins, two-photon imaging can be used to make recordings from the retina without bleaching rod and cone photoreceptors. Intriguingly, tissue-clearing methodologies such as SCALE, CLARITY, SeeDB, CUBIC, and PACT have rendered the endogenous expression of fluorescent proteins and organ-wide mapping at cellular and subcellular resolution by whole brain optically transparent, permitting circuit- and CLARITY, SeeDB, CUBIC, and PACT have rendered the endogenous expression of fluorescent proteins and organ-wide mapping at cellular and subcellular resolution by whole brain optically transparent, permitting circuit- and CLARITY, SeeDB, CUBIC, and PACT have rendered the endogenous expression of fluorescent proteins and organ-wide mapping at cellular and subcellular resolution by whole brain optically transparent, permitting circuit- and CLARITY, SeeDB, CUBIC, and PACT have rendered the endogenous expression of fluorescent proteins and organ-wide mapping at cellular and subcellular resolution by whole brain optically transparent, permitting circuit- and

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