Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus

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We describe a powerful system for revealing the direct monosynaptic inputs to specific cell types in Cre-expressing transgenic mice through the use of Cre-dependent helper virus and a modified rabies virus. We generated helper viruses that target gene expression to Cre-expressing cells, allowing us to control initial rabies virus infection and subsequent monosynaptic retrograde spread. Investigators can use this system to elucidate the connections onto a desired cell type in a high-throughput manner, limited only by the availability of Cre mouse lines. This method allows for identification of circuits that would be extremely tedious or impossible to study with other methods and can be used to build subcircuit maps of inputs onto many different types of cells within the same brain region. Furthermore, by expressing various transgenes from the rabies genome, this system also has the potential to allow manipulation of targeted neuronal circuits without perturbing neighboring cells.

transsynaptic | pseudotyped virus | adeno-associated virus | EnvA | TVA

One of the most intractable problems in systems neuroscience has been the systematic description of neural connectivity in the intact mammalian brain. Many different types of neurons, each with distinct connectivity and function, can inhabit the same brain region. Even within a single neocortical column, dozens of types of projection neurons and local interneurons perform the computations that ultimately lead to the spiking output of that column. Through painstaking studies using molecular and cell biology techniques, electron microscopy, and electrophysiology, we are beginning to understand how a neuron's connectivity contributes to its function in the circuit in which it is embedded, but we lack efficient means for performing circuit-level analyses in vivo. Especially in brain regions with considerable neuronal heterogeneity, we are still greatly limited in our ability to study how groups of cells form their fine-scale connections, how these connections change over time, and how this plasticity affects a cell type's computational role in a dynamic circuit.

Standard anterograde and retrograde neuronal tracers can reveal the locations of neurons projecting to or from particular brain regions, but fail to identify the cell types that actually receive the synaptic connections. This difficulty can be overcome by combining anterograde tracing with electron microscopy, but such studies are extremely tedious, often requiring years to elucidate just a small fraction of the possible connections. Furthermore, even EM studies do not readily reveal which cell types contribute to anterogradely labeled synapses or which cell types receive these connections. Over the last two decades, electrophysiological experiments in living brain slices have made it possible to more readily assay connections at high resolution. Although still relatively cumbersome, these studies have revealed that connectivity is extremely precise, allowing investigators to decipher many of the rules of local circuit connectivity. However, slice studies are limited in their ability to reveal connections between distant neurons, which often account for the majority of synaptic inputs onto a given cell.

Modern advances in virology and genetic technology are now beginning to complement more traditional approaches and promise to revolutionize our understanding of the precise organization of neural circuits in complex brain structures (1). For example, it has recently become possible to express channelrhodopsin in axons originating from a known source and cell type, and then test possible postsynaptic targets for functional connectivity through intracellular recording in brain slices (2). Cre-dependent expression of wheat germ agglutinin (WGA) or tetanus toxin C-fragment can allow transneuronal tracing of connections to or from specific cell types in Cre-expressing mouse lines (3, 4); however, the lack of amplification following transneuronal spread might limit the detection of weak connections, and polysynaptic spread creates ambiguity about what cells are directly connected versus indirectly connected. Generation of a retrograde transsynaptic herpes virus ("pseudorabies virus," PRV) whose replication is Cre-dependent (5) and expression of WGA-Cre fusion protein (6) provide systems that can amplify and thus are likely to be more sensitive. Although potentially powerful, these systems do not distinguish between neurons connected directly to the starting population and neurons connected indirectly. Furthermore, even attenuated PRV strains kill neurons within just a few days (7), and WGA can spread transneuronally in both the anterograde and retrograde directions (3, 6, 8).

Our aim in the present work was to use transgenic and knock-in mice that express proteins in a cell type–specific manner, in conjunction with a modified rabies virus, to produce an efficient system for labeling direct, monosynaptic inputs onto defined cell populations. Previous studies demonstrated that infection with glycoprotein gene–deleted (ΔG) rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA (EnvA-ΔG rabies virus) can be restricted to neurons in brain slice culture that express TVA, an avian receptor protein that is absent in mammalian cells unless provided through gene delivery vectors (9). Retrograde spread of the virus can be restricted to directly presynaptic neurons by separately expressing rabies glycoprotein (G) selectively in the same cells. Expression of a reporter gene from the rabies genome directly reveals the synaptically connected neurons (9), and infected neurons remain viable for weeks (10).

Our ultimate goals were to expand this technique to develop a robust, reliable, and accurate means for identifying direct inputs onto specific cell populations in vivo, and to make this

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technique generically applicable across a wide variety of brain regions and cell types. Toward these aims, we developed Cre-dependent viral vectors that express the required proteins for rabies virus infection and transsynaptic spread. We chose this approach because stereotaxic injection of Cre-dependent viruses provides a means for delivering genes of interest in a spatially localized manner, allowing us to target a specific cell type in a defined brain region of our choosing. Furthermore, it was recently demonstrated that adeno-associated viruses (AAVs) with Cre-dependent gene cassettes can restrict transgene expression to cells expressing Cre recombinase in vivo (11–13). This strategy allows investigators to use hundreds of existing mouse lines that express Cre in a restricted manner, as well as many other Cre lines that are in active development. High-throughput examples of Cre mouse generation include efforts at the Allen Institute (14), as well as the GENSAT project (15).

In this paper, we describe a two-virus system that can reliably target specific cell types in the brain and label their direct, monosynaptic connections in vivo. This system can be applied to many different brain regions with minimal modification and provides an appealing approach to performing circuit-level investigations in the mammalian brain. It potentially can deliver a variety of genetically encoded tools to targeted networks without perturbing neighboring neuronal circuits, further expanding its utility to the neuroscience community.

Results

To take advantage of mouse lines expressing Cre in specific cell types, we developed helper viruses that express the genes necessary for the infection, complementation, and monosynaptic retrograde spread of EnvA-ΔG rabies viruses (Fig. 1*B*). These helper viruses selectively express their gene payloads in cells expressing Cre recombinase, and use the FLEX double-floxed system to reliably express genes in a Cre-dependent manner (13). Because a miniscule amount of leaked TVA is sufficient to facilitate EnvA-pseudotyped virus infection (16), we also deleted the ATG start codon from all genes within the Cre-dependent cassette, and placed a single Kozak sequence leading the FLEX cassette. Cre recombinase acts to reverse-complement the gene sequence and lock the cassette in the proper orientation and frame for transcription (Fig. 2). We linked multiple genes using foot-and-mouth disease type 2A elements (17, 18) to allow for the production of multiple protein products from a single ORF. We packaged the Cre-dependent cassettes into an AAV serotype 9 vector (AAV9) because of this serotype's broad infectivity potential in the mouse brain.

We then used this system, as schematized in Fig. 1*A*, to directly demonstrate rabies virus infection and monosynaptic retrograde spread from a specified postsynaptic neuronal population. We developed Cre-dependent helper viruses coding for both the avian viral receptor TVA and rabies G, and infected a brain region of interest (Fig. 1 *A* and *B*; "helper virus"). Following expression of TVA and G in Cre-expressing neurons, we injected EnvA-ΔG rabies expressing a fluorescent protein into the same brain region (Fig. 1 *A* and *C*; rabies virus). This rabies virus can only infect cells expressing the EnvA binding partner, TVA (9), thus targeting rabies virus infection to Cre-expressing cells. Newly synthesized rabies virus particles can then complement with the rabies glycoprotein expressed in these cells, allowing the rabies virus to spread retrogradely across synaptic contacts, directly labeling presynaptic neurons. Rabies-infected cells are uniquely labeled by the fluorescent protein expressed from the rabies genome (Fig. 1*A*, red neurons). Because the presynaptic neurons do not express rabies glycoprotein, these cells cannot produce infectious rabies virus particles, restricting infection to the targeted cell type and its direct monosynaptic inputs (9, 10, 19).

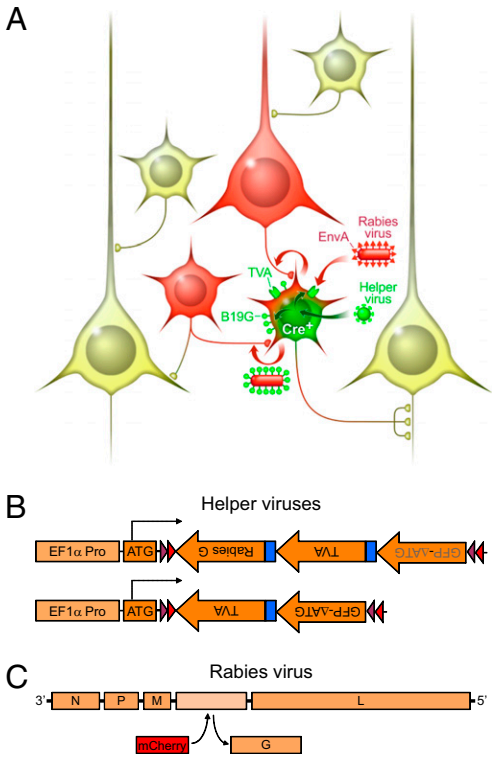

Fig. 1. Using a Cre-dependent helper virus to target rabies virus infection and monosynaptic retrograde spread. (*A*) Cre-dependent helper virus (green) is injected into the brain of a mouse that expresses Cre in a specific cell type (*Cre+*). Following Cre recombination, this helper virus expresses two proteins that are necessary for subsequent rabies virus infection (TVA) and monosynaptic retrograde spread (the rabies glycoprotein B19G). TVA is an avian receptor protein that confers infection capability to rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA (red). EnvA-pseudotyped rabies virus is incapable of infecting mammalian neurons in the absence of TVA, restricting rabies virus infection to TVA-expressing cells. The rabies virus has its own glycoprotein gene deleted from its genome, which renders the virus incapable of spreading retrogradely in the absence of another source of rabies glycoprotein. The helper virus complements this deficiency, allowing the rabies virus to spread one synapse retrogradely. This presynaptic neuron population does not express the rabies glycoprotein, so the rabies virus cannot spread any further, limiting rabies virus infection to a small population of Cre-expressing cells and their directly presynaptic partners. (*B*) Cre-dependent, AAV serotype 9 helper virus vectors were generated to express either GFP and TVA (AAV9-pEF1α-FLEX-GT; *Upper*) or TVA and Rabies G (AAV9-pEF1α-FLEX-GTB; *Lower*) in targeted cell types. Cre-dependence was conferred using the FLEX double-floxed system, in which Cre recombinase acts on mutually exclusive sets of lox sites [lox2272 (purple triangles) and loxP (red triangles)] to lock the reverse complemented gene sequence into the proper orientation for transcription. Start ATGs were removed from all genes, and a single Kozak sequence was added outside of the double-floxed cassette, to eliminate any remaining leak expression. To express multiple genes under the control of a single promoter, coding sequences were linked by foot-and-mouth disease virus type 2A elements (blue boxes). (*C*) The rabies virus genome has been modified such that the glycoprotein gene (G) is replaced with a fluorescent reporter, mCherry. The rabies virus is then pseudotyped with EnvA to produce (EnvA)SAD-ΔG-mCherry.

For proof-of-concept purposes, we first used this system to recapitulate known circuitry in the mouse cerebellum. To demonstrate that we could target helper virus gene expression and, by extension, infection of the EnvA-ΔG rabies virus to specific cell types, we injected 180 nL of Cre-dependent helper virus expressing GFP and TVA linked by the T2A element (AAV9-pEF1α-FLEX-GT) into the cerebellum of mice expressing Cre almost exclusively in cerebellar Purkinje cells [L7-Cre mice (20)]

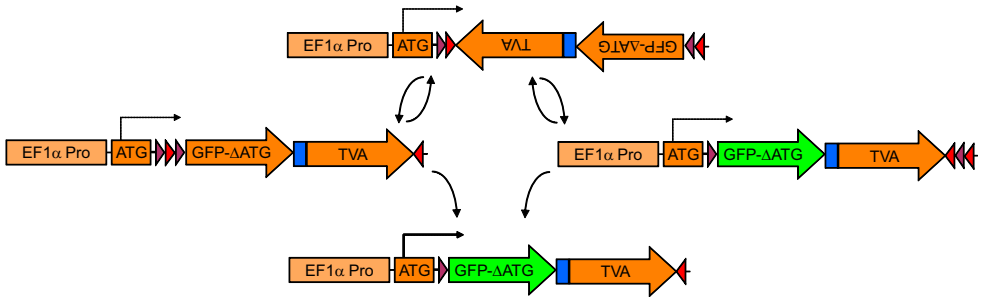

Fig. 2. Cre-dependent recombination of helper virus gene cassette. The helper viruses carry reverse-complemented gene sequences flanked by two mutually exclusive sets of lox sites, termed FLEX (13). Cre can act on either pair of lox sites, lox2272 (purple triangles) or loxP (red triangles), but cannot mediate recombination across 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 high levels of gene expression.

(Fig. 3*A*). Then, 3 wk later, we injected 180 nL of mCherry-expressing (EnvA-ΔG-mCherry) rabies virus into the same location (Fig. 3*B*). Brain tissue was extracted and processed 1 wk after the rabies virus injection. As expected, Cre-dependent AAV expressed GFP almost exclusively in Purkinje cells (Fig. 3 *A* and *B*), and the EnvA-pseudotyped rabies virus was only capable of infecting these TVA-expressing cells. In a representative animal, counted across every sixth section, 151/151 cells labeled with mCherry also were positive for GFP, 145 (96%) of which were Purkinje cells. In the absence of rabies glycoprotein, the rabies virus could not spread transsynaptically (Fig. 3*B*). Furthermore, reporter gene expression was almost completely undetectable from both the AAV and rabies virus in WT animals, with no cells expressing GFP and 8.0 ± 8.0 cells (mean ± SEM) expressing mCherry (n = 2 animals) (Fig. 3*C*). These results, combined with those from further control experiments (Fig. S1), demonstrate that the EnvA-ΔG rabies virus is incapable of infecting neurons in the absence of TVA, that the Cre-dependent helper virus is capable of delivering TVA to specific cell types in vivo, and that the EnvA-ΔG rabies virus can infect only those TVA-expressing cells.

We next wished to demonstrate complementation and monosynaptic retrograde spread of EnvA-ΔG rabies virus in the presence of rabies glycoprotein provided by the helper virus. Because the inputs onto Purkinje cells are known, we were quickly able to assess the effectiveness of this strategy. We produced a helper virus expressing GFP-T2A-TVA-E2A-rabies G (AAV9-pEF1α-FLEX-GTB) and injected this virus into the cerebellum of L7-Cre mice, followed 3 wk later by injection of EnvA-ΔG-mCherry rabies virus (Fig. 3 *D* and *E*). GFP was not reliably detectable using this helper virus, but both TVA and rabies G were functionally

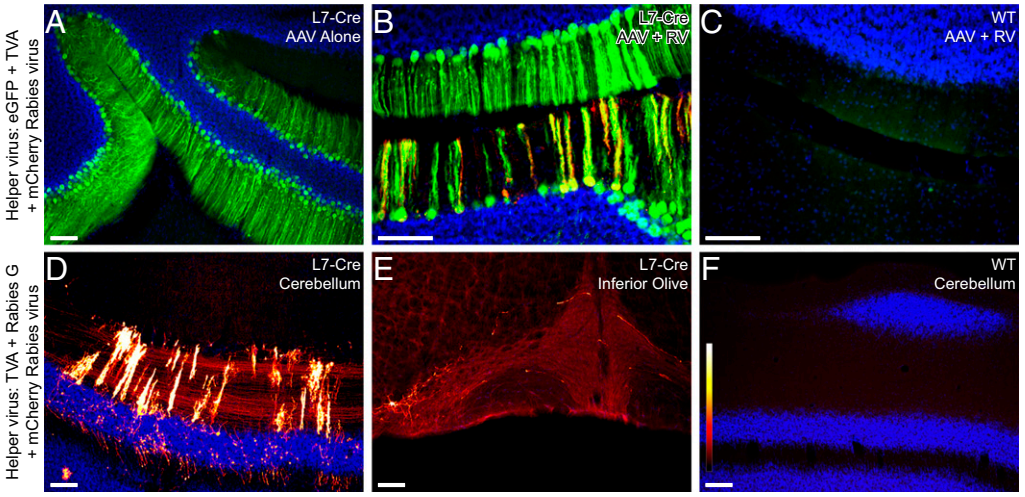

Fig. 3. Helper virus mediation of rabies virus infection and spread in the cerebellum of L7-Cre mice. (*A–C*) A helper virus expressing Cre-dependent eGFP and TVA (AAV9-pEF1α-FLEX-GT) was injected into the cerebellum of L7-Cre mice (*A* and *B*) and WT mice (*C*) and allowed to express for 3 wk, either in the absence of rabies virus (*A*) or followed by injection of rabies virus expressing mCherry [(EnvA)SAD-ΔG-mCherry] (*B* and *C*). (*A*) As expected, the Cre-dependent AAV expresses GFP almost exclusively in Purkinje cells in the L7-Cre mouse. (*B*) (EnvA)SAD-ΔG-mCherry can only infect cells that are expressing GFP and TVA. In the absence of rabies glycoprotein, the rabies virus is unable to spread from the Purkinje cells to the presynaptic granule cells, labeled densely by blue DAPI stain directly below the Purkinje cell layer. The top row of green Purkinje cells is outside the core region of the rabies virus injection, whereas the bottom row is within the boundary of the rabies virus injection sphere. (*C*) Cre-dependent AAV does not detectably express GFP, and (EnvA)SAD-ΔG-mCherry is incapable of infecting neurons in the WT animal, indicating that the Cre-dependent AAV effectively restricts TVA expression to only Cre-positive cells. (*D–F*) A Cre-dependent AAV expressing TVA and rabies glycoprotein (AAV9-pEF1α-FLEX-GTB) (eGFP is in the genome but not detectably expressed) was injected into the cerebellum of L7-Cre mice (*D* and *E*) and WT mice (*F*), followed 3 wk later by injection of (EnvA)SAD-ΔG-mCherry. (*D*) (EnvA)SAD-ΔG-mCherry can directly infect Purkinje cells and spread transsynaptically to directly presynaptic interneurons and granule cells in the presence of TVA and rabies G in the L7-Cre mouse. The rabies label in *D–F* is visualized using the Redhot lookup table in *F* and overlaid onto the DAPI signal (blue), which is used to visualize cerebellar layers. (*E*) (EnvA)SAD-ΔG-mCherry can spread from directly infected Purkinje cells across the climbing fiber synapse, and can directly label presynaptic cells in the inferior olive in the L7-Cre mouse. (*F*) As expected, no rabies virus infection is detectable in the WT animal, confirming that TVA expression is properly restricted in the absence of Cre. (Scale bars: 100 μm.)

expressed. As predicted, a bright mCherry signal was detected in Purkinje cells and their known direct monosynaptic inputs. Locally, thousands of granule cells and their parallel fibers were labeled (Fig. 3*D*), as were locally connected cerebellar interneurons. An example animal, sampled across every sixth section, contained 63 mCherry-expressing Purkinje cells, as well as many thousands of granule cells with average density of 85 cells/100 μm^3 region, spread across a band of ≈2 mm × 1 mm × 250 μm. In addition, we could reliably detect brightly labeled cells in the inferior olive, demonstrating that rabies virus can efficiently infect and travel retrogradely across long-distance connections (Fig. 3*E*). Rabies virus infection was almost completely undetectable in the absence of Cre (0.5 ± 0.5 cells; $n = 2$; Fig. 3*F*), and no unexpected connections (i.e., neurons that give rise to the cerebellar mossy fiber inputs) were detected in Cre mice, demonstrating that the rabies virus does not spread spuriously or extrasynaptically. These results demonstrate that EnvA-ΔG rabies viruses can infect specified cell populations and selectively label their direct, monosynaptic inputs in the presence of Cre-dependent TVA and rabies G.

To demonstrate the utility of this two-virus system in mouse neocortex, we began by injecting AAV9-pEF1α-FLEX-GT (expressing GFP and TVA) into the barrel cortex of parvalbumin (PV)-Cre mice, followed 3 wk later with EnvA-ΔG-mCherry rabies virus. As expected, we detected fluorescent protein expression from both viruses primarily in PV^+ interneurons, but also observed viral protein expression in a small population of layer 5 pyramidal cells (Fig. S2). Although these results were initially perplexing, a recent paper (14) reported that some GABA-negative cells in layer 5 of this mouse line do in fact express low levels of PV and, as a result, express Cre as well.

To demonstrate that we could then label the monosynaptic inputs onto PV^+ cells in the neocortex, we injected AAV9-pEF1α-FLEX-GTB (expressing TVA and rabies G) into the barrel cortex of PV-Cre mice (21), followed 3 wk later with EnvA-ΔG-mCherry rabies. As expected, we detected thousands of neurons, both primary and retrogradely infected, near the injection site (Fig. 4*A*). Furthermore, the retrograde label was detected in brain regions known to project to the barrel cortex, including contralateral S1, ipsilateral S2 (Fig. 4*C*), and ipsilateral thalamic nuclei VPm and Po (Fig. 4*E*). An example PV-Cre animal, sampled across every sixth section, was found to contain ∼600 locally infected neurons and ∼20 long-distance projection neurons. Leaking expression of mCherry was rarely detected in the absence of Cre (9.3 ± 2.6 cells; $n = 6$ animals), and these cells were observed only in the immediate vicinity of the injection site (Fig. 4*B*). No long-range projections were labeled in the absence of Cre (Fig. 4 *D* and *F*). These results demonstrate that this system also functions effectively to target cell types and selectively label their monosynaptic connections in the neocortex.

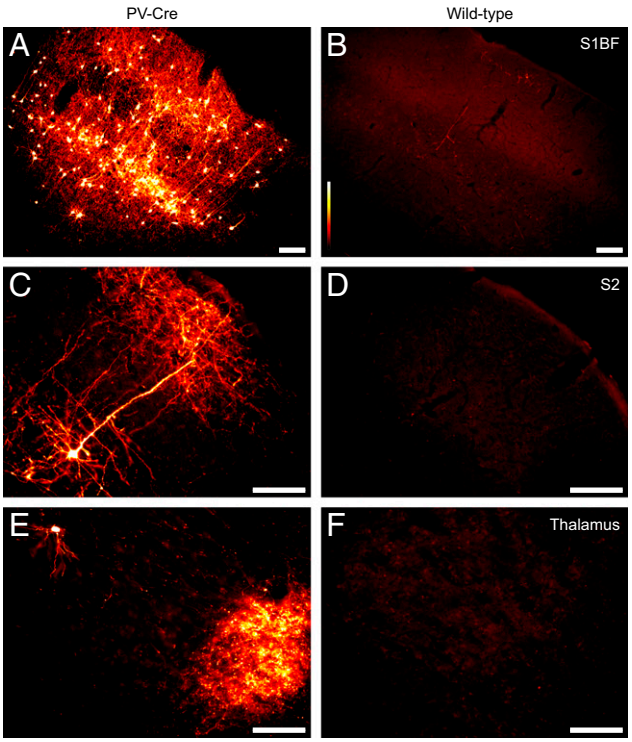

Fig. 4. Rabies virus labels direct inputs to PV^+ cells in the barrel cortex of PV-Cre mice. Helper virus (AAV9-pEF1α-FLEX-GTB) and rabies virus [(EnvA)SAD-ΔG-mCherry] were injected into the barrel cortex of PV-Cre mice or into WT control animals. mCherry signal is visualized using the Redhot lookup table in *B*. (*A*) Rabies virus can infect PV cells and their retrogradely connected partners in the primary somatosensory cortex. (*B*) In contrast, rabies infection is almost completely absent in the WT animal. (*C* and *D*) Retrogradely infected cells can be detected in secondary somatosensory cortex in the PV-Cre mouse (*C*), but never in the WT animal (*D*). (*E*) Retrograde label in the thalamus of PV-Cre mice. Both somata and dense axon termini are reliably detected in thalamic nuclei VPm and Po, which are known to project into barrel fields of the primary somatosensory cortex. Axon terminal labeling arises from corticothalamic projection cells that are retrogradely labeled in *A*. (*F*) As expected, there is no mCherry signal in the thalamus of WT animals. (Scale bars: 100 μm.)

Discussion

We demonstrated that a combination of Cre-dependent helper virus and EnvA-pseudotyped, G-deleted rabies virus can be used to effectively identify the direct local and long-distance connections to Cre-expressing neurons. We showed that Cre-dependent helper viruses can be used to target EnvA-ΔG rabies virus infection to specific cell types in the brain, and that expression of rabies G in a Cre-dependent manner then permits the rabies virus to spread retrogradely from a predefined cell population to its directly connected partners. Our findings also provide strong evidence that EnvA-ΔG rabies virus cannot infect cells in vivo in the absence of TVA and that rabies virus spreads specifically only to known synaptic inputs when complemented with rabies G.

Using these approaches in various mouse lines that selectively express Cre recombinase in specific types of neurons will make it possible to identify the sources and cell types providing direct monosynaptic input to any cell type for which relevant mouse lines are available. However, our observation regarding the small number of layer 5 pyramidal cells in the PV-Cre line demonstrates an important point when using this circuit-tracing system: Labeling specificity will be only as tight as the Cre mouse line in which the experiments are performed. Because Cre-dependent viruses can convert widely varying levels of Cre recombinase expression into high levels of transgene expression, this system may not fully recapitulate previous characterizations of Cre expression in other mouse lines. Thus, it is necessary to characterize the mouse line using the experiments and controls described in *Results* to fully describe targeted cell types and their inputs.

Although the GFP was not readily detectable from the FLEX-GTB helper virus, we have demonstrated that this virus functionally expresses both TVA and rabies glycoprotein, allowing EnvA-pseudotyped virus to specifically infect and spread from Cre-targeted cells. However, in the helper virus' current form, distinguishing presynaptically connected cells from the postsynaptic starting population might be difficult in certain systems. Any labeled neurons remote from the rabies virus injection site will be presynaptically connected by necessity, but cells near the injection site may be harder to identify. Characterization of a Cre line using the TVA-only helper virus provides good insight into

the spatial range, rough number, and types of cells that will be infected initially; however, the lack of functioning fluorophore in the FLEX-GTB helper virus makes it impossible to quantitatively determine the number of postsynaptic cells and the relative abundance of locally connected cell populations. Although the lack of GFP expression from this helper virus was initially confusing, multiple studies have encountered similar findings in which fluorescent proteins linked to other genes through C-terminal 2A elements failed to fluoresce (22, 23). This discrepancy could possibly be rectified in the future by using improved 2A elements (24) or by reordering the genes within the Cre-dependent cassette. Commercially available antibodies are currently incapable of specifically recognizing TVA or rabies G, so having a functional fluorophore or antibody tag will be useful in future iterations of this system.

It also is important to note that the retrogradely labeled neuronal population underrepresents the full complement of connections. A recent paper using the same strain of rabies virus demonstrated that ≈50 cells were retrogradely infected from a single cortical neuron in vivo (25). However, our system benefits from the fact we start with a comparatively large postsynaptic population, allowing labeling of even sparse connections. Although rabies virus has broad neurotropism, injecting nonpseudotyped rabies virus directly into a brain region of interest also can be useful for identifying the complement of synapses that can be infected by the rabies virus.

Our proposed two-virus system provides a powerful means for understanding brain circuitry by elucidating the direct inputs onto known cell types in a variety of brain areas, providing a flexible and generically applicable means for building circuit-level models of neuronal connectivity. Furthermore, recent advances in molecular biology, virology, and genetics will allow investigators to extend their studies beyond the realm of neuroanatomy. Because the rabies virus itself is a gene delivery vector, the rabies genome can be manipulated so that infected neurons will express genes other than the fluorescent reporters illustrated herein. Because rabies virus–infected cells remain viable for weeks (10), it is possible to combine circuit tracing with functional studies in vivo. For example, by expressing channelrhodopsin in the direct inputs onto a defined cell type, investigators can control the activity of this connected neuronal population without activating neighboring, unconnected neurons, allowing for more precise analysis of the role of direct synaptic input in regulating the activity of cells of interest. Using the full range of genetic tools that can monitor or manipulate infected neurons (1), investigators should be able to modify this system to probe a variety of circuit-level questions in vivo that were previously unanswerable using standard electrophysiological techniques.

Materials and Methods

All experiments using live animals were performed in accordance with protocols approved by the Salk Institute's Institutional Animal Care and Use Committee.

AAV Production. The initial AAV gene delivery plasmid (pAAV-FLEX-hGTB; #26196) was generated de novo by Mr. Gene. We generated the EF1α promoter plasmids used in this study (pAAV-EF1α-FLEX-GTB; Addgene #26197 and pAAV-EF1α-FLEX-GT; #26198; Addgene) by deleting one or more elements from the pAAV-FLEX-hGTB plasmid and then subcloning the double-floxed gene cassette into an existing vector, pAAV-EF1α-double flox-eYFP-WPRE (26). These plasmids use the FLEX double-floxed sequence rather than the DIO sequence used in previous work (11). Both of the EF1α promoter AAVs were isolated through transfection of a single 15-cm plate of HEK293T cells, followed by freeze–thaw lysis of the cells and crude extraction of the intracellular contents. Genomic titers were both approximately $\sim 10^8$ genome copies per milliliter.

Rabies Virus Production. EnvA-pseudotyped, G-deleted rabies viruses were produced in a manner similar to that described previously (27). (EnvA)SAD-ΔG-mCherry had functional titers of $\sim 2 \times 10^{10}$ infectious units per milliliter as determined through infection of TVA-expressing HEK293T cells.

Animal Surgery and Viral Injection Parameters. L7-Cre (20) and PV-Cre mice (21) were maintained in a C57BL/6 background and selected for experiments when animals were 2–6 mo of age. For all experiments, age- and sex-matched C57BL/6 mice were used as controls. To perform stereotaxic viral injections into the brain, mice were briefly induced under isoflurane and then injected with ketamine/xylazine (100 mg/kg ketamine and 10 mg/kg xylazine in sterile saline) to achieve stable anesthesia. The mice were then mounted in a rodent stereotax (David Kopf Instruments model 900 series) and head-fixed using ear bars, a bite bar, and a nose clamp. A small incision was made in the skin over the skull to expose the bregma, lambda, and desired injection site. A three-axis micromanipulator was used to measure spatial coordinates for the bregma and lambda. The injection site was calculated relative to these landmarks, using canonical coordinates. The following injection coordinates were used (all values given relative to the bregma): cerebellum (anteroposterior, −5.88 mm; lateromedial, +0.75 mm; dorsoventral, −1.50 mm); barrel fields of the primary somatosensory cortex (anteroposterior, −0.75 mm; lateromedial, +3.00 mm; dorsoventral, −1.50 mm). A small drill hole was made in the skull over the injection site, exposing the brain. A pulled glass pipet (tip diameter, ≈30 μm) was loaded with virus and then lowered into the brain to the appropriate coordinates. A Picospritzer (General Valve) was used to pulse virus into the brain.

A total of 180 nL of helper virus was injected into the brain at a rate of 15–20 nL/min, with ≈350 pL delivered per pulse. To prevent backflow of virus, the pipet was left in the brain for 5 min after completion of the injection. Once the injection pipet was removed, the mouse was removed from the stereotax, and the incision was closed with wound clips. Mice were allowed to recover for 3 wk before rabies virus infection. In these experiments, rabies virus was injected under the same conditions and injection volume as the for initial AAV injection. The rabies virus was allowed to replicate and spread for 7 d before perfusion and tissue processing.

Tissue Processing and Imaging. To preserve brain tissue for imaging and subsequent analysis, mice were intracardially perfused with 50 mL of a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). After perfusion, the brain was isolated and transferred to a postfixative solution containing 4% paraformaldehyde and 30% sucrose in PBS, and then incubated overnight at 4 °C on a rotating shaker.

The next day, 40- to 50-μm coronal brain sections were prepared using a microtome with freezing stage, and tissue was separated into between four and six groups to allow for multiple tissue manipulations. Tissue groups that were not used immediately were placed in a cryopreservative solution (30% glycerol and 30% ethylene glycol in PBS) and stored at −20 °C.

Fixed tissue was immunostained following a standard protocol. To preserve the GFP signal, we used a chicken primary antibody against GFP (1:500; Aves Labs) and amplified with a Cy2-conjugated anti-chicken secondary antibody (Jackson ImmunoResearch). To preserve the mCherry signal, we used a rabbit polyclonal antibody against DsRed (1:250; Clontech) and amplified with a Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). To label cell bodies, tissue was stained with 10 μM DAPI for 10 min after any secondary antibodies were rinsed off. A mouse monoclonal antibody against PV (1:1,000; Sigma-Aldrich) was used to label PV^+ cells in the neocortex.

Immunostained tissue was mounted on chrome-gelatin subbed slides and allowed to dry overnight. The tissue was dehydrated and defatted using a series of ethanol and xylene immersion steps. Slides were then coverslipped using Krystalon (Harleco) mounting medium and glass coverslips. Images were acquired with a modified Olympus BX51 microscope, globally γ-adjusted to reduce background, and pseudocolored using ImageJ (National Institutes of Health). Images showing quantitative colocalization were produced by merging color channels in ImageJ. Images that made use of DAPI only to indicate underlying brain structure were overlaid in Photoshop (Adobe Systems) with DAPI as the bottom layer.

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