UNIVERSITY OF CALIFORNIA, SAN DIEGO

TRANSSYNAPTIC TRACING BY *IN SITU* COMPLEMENTATION OF A DELETION MUTANT NEUROTROPIC VIRUS

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in

Neurosciences

by

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2006

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ABSTRACT OF THE DISSERTATION

TRANSSYNAPTIC TRACING BY *IN SITU* COMPLEMENTATION

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Ian R. Wickersham

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Professor Edward M. Callaway, Chair

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We have developed two new tools for tracing neural connections. The first is a means of identifying cells that project to a region of interest within the brain, illuminating these cells with intense fluorescence and allowing their finest morphological details to be seen. This permits either highly detailed reconstruction of the cells' anatomy or their targeting, in live tissue, on the basis of their morphology for physiological study. The second tool is a means of identifying, on a large scale, neurons which are directly presynaptic to either a targeted cell type or indeed a single neuron. Because this technique also results in intensely fluorescent cellular details, this too could be used either for detailed anatomical studies or for physiological ones. This should allow a significantly more precise

understanding of the organization of nervous systems than has previously been possible.

I. INTRODUCTION

Advances in neuroscience often stem from advances in technology: experimentation with static electricity led to the discovery of electrical transmission in nerves[1]; Cajal's detailed descriptions of neuronal anatomy quickly followed Golgi's discovery of the silver chromate staining reaction[2]; single unit studies in cortex became possible only after systematic development of the tungsten microelectrode[3].

The rise of molecular biology in the past several decades has provided neuroscience with powerful tools that are rapidly advancing understanding of nervous systems and in turn allowing the development of yet more tools to advance it even further. The work described in this dissertation comes out of these advances and would not have been possible a little over a decade earlier, because it makes use of very recent advances in virology, most importantly the development of the ability to manipulate the genome of rabies virus[4].

The dissertation describes two new techniques. The first is the use of a recombinant rabies virus for retrogradely labeling neurons that project to an injection site, filling their cytoplasms with high levels of enhanced green fluorescent protein and thereby allowing either the detailed reconstruction of their anatomy or their identification in live tissue for subsequent electrophysiological study. The second technique, likely to be of significantly greater impact than the first, is a means of labeling on a large scale neurons that are directly presynaptic to a cell type of interest or to a single cell. Since it uses the virus just described,

it too illuminates the cells in question in vivid fluorescent detail and therefore also allows their detailed anatomical or physiological study. Our hope and expectation is that the techniques described below will be useful.

II. INTENSELY FLUORESCENT COMPLETE CELL FILLS OF PROJECTION NEURONS BY RETROGRADE INFECTION WITH A DELETION MUTANT RABIES VIRUS

ABSTRACT

We have constructed a deletion mutant rabies virus encoding EGFP and find it to be an excellent retrograde tracer for studying neural connectivity, apparently vastly superior to any other currently available. Due to the natural axonal tropism of rabies virus, the virus efficiently labels large numbers of distant cells that project to injection sites within the brain. However, the most critical difference between this mutant and other viruses previously described as effective for retrograde gene delivery is the nature of the particular deletion from its genome: missing only its envelope glycoprotein gene, the virus is unable to spread beyond initially infected cells yet can still replicate its core within them. Because of this, any infection of a cell results in inevitable proliferation of the viral core to maximally high copy numbers and the concomitant expression of extraordinarily high levels of EGFP. Infected cells are filled in every detail

of their dendritic and axonal trees with zero background from partially or faintly labeled cells. Since infected cells survive for weeks despite the viral core's intact replication, cells can be targeted on the basis of their detailed morphology for physiological investigations. We suggest that this and similar recombinant rabies viruses will be extremely useful tools for studying the detailed anatomy and physiology of identified projection neurons.

INTRODUCTION

One of the most fundamental questions regarding the organization of the brain is how the various structures are connected to each another. A basic understanding of which regions connect to which has been a longstanding goal of neuroscience, as has an understanding of the more detailed issue of which of the often myriad cell types within each region project to a given structure and what their physiological properties are. Many techniques have been used to answer such questions, most being variations of the general technique of injecting into a region of interest some "retrograde tracer": a substance which is taken up by the axon terminals of neurons that project to the injection site and which subsequently allows either visualization of their anatomy or, in the case of fluorescent tracers, their targeting for physiological study. Such techniques have been tremendously useful and have provided the majority of our understanding of the long-range connectivity of nervous systems.

An ideal tracer would fill cells in every detail, so that their complete morphology can be seen, and moreover would do so in a vividly fluorescently manner and leave the cells alive and functioning, so that neurons could be identified on the basis of their detailed anatomy for physiological study.

There is no tracer currently available that fills these requirements in a satisfactory way. Fluorescent microspheres only label somata and proximal dendrites; carbocyanine dyes such as DiI and DiO work far better in fixed or immature tissue than in live adult animals[5, 6]. Even in the domain of purely anatomical tracers that require postprocessing for visualization, there is significant room for improvement: all conventional tracers, such as the fluorescent ones just mentioned as well as others such as biotinylated dextrans, cholera toxin subunit B, and wheat germ agglutinin [7, 8], are afflicted by the absence of any amplification within labeled cells. As a result, while the most effective of these can label axonal and dendritic details under optimal conditions [9], any complete cell fills that may be achieved inevitably compete with a background of poorly and partially filled cells [7, 10].

These perennial problems have motivated the development of multi-step approaches combining an initial injection of a fluorescent tracer with subsequent *ex vivo* treatment of retrogradely labeled cells to result in visualization of their detailed morphologies. The first such approach, taken by Larry Katz, relied on axonal uptake of fluorescent microspheres followed by targeting of retrogradely labeled somata for intracellular filling with Lucifer Yellow[11]. Very recently, and explicitly to solve the problem of fluorescently identifying cellular details in live

tissue for subsequent targeting for physiological study, Dennis Dacey and colleagues presented a system in which cells retrogradely labeled with rhodamine dextran are “photostained” by illuminating the live tissue to cause intracellular dispersal of the dye to fill the cytoplasm[12]. However, both this and the Katz technique are complicated and laborious processes requiring secondary staining steps following production of live brain slices or other dissection of tissue, which in any case makes labeling distant arborizations that are inevitably cut off by such manipulations impossible.

For these reasons, there has been a clear need for a tracer that is efficiently taken up retrogradely, labels fine details of both axonal and dendritic arbors, and that moreover leaves cells brightly fluorescent and alive for physiological investigations. The ascendance of molecular biology suggests the possibility of using retrogradely infectious viruses, perhaps expressing a genetically-encoded fluorophore, as retrograde tracers, and several have been used as such[13-15]. In no published cases, however, has the resulting fluorescence been bright enough to provide consistently detailed anatomical information without immunohistochemical amplication and therefore to permit high-resolution identification of live labeled neurons for subsequent physiological study.

Here we present a novel virus that, because of several unique characteristics, does achieve these goals and, we suggest, is the best available means of revealing detailed morphology of retrogradely labeled neurons.

RESULTS

Rabies virus, which has been used with great success as a transsynaptic tracer [16-19], is a neurotropic virus that infects neurons via axon terminals, replicates in the cytoplasm, and spreads between synaptically coupled neurons in an exclusively retrograde direction. It is superior to the other widely used family of neurotropic tracing viruses, the alpha-herpesviruses, in at least two ways: first, it is far less cytopathic, so that infected cells survive for much longer [17], and, second, it is far more efficient: whereas several tens of thousands of particles of a herpesvirus must be injected into the brain before any infection can be initiated [20-22], only a single infectious unit of rabies virus must be introduced into a brain to result in a full-scale case of rabies [23, 24]. Rabies virus therefore represents an already very powerful and high-precision tool that could potentially be harnessed for fine-scale mapping of long-range connections within the brain.

However, the “wild-type” virus typically used in transsynaptic tracing studies is unsuitable as a direct retrograde tracer - that is, to identify those cells in particular that project directly to an injection site - for two reasons. First, the virus spreads transsynaptically, which of course is the main reason for its use, but moreover does so in asynchronous fashion, like all transsynaptic tracers [17]. This makes unambiguous identification of only directly projecting cells, as opposed to cells that project via intervening synaptic steps, impossible. Second, since the virus does not contain the gene for a marker that fills infected cells, it

provides only limited morphological information, because the only avenue for visualization - immunostaining for viral proteins – results in crude label only enabling resolution of cell bodies and proximal dendrites [18]; such examination, furthermore, is obviously restricted to dead and processed tissue and is therefore not useful for physiological investigations.

Addressing each of these two problems - disabling the virus' ability to spread beyond initially infected cells, and introducing a marker gene that would yield improved morphological information – requires modification of the viral genome. Because rabies virus, as a member of the rhabdovirus family, is a negative-stranded RNA virus whose genome neither serves directly as mRNA nor passes through any DNA intermediate state in the natural replication cycle, construction of recombinant versions has historically been unattainable but has become possible within the last few years following work in one of our laboratories (K.K. Conzelmann) [25, 26].

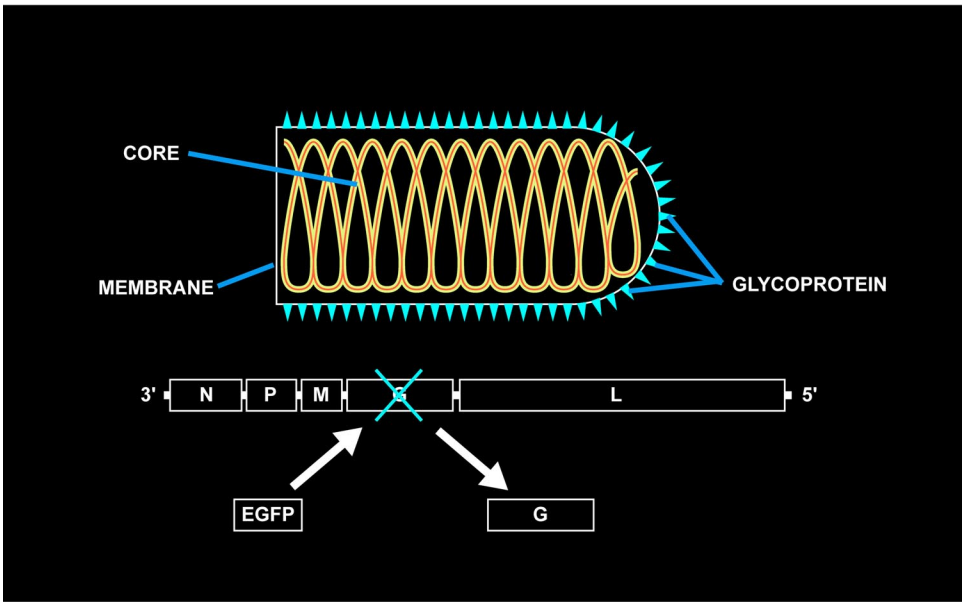

Fig. 1. The rabies virion & genome. Top: The rabies virion. The viral core consists of the helically wound RNA genome encapsidated by the nucleocapsid protein (N) and associated with the matrix protein (M) and with the viral polymerase composed of the phosphoprotein (P) and large protein (L). The core is surrounded a host-cell-derived membrane in which is embedded trimeric spikes of the viral glycoprotein (G). Bottom: The viral genome, showing the substitution of the EGFP gene in place of the coding sequence for the viral glycoprotein.

The rabies viral particle or virion is depicted schematically in figure 1. The viral core is composed of the single-stranded, negative-sense RNA genome wound into a helix and tightly associated with the nucleocapsid protein (N), as well as the matrix protein (M) and viral polymerase consisting of the phosphoprotein (P) and large protein (L). Surrounding this core is a host-cell-derived phospholipid bilayer envelope or membrane, and embedded in the membrane is the virus' envelope glycoprotein (G)[27].

The glycoprotein is not involved in transcription of the viral genes or replication of the viral core. Instead, its role is to facilitate "budding" of completed cores through the host cell membrane to produce the final enveloped virus coated in trimeric "spikes" of glycoprotein, which then attach to receptors on presynaptic terminals of synaptically coupled cells and mediate penetration of the viral core into the cytosol following endocytosis [27].

In earlier work (K.K. Conzelmann lab), we produced a virus with the glycoprotein gene deleted from its genome but that was grown in phenotypically complementing cells so that the glycoprotein itself, provided *in trans* by the producer cells, was incorporated into the viral particles' membranes despite the lack of its coding sequence in the viral genome[28]. Such a virus can infect contacted cells normally and, because the glycoprotein plays no role in transcription and replication, can still express its remaining genes and proliferate its viral core within initially infected cells. However, with no means of synthesizing glycoprotein in these cells, the newly created progeny were found to be greatly diminished in their ability to bud out through the host cell membrane

into the extracellular space, and in any case the few that managed to do so were “sterile”, with membranes devoid of their glycoprotein, unable to bind to receptors on the presynaptic cells and penetrate them to begin a new round of infection[28, 29].

Deleting the glycoprotein gene, therefore, constitutes an absolute block to transsynaptic transfer, solving the first problem described above, namely preventing the virus from spreading beyond directly infected cells. To solve the second problem, that of providing some cell-filling marker to reveal detailed morphology, we substituted the gene for enhanced green fluorescent protein (EGFP) in place of the gene for the glycoprotein (figure 1). We then grew virus with this genome on glycoprotein-complementing cell line to produce particles with the glycoprotein present on their membranes, so that they can infect initially contacted cells, in which we hypothesized that the cores should replicate to high copy number and high levels of EGFP should be expressed, but beyond which the virus would not be able to spread (figure 2). We termed this virus, which was derived from the SAD B19 strain of rabies virus, SADΔG-EGFP.

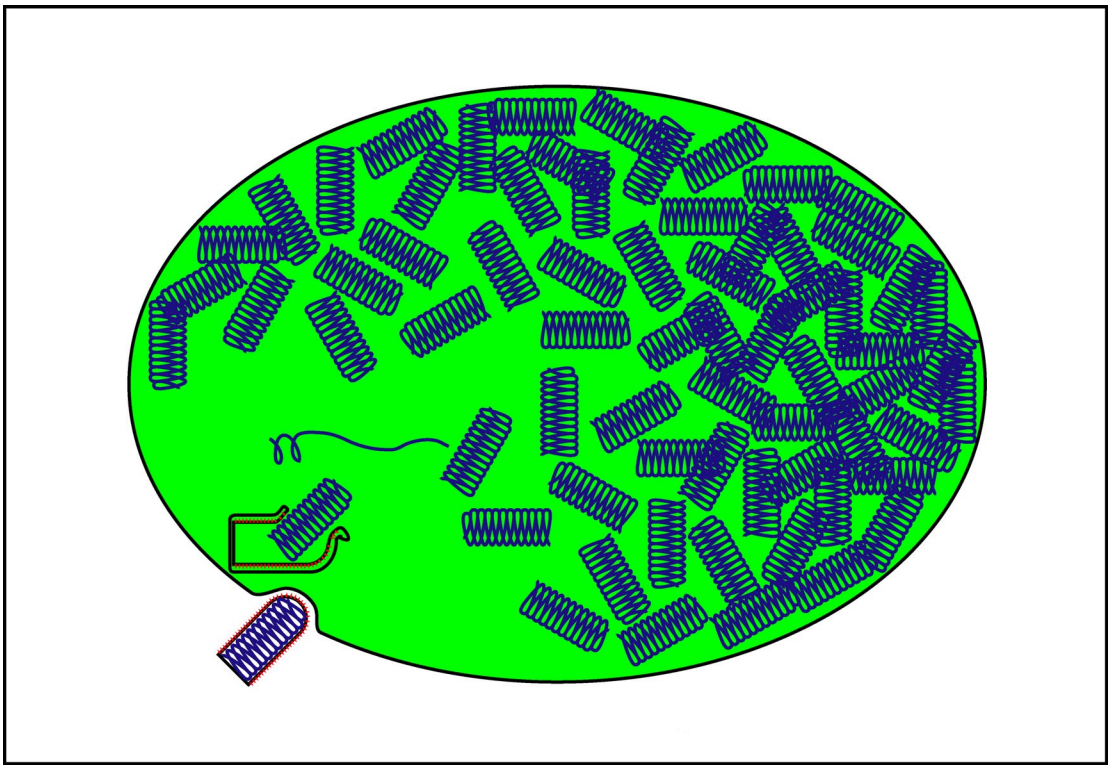

Fig. 2. The glycoprotein-deleted virus should replicate its core to high copy number within initially infected cells and strongly express the transgene, EGFP, following penetration by an arbitrarily small number of viral particles. These newly synthesized viral cores will be unable to spread beyond the initially infected cells.

An example of results from stereotaxic injection of SADΔG-EGFP into mouse brains is shown in figure 3. These images are of intrinsic EGFP fluorescence in unstained tissue, 6 days following injection in the thalamus. At the injection site (inset), neurons and glia are both illuminated with large amounts of EGFP. Large numbers of neurons in regions of the brain that project to the thalamic injection site are labeled with similarly brilliant fluorescence. Numerous pyramidal cells in the deep layers of ipsilateral cortex were retrogradely labeled.

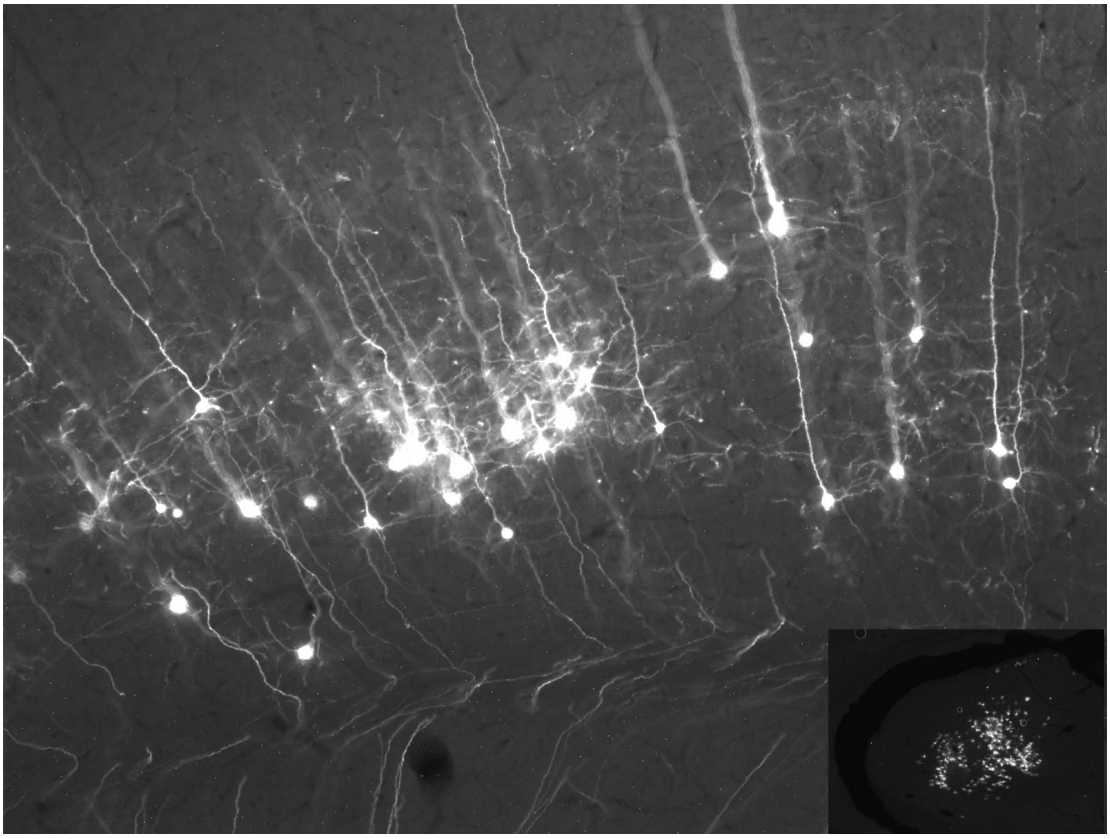

Fig. 3. An example of retrograde labeling with SADΔG-EGFP. This is a widefield microscopic image at 20X of intrinsic EGFP fluorescence in unstained tissue. Top: deep layer cortical pyramidal cells infected following injection in thalamus (20X). Inset: injection site (10X).

Of cortical cells, only layer 5 and layer 6 pyramidal cells, which are both well established as providing the sole cortical projection to thalamus, are infected, expressing EGFP at levels high enough to easily resolve the fine processes of both dendrites and axons as seen in the confocal images of unstained tissue in figures 4 and 5.

Infected cells invariably display uniform fluorescence filling of all of their processes; label is visible at 2 days postinjection with immunostaining for EGFP but steadily increases in brightness thereafter. The confocal images shown in figures 4 and 5 are of intrinsic EGFP fluorescence, with no amplification, at 6 days postinjection. Labeled cells are also found in myriad other brain regions that project to the thalamus. Similar results are obtained from injections in striatum, superior colliculus, and various cortical areas (data not shown).

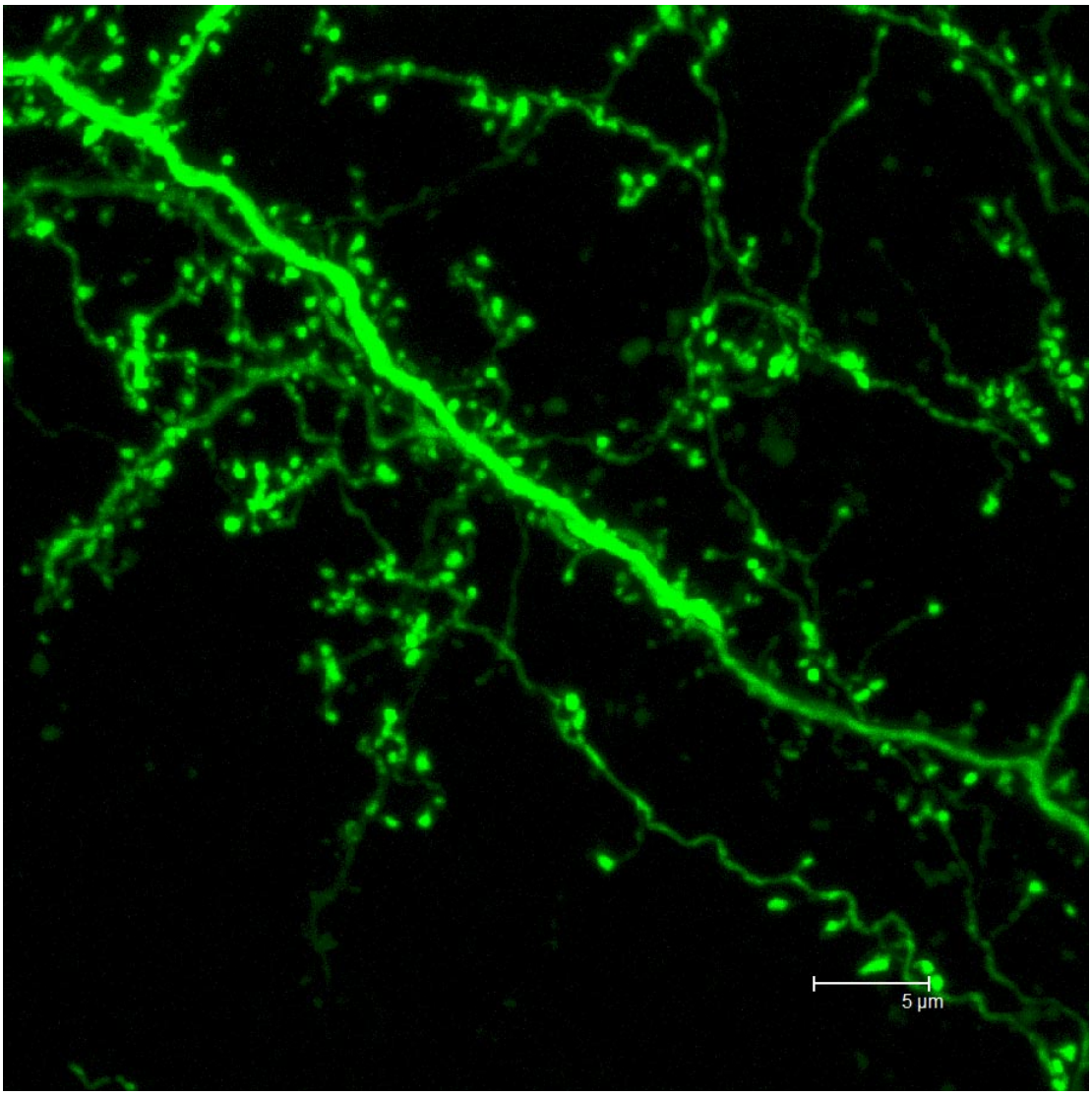

Fig. 4. Confocal image of apical dendrites and spines of cortical pyramidal cells retrogradely labeled with SADΔG-EGFP. Intrinsic fluorescence; no amplification.

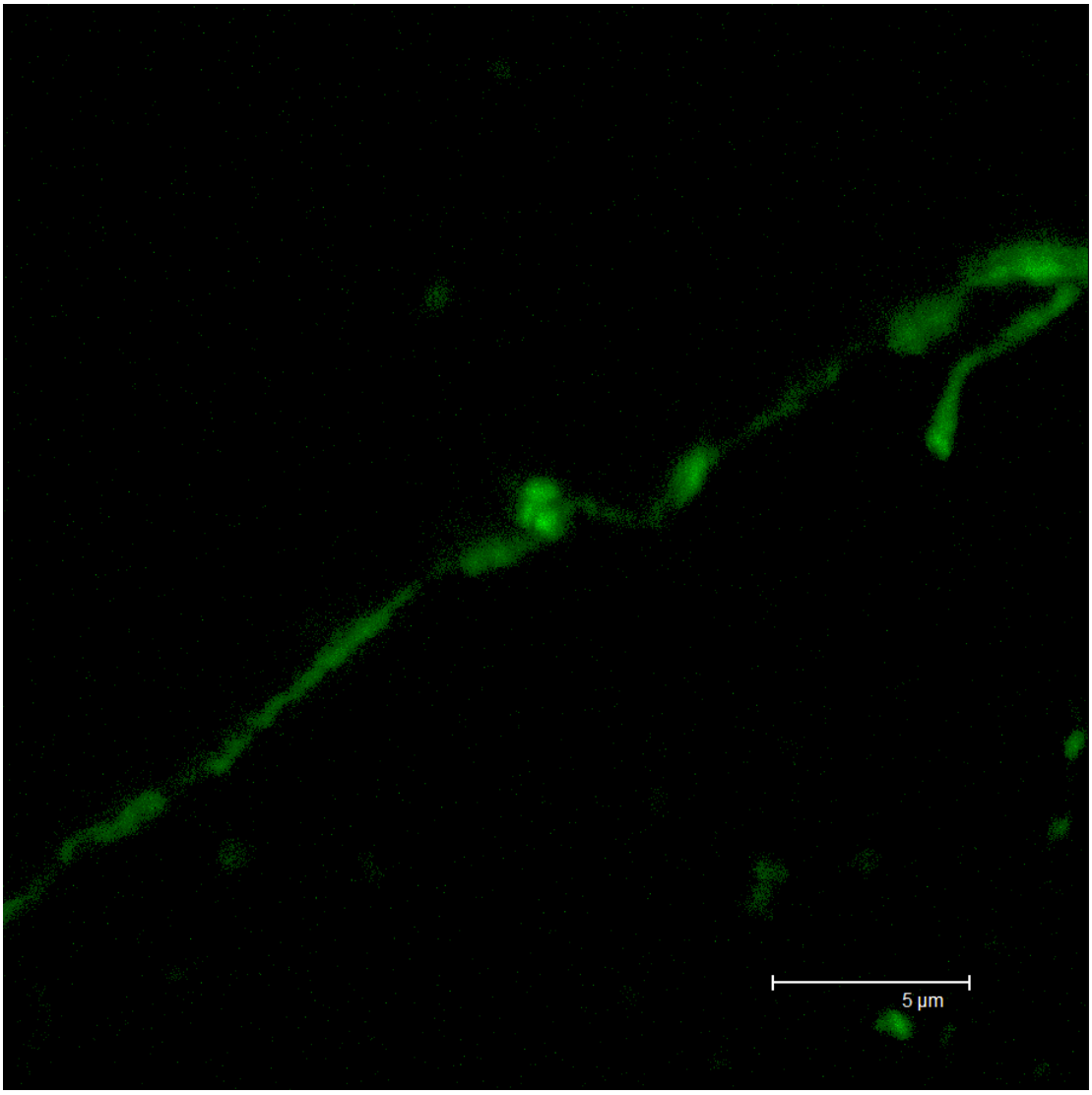

Fig. 5. Confocal image of an axon and associated en-passant synaptic boutons of cortical pyramidal cell retrogradely labeled with SADΔG-EGFP. Intrinsic fluorescence; no amplification.

To determine the effect of the recombinant rabies virus on the membrane properties of infected neurons, we performed patch-clamp recordings on fluorescent cortical cells in brain slices prepared from rats injected with SADΔG-EGFP into thalamus 5-12 days previously (figure 6).

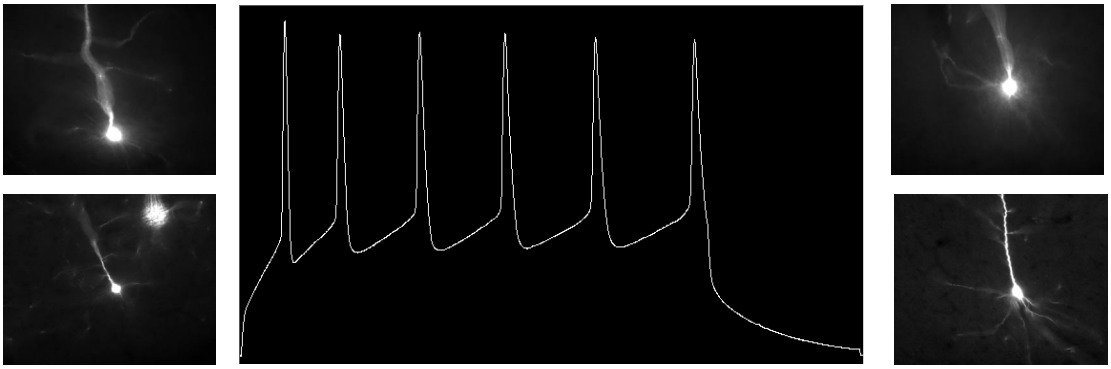

Fig. 6. Bright intrinsic fluorescence allows targeting of infected neurons for physiological recording. Deep layer pyramidal cells (side panels) retrogradely infected from virus injected into the thalamus. Middle panel: whole cell recording of one such fluorescent cell.

We found no difference in gross electrophysiological properties of infected cells versus those of nearby nonfluorescent cells. The resting membrane potential of infected cells was -49.6±8.0 mV (mean ± SD; n=14) while that of noninfected cells (n=9) was -50.7±6.0 mV; action potential thresholds were -36.4±9.2 mV and -39.3±10.1 for the infected and control cells respectively. These differences were not significant ($p = 0.73$ and 0.48, respectively; two-tailed Student's t-tests), indicating that gross membrane properties of infected cells are preserved within the postinfection window examined.

To estimate the time course of survival of neurons infected with the recombinant rabies virus, we injected thalami of 18 mice with equal volumes each of virus solution and sacrificed them at 2 day intervals postinjection to count fluorescent cells in overlying cortex.

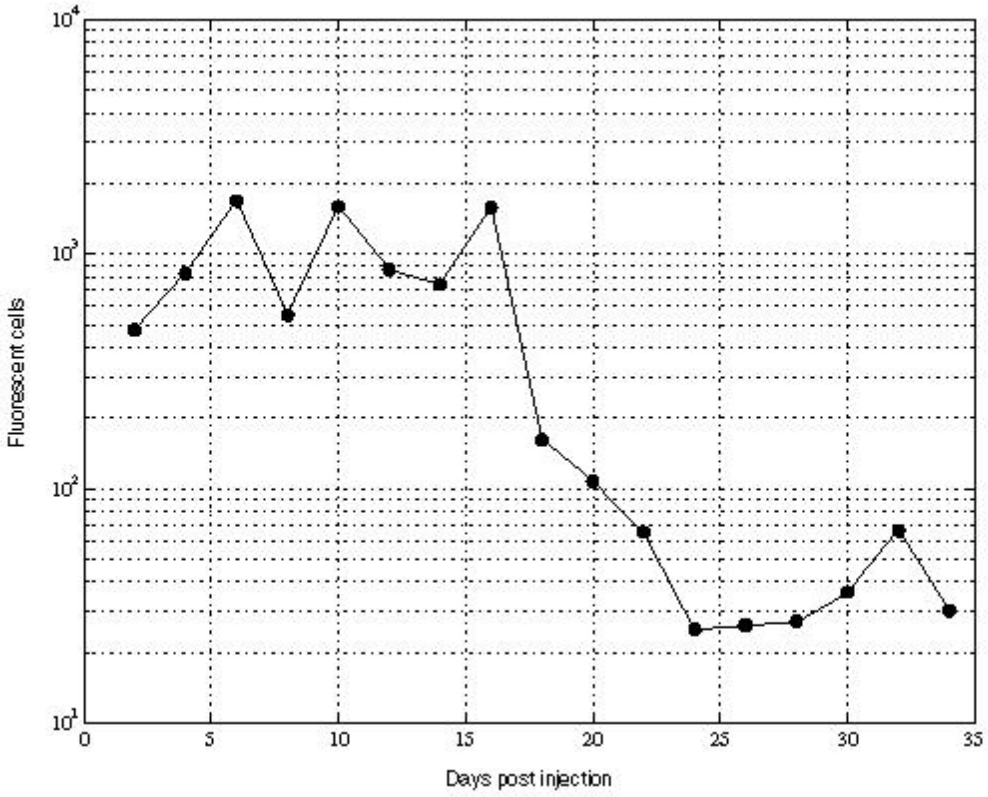

Fig. 7. Survival time course of neurons infected with SADΔG-EGFP.

As seen in figure 7, the number of extant fluorescent cells remains roughly constant out to 16 days, then drops significantly to a much lower but persistent number thereafter. This dropoff was accompanied by morphological changes in many surviving neurons, such as blebbing of processes and the appearance of "bifurcating" somata, indicative of cytotoxicity.

One mouse was found dead after 8 days' survival; although exact counts could not be obtained for this individual, fluorescent cells were still found, in cortex, only in deep pyramidal cells, and elsewhere in the brain did not appear significantly more widespread than in the other animals in the cohort. This mouse therefore evidently did not die from spread of the virus but from some unknown cause; in our hands, injections of other non-toxic vectors into mouse thalamus also result in occasional mortality, apparently due to complications from the injection process itself.

Other viruses besides rabies virus are also known to infect retrogradely, among them adenovirus, alpha-herpesviruses and lentiviral vectors pseudotyped with envelope glycoproteins of various strains of rabies virus[13, 15, 30-33]. In informal comparison with either adenovirus or pseudorabies virus encoding EGFP driven by the synapsin and human cytomegalovirus (HCMV) promoters, respectively, both the number of retrogradely infected cells and the intensity of fluorescence were dramatically higher with the modified rabies virus (data not shown). We did not further pursue comparison with these two other viruses.

However, for direct quantitative comparison with the performance of our recombinant rabies virus, we constructed an HIV-1 lentiviral vector encoding

EGFP under the control of the HCMV promoter. This virus was pseudotyped with the envelope glycoprotein from the same strain of rabies virus (SAD B19) used to derive our recombinant version, so that both the lentivirus and rabies virus contained the same envelope glycoprotein in their membranes. We equalized the titers of the two viruses and injected equal volumes of virus solution into the thalami of 4 mice each, then examined the brains 6 days later.

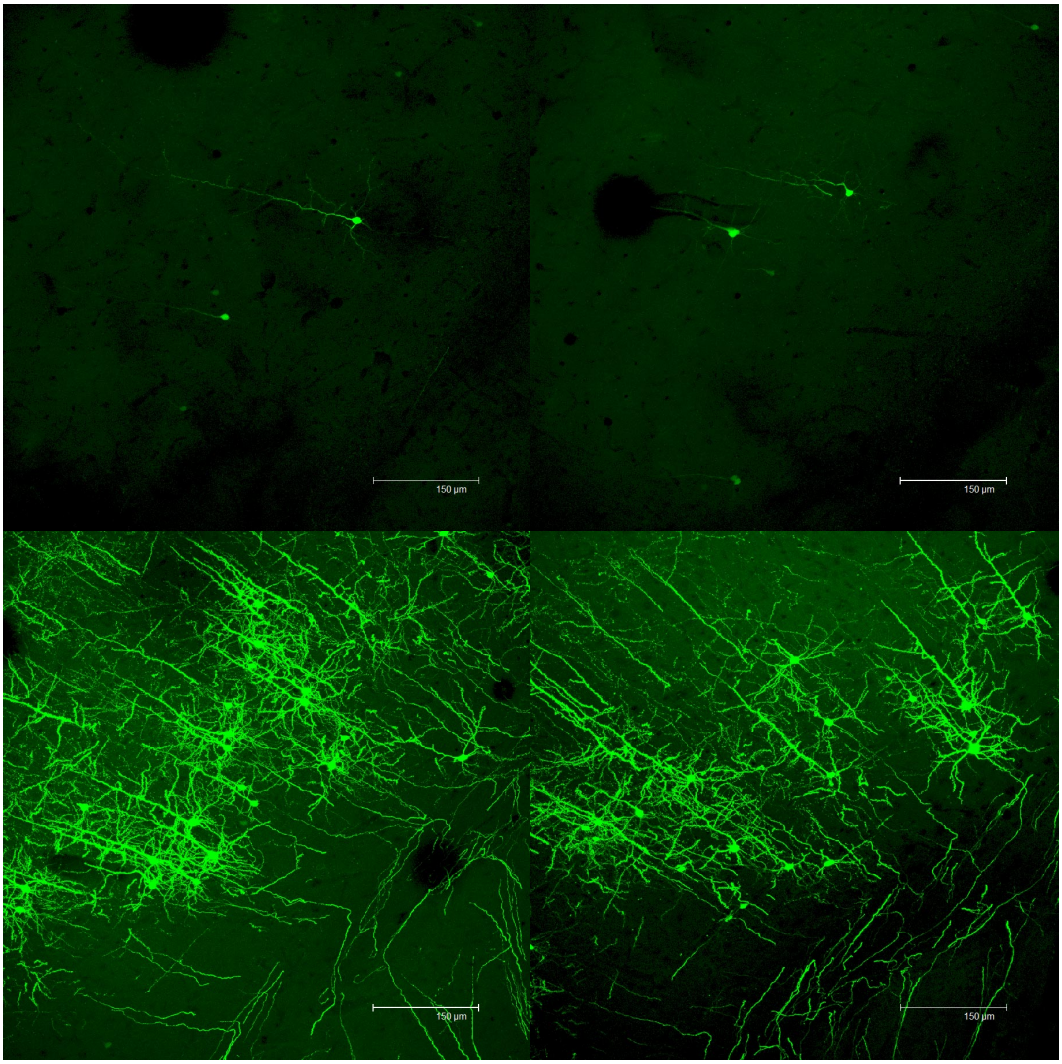

Fig. 8. Comparison of SADΔG-EGFP with lentivirus expressing EGFP under the cytomegalovirus promoter, pseudotyped with the rabies glycoprotein. Titers were equalized and equal volumes were injected. These are confocal images, with identical instrument settings, of unstained tissue. Top: Lentivirus. Bottom: Rabies virus.

As shown in figure 8, cells retrogradely infected with the lentivirus are markedly less impressive, by eye, than those backlabeled with the rabies virus. The four images in the figure were taken back-to-back with identical exposure parameters. The lentivirus-infected neurons also exhibit considerable heterogeneity in fluorescence intensity, with some cells quite bright but most extremely faint even following signal amplification by immunostaining for EGFP. Without antibody staining, in fact, very few cells were visibly infected with the lentivirus at all – those shown in the top panels of figure 8 were the brightest available - whereas native fluorescence from the rabies virus, as seen in the bottom panels - was invariably brilliant and unmistakable.

We counted labelled cells in cortex ipsilateral to the injection site, following amplification by immunostaining, in every sixth section (see Methods). For both viruses these were pyramidal cells in the deep layers of cortex, without exception. In the brains injected with rabies virus, there were 210.5±35.4 detectable cells (mean ± SD); in the lentiviral case, there were 107.3±33.4. The lentiviral number is doubtless an underestimate, because many of the cells were so faintly labeled as to be barely discernable from background. The numbers of retrogradely infected cells, therefore, are similar for the two viruses.

The difference in fluorescence intensity, however, was dramatic. To quantify this, we used a confocal microscope to image unstained fluorescent cells from both sets of injections under identical illumination and exposure parameters. Figure 8 shows the confocal images of the unstained tissue used

for the analysis. Individual somata of retrogradely infected cells were manually identified and the maximum fluorescence intensity was taken within each of these somata.

This comparison was heavily handicapped in favor of the lentivirus, for two reasons. First, there was no set of parameters that was sufficient to effectively image the cells infected with lentivirus that did not cause saturation of the photomultiplier tube from most of the much brighter cells infected with rabies virus. This artificially lowered the measured intensity of the rabies virus-mediated fluorescence as compared to that caused by the lentivirus. Second, since far fewer lentivirus-infected cells were visible by intrinsic fluorescence than became apparent in antibody-amplified alternate sections from the same animals, only the brightest of these cells were able to be used for the quantification, artificially raising the mean intensity of the lentiviral-mediated fluorescence.

Despite this bias, the mean fluorescence intensity for the lentivirus-infected cells (n=18) was 499.2 +/- 556.4 (arbitrary units; see Methods). The corresponding value for the rabies virus-infected cells (n=23) was 3938.6 +/- 528.1 ($p < 5 \times 10^{-21}$; two-tailed Student's t-test). In other words, notwithstanding the facts that the lentiviral mean was artificially raised by selection of the few visibly bright cells and that the rabies viral mean was artificially lowered by instrument saturation, the fluorescence from the rabies virus was calculated to be eight times brighter.

DISCUSSION

We propose that the recombinant rabies virus presented here is the best retrograde tracer for revealing detailed neuronal morphology that has been available to neuroscientists to date. Because rabies virus specializes in infection via axon terminals and long-range intracellular transport, our recombinant version is extremely efficient at retrograde labeling. Because the genomic deletion of this mutant is of a gene essential for cell-to-cell spread but irrelevant to transcription and genome replication, infection occurs in an all-or-nothing fashion resulting in cells labeled brilliantly and in great detail if they are labeled at all. These results are consistent with the ability of a single rabies particle to amplify sufficiently for maximal EGFP expression. Because of the much slower and less severe cytopathic effect of rabies virus compared to the herpesviruses and adenovirus, this mutant nevertheless affords potentially several weeks of anatomical or physiological study of infected cells.

Of course, for very long-term study of neurons with minimal impact on their health, lentivectors are the more appropriate choice. Intriguingly, the fact that the numbers of retrogradely infected cells were similar for the rabies virus and for the titer-equalized lentivirus pseudotyped with the rabies virus glycoprotein may indicate that the glycoprotein is the sole determinant of rabies virus' ability to retrogradely infect cells once in contact with their axons. This probably also means that any enveloped virus that can be pseudotyped with the

rabies virus glycoprotein would be as effective as any other at retrogradely infecting cells in terms simply of numbers of transduced neurons.

However, our results suggest that for shorter-term studies where number of visibly labeled cells and intensity of label are at all important, there is no comparison whatsoever to the recombinant rabies virus, infection with which causes every cell to be illuminated in great detail, a very far cry from the effect of the lentivirus.

The mutant described here, moreover, may be viewed as a first-generation version that could potentially be improved upon by deleting a second gene, the coding sequence for the matrix protein (M) from its genome. This would not significantly affect transgene expression[34] but is likely to significantly reduce cytotoxicity due to the matrix protein's role in shutting down nuclear pore transport [34-38]. In addition, the level of EGFP expression could be considerably increased, if any increase over the already extremely high level were desired, simply by changing the position of the EGFP gene within the viral genome: because rhabdoviral genes are transcribed serially in quantities decreasing monotonically from the 3' end (see Fig 1), placing a transgene farther upstream relative to the other genes results in its increased expression[27]. Also, of course, the system could be used for retrograde expression of any other gene besides the coding sequence for EGFP, suggesting a variety of physiological studies using fluorescent reporters of activity or photosensitive proteins for optical perturbation of membrane potential of cells targeted on the basis of their axonal projection targets.

METHODS

Production and purification of rabies virus

The pSADΔG-EGFP cDNA plasmid was constructed by replacement of the G-gene of pSAD L16 [4] with the EGFP reporter gene (Clontech), resulting in the genome organization 3'-N-P-M-EGFP-L-5'. A detailed description of all cloning steps and the final sequence is available from the authors by email.

The cDNA construct pSADΔG-EGFP was rescued into functional RNPs in cells expressing RV N, P, and L proteins from transfected plasmids as described previously [39]. After 3 days of incubation, the cells were repeatedly transfected with G-encoding pTIT-G plasmid (5µg cDNA/10^6 cells) until spread of virus was detectable by GFP expression. The virus-containing cells were trypsinized and were mixed with a hygromycin-resistant BSR cell line expressing RV G and M proteins after induction with doxycyclin (clone MG139). Two days after cell mixing, BSR T7/5 cells were eliminated by adding hygromycin at 1 mg/ml. Expression of RV G in MG139 cells was repeatedly induced until approximately 50% of the cell culture was infected with SADΔG-EGFP, as determined by EGFP fluorescence. Cell culture supernatants were transferred on fresh MG-on cells for SADΔG-EGFP stock production.

Virus stocks were clarified from cell debris by low speed centrifugation, and the viruses were purified by centrifugation in an SW 28 rotor (27,000 rpm, 2 hrs, 4°C) through 20% sucrose onto a 60% sucrose cushion prepared in TEN

buffer (10 mM Tris {pH 7.4}, 50 mM NaCl, 1 mM EDTA). The purified viruses were collected from the top of the 60% sucrose layer.

Production and purification of lentivirus

HEK-293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Essential Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone), 2% penicillin/streptomycin (Invitrogen) and 1% Fungizone (Invitrogen) in a 5% CO_2 incubator at 37^{o}C.

A third-generation, four-plasmid HIV-1 lentivector production system was used, with plasmids generously provided by Dr. Inder Verma (Salk Institute). Cells were transfected in 10 cm plates at 95% confluency using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the following DNA quantities: the fourth generation transfer vector p156RRLsinPPTCMV-GFP WPRE, 10 µg per plate; the rev-deleted packaging vector pMDL.gp.RRE.Oded, 6.5 µg per plate; the supplementary rev plasmid pRSV-Rev, 2.5 µg per plate; and the SAD B19 glycoprotein expression vector pHCMV-RabiesG[40], 3.5 µg per plate. Medium was changed to OptiMEM (Invitrogen) immediately prior to transfection and back to complete medium 4-6 hours afterwards.

Supernatants were collected 48 hours following transfection and centrifugated in an SW28 rotor (19400 RPM, 2hrs, 4°C). Pellets from 6 buckets were resuspended in a total of 3.5 ml PBS, then layered on top of 20% sucrose and spun in an SW55 rotor at 21000 RPM for 2 hrs at 4°C. The final pellet was resuspended in 100 µl PBS.

Determination of virus titers and fluorescence analysis

Virus titers were determined by serial dilution and overnight infection of HEK-293T cells followed by fluorescence activated cell sorting on a FACscan (BD Biosciences, San Jose, CA) three days later. Data were subsequently analyzed with CellQuest software (BD Biosciences) to determine the fraction of fluorescent cells and the titer taken as $-\ln(1-p)*N_o/v$, where p is the fraction of fluorescent cells, N_o is the number of cells at infection, and v is the volume of applied virus. Both viruses were titered simultaneously in equivalent wells, the average of triplicate conditions for each virus was taken as its titer, and the ratio of these titers was used as the dilution factor to equalize the two stocks' titers for stereotaxic injection.

Stereotaxic injections and perfusions

Borosilicate glass capillaries (Sutter, Novato, CA) of 0.5 mm inner diameter were pulled to a fine taper on a P-97 micropipette puller (Sutter) and snipped under a dissecting microscope to a tip inner diameter of 30-50 um. Mice (C57B/6 from Harlan, Indianapolis, IN) were anesthetized with a mixture of ketamine (100 mg/kg IM) and xylazine (10 mg/kg IM); for rats (Long-Evans, also from Harlan), a mixture of ketamine (100 mg/kg IM), xylazine (5 mg/kg IM), and acepromazine (1 mg/kg IM) was used. Animals were mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA). Virus was loaded into pulled pipettes and injected using a Picospritzer III (Parker Hannifin/General Valve Corporation,

Fairfield, NJ) at approximately 20 nl/min. For targeting injections to thalamus, stereotaxic coordinates, in millimeters relative to bregma, were as follows. For slice physiology: -3.6 AP, +2.4 AP, -5.8 DV (rat). For neuronal survival time course study: -1.82 AP, +1.25 LM, -3.5 DV. For comparison of rabies virus and lentivirus (cell counts and fluorescence quantification): -1.82 AP, -1.167 LM, -3.783 DV with an approach angle of 45^{o} in the coronal plane to avoid traversing ipsilateral cortex. The pipette was left in place for 5 minutes following cessation of injection, then withdrawn; the scalp was subsequently closed with wound clips and the animals housed singly thereafter. All animal work was performed in accordance with NIH guidelines and approved by the Salk Institute's Institutional Animal Care and Use Committee.

At 2-34 days postinjection, for the anatomical studies, animals were deeply anesthetized with inhaled 4% isoflurane and perfused transcardially with 4% paraformaldehyde in PBS. Brains were postfixed and cryopreserved overnight in 4% paraformaldehyde/30% sucrose in PBS, then kept in 30% sucrose in PBS until sectioning.

Sectioning, histochemistry and microscopy

Brains were sectioned on a freezing microtome (Microm, Heidelberg, Germany) at 50-80 um, then stored in cryoprotectant solution (30% ethylene glycol/30% glycerol/40% PBS) at -20°C until further treatment.

Sections to be immunostained were washed at room temperature with 30-45' of 5-10' washes of 0.5% Triton-X in PBS, then incubated for 2 hours at room

temperature in blocker consisting of, in PBS, 2% bovine serum albumin (SeraCare, Oceanside, CA), 0.5% Triton-X, 0.45% gelatin (Sigma), and 10% normal donkey or goat serum, depending on the secondary antibody to be used. Sections were incubated overnight at 4°C with a chicken anti-GFP polyclonal (Aves) at 1/500 dilution in blocker, followed by 30' at room temperature the next morning. 45' of washes in 0.5% Triton were then followed by 2 hours incubation at room temperature in secondary solution consisting either of Cy2-conjugated donkey anti-chicken polyclonal (Jackson ImmunoResearch) or AlexaFluor 555-conjugated goat anti-chicken (Invitrogen) at 1/100 dilution in blocker. Following 15'-20' of subsequent washes in PBS, sections were stained for 15' with 10 uM DAPI (Sigma-Aldrich) in PBS, then rinsed with a further 30' of PBS washes before being mounted and coverslipped with Krystalon (Harleco).

Microscopy & fluorescence quantification

Fluorescence images were taken with either a standard widefield (Olympus) or confocal (Zeiss) microscope. To quantify fluorescence intensities, lentivirus- and rabies virus-infected samples were imaged on the confocal with identical instrument settings. Maximal 2-d projections were computed. Figure 8 depicts the fields analyzed; the actual images used for analysis were taken at lower gain to decrease saturation of the rabies virus case while maintaining visibility of lentiviral-infected cells. Using Slidebook analysis software (Intelligent Imaging Innovations, Inc., Denver, CO). cells were identified visually and their somata marked as regions of interest. The maximal intensity pixels within these

were taken as the dependent measure and the means and standard deviations of intensity, in arbitrary units, were calculated from these.

Physiological recordings

Vibratome-cut 400 μm thick brain slices were prepared from rats injected with SADΔG-EGFP 5-12 days previously at postnatal day 23. Slices were cut in ice-cold artificial cerebrospinal fluid (ACSF) consisting of 124 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1 mM H_2PO_4, 26 mM $NaHCO_3$, and 11 mM dextrose and maintained afterward submerged in room temperature ACSF aerated with 95% oxygen/5% CO_2 for at least one hour, then transferred to a recording chamber, continually perfused with aerated room temperature ACSF, mounted on an infrared DIC microscope (Olympus). Glass recording electrodes (8 -12 MΩ resistance) were filled with intracellular solution (140 mM potassium gluconate, 8 mM NaCl, 10 mM HEPES, 1.3 mM EGTA, 2 mM ATP, and 0.3 mM GTP, adjusted to pH 7.7 with KOH).

CHAPTER I ACKNOWLEDGEMENTS

The text of chapter I, in full, consists of material that is in preparation for submission for publication. The dissertation author was the primary researcher and author, and the following co-authors contributed to the research which forms the basis for this chapter: Stefan Finke, Karl-Klaus Conzelmann, and Edward M. Callaway.

III. MONOSYNAPTIC RESTRICTION OF TRANSSYNAPTIC TRACING BY *IN SITU* COMPLEMENTATION OF A DELETION MUTANT NEUROTROPIC VIRUS

ABSTRACT

We have developed a transsynaptic tracer that crosses one synaptic step and then stops, allowing unambiguous identification of monosynaptically connected populations of neurons within a circuit. The essential principle is to use a tracing virus with one or more genes that are required for transsynaptic spread deleted from its genome, with the missing viral gene(s) supplied in *trans* into this and only this population. We have implemented this idea using rabies virus with its envelope glycoprotein gene deleted. The virus was pseudotyped with the envelope glycoprotein of an avian retrovirus, subgroup A avian sarcoma and leukosis virus (ASLV-A), allowing initial infection to be targeted specifically to cells that express the alien viral receptor. Here we present *in vitro* results from

cultured brain slices, with paired patch recordings confirming that viral spread is overwhelmingly if not entirely restricted to synaptically connected neurons. This method therefore allows identification of cells presynaptic to a single neuron.

INTRODUCTION

Determining the connection patterns of the thousands of identifiable neuronal cell types is one of the most basic outstanding problems of neuroscience. Our ignorance as to which cell types connect to which, and, at a finer level of resolution, how an individual cell of each cell type is connected to the rest of the network, is a massive stumbling block to understanding the neural basis of cognition and behavior. Our existing knowledge of these interconnections is precious and hard-won and is based on many techniques, such as staining tissue and examining overlap of axons and dendrites[41, 42], simultaneous patch recording from pairs of cells[43], photostimulation-based mapping of connections in brain slices[44-46], electron microscopy[47, 48] as well as many others. Although these techniques have been extremely valuable and in many cases provide information that could be gained by no other means, all of them are limited in various ways. There has never been a wholesale way of identifying *en masse* the cadre of cells which are connected either to some other cell group or, especially, to a single cell.

Transsynaptic tracers, out of all the available techniques, might at first pass appear to offer a solution to this problem. By introducing a tracer into a particular cell or cell type, synaptically connected cells should be labeled by the tracer and therefore be identifiable as those in synaptic contact with the starting cells in question. Several approaches to selectively introducing either conventional or viral tracers into particular genetically identified populations have been taken[49-52]. These techniques represent a huge advance and have had great success in identifying cells that are connected, by one or more synaptic steps, to the starting population. The same is true for the more established techniques of simply injecting conventional or viral transsynaptic tracers into particular nuclei or areas of the brain, or into muscles, which have provided much important information about the organization of nervous systems[16-18, 20, 53-56].

No such method has been sensitive enough to label cells that connect to a single starting cell. There is, however, a more fundamental problem that makes them unable to answer the question as to which cells are directly connected to a population of interest. There is no way with any of these techniques, without additional information, to unambiguously identify monosynaptically connected cells. The reason is that all existing tracers, without exception, are afflicted by a fundamental flaw - or feature - that makes them in general incapable of determining exactly how many synaptic steps have been crossed to label any given cell (figure 9).

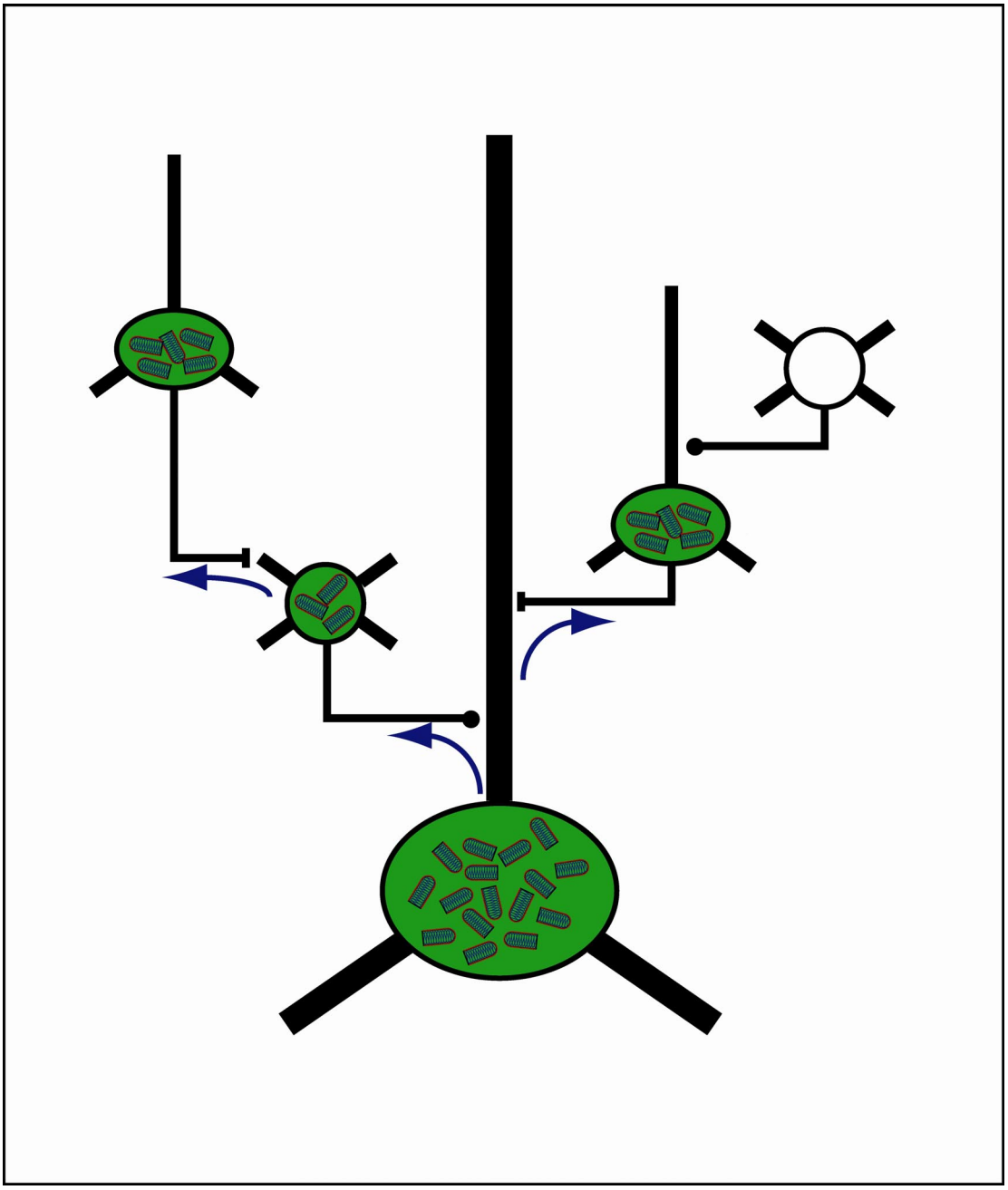

Fig. 9. The problem with transsynaptic tracers. 1) They don't stop after crossing to connected cells, and 2) They cross different synapses at different rates. As a result, they are incapable of distinguishing weak direct connections from strong indirect ones.

This feature has two components:

1) Transsynaptic tracers don't stop after crossing a synapse, but continue spreading beyond transsynaptically labeled cells to label cells that are connected to them in turn.

This of course can be beneficial, since higher-order connections can be determined, but, for identifying *monosynaptically* connected cells, it becomes a huge problem, because of the second component, namely that

2) Transsynaptic tracers cross different synapses at different rates, with bigger, stronger, denser connections being crossed faster than smaller, weaker, sparser ones.

All transsynaptic tracers, conventional and viral alike, share this feature, and the reason is simply that, since they universally depend on cellular machinery for transport to and across synapses [6], the more hardware servicing a given connection, the more efficiently it will be traversed by the tracer.

The effect of these two components taken together is the following. A tracer starting off in a given population spreads initially to the cells that are connected most strongly to it. The tracer that accrues in these transsynaptically labeled cells will then begin spreading to the cells that are connected to them, and in fact can label the most strongly connected of these *even before* weakly connected synaptic partners of the starting population. This has been found to be true for the alpha-herpesviruses as well as for rabies virus[20] [17].

The result is an inescapable ambiguity in the number of synapses crossed, namely that

➡ *Transsynaptic tracers are incapable of distinguishing strong indirect connections from weak direct ones*

and therefore cannot be used in their current form to determine monosynaptic connections in any kind of comprehensive way.

Instead, what is needed is a tracer that crosses one synaptic step, to cells directly connected to the starting population, and then stops, unable to spread beyond them to secondarily connected cells. Monosynaptically connected cells would therefore be labeled unambiguously.

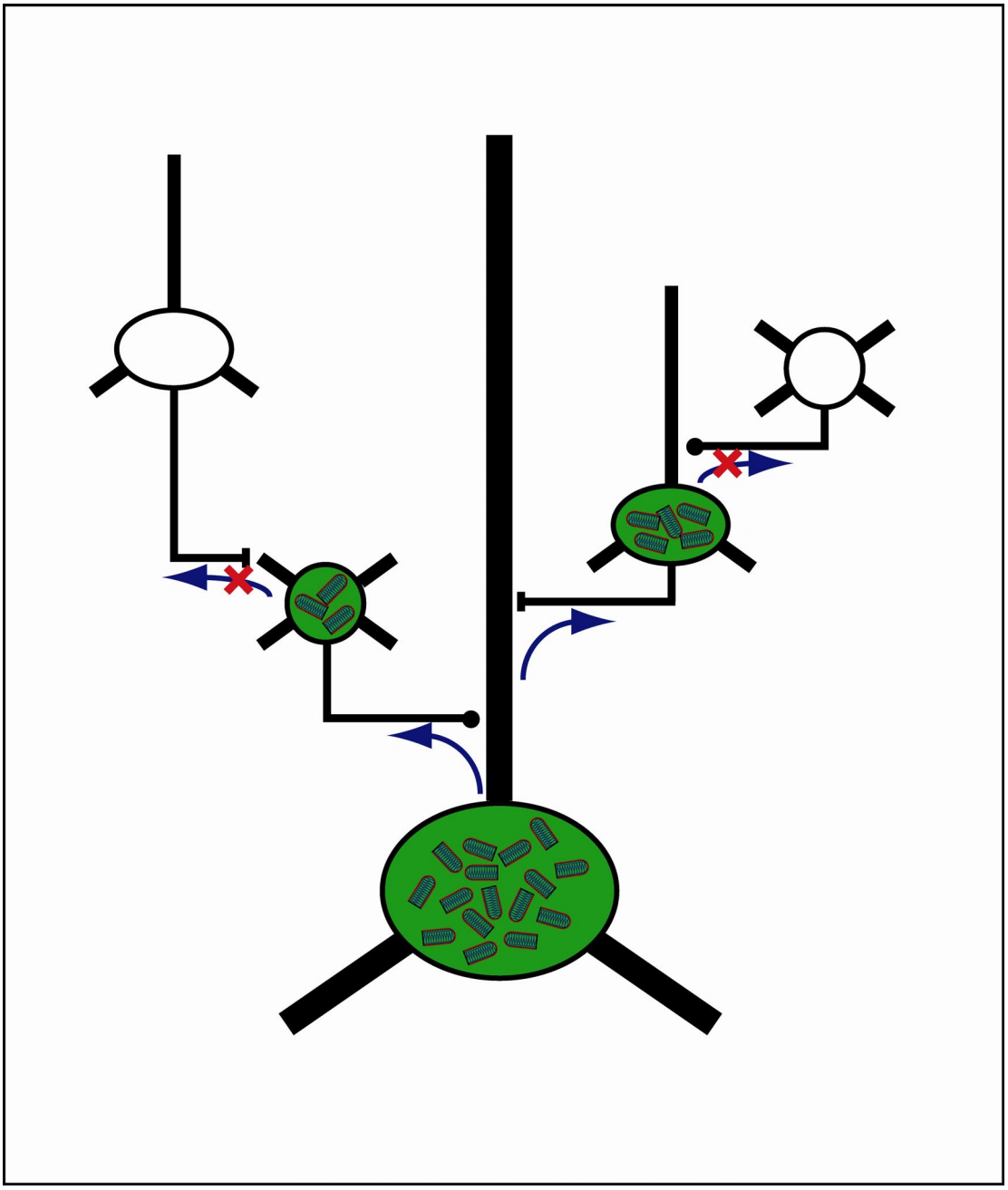

Fig. 10. What is needed is a tracer that crosses one synapse and stops, so that all labeled cells are monosynaptically connected to the starting cell or cell type of interest.

RESULTS

Core concept: *in situ* complementation

Figure 11 illustrates the core idea of the system.

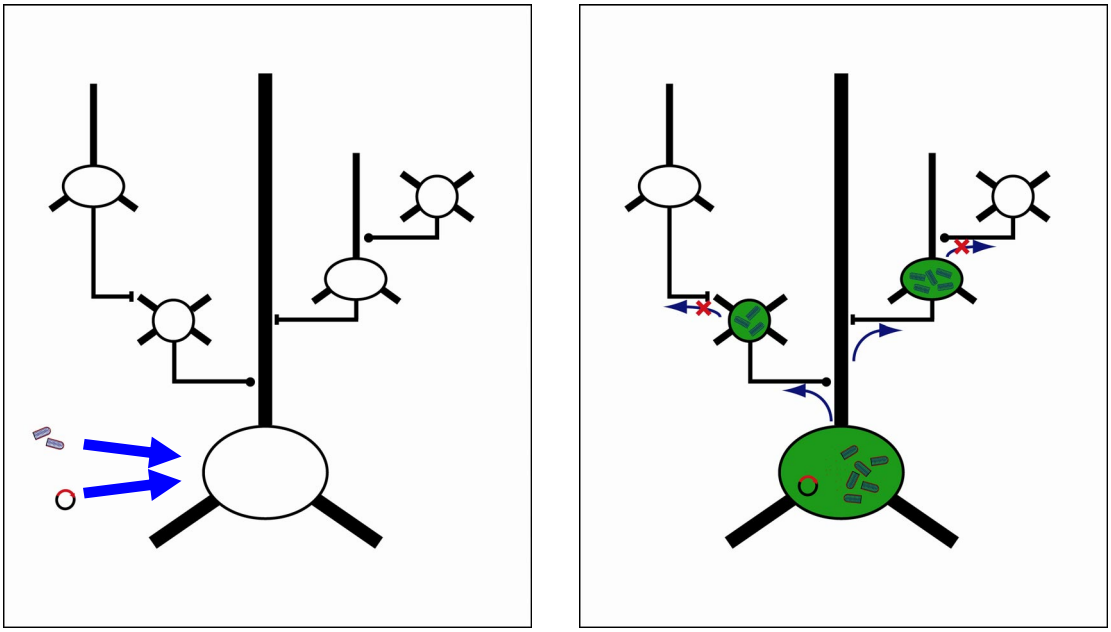

Fig. 11. Transsynaptic tracing by *in situ* complementation: core concept. Left: Into cell or cell type of interest, introduce 1) a deletion mutant virus missing one or more genes required for transsynaptic spread and 2) the missing viral gene(s). Both the initial infection and the complementing viral genes must be restricted to the neuronal population of interest. Right: In the initially infected cells ("first-order cells"), all viral genes are present due to the complementation of the deleted genes. The virus can therefore spread transsynaptically to cells in synaptic contact with the starting cells. Since the missing viral genes are not present in these cells, the virus cannot spread beyond them. The result is a virus that crosses only one synaptic step and stops in monosynaptically connected cells.

It has two components:

1) In the starting cell or cell type of interest, initiate an infection by a tracing virus that is missing one or more genes that are required for transsynaptic spread.

These could be genes needed for replicating the viral genome, packaging the virus, budding from a cell membrane or subsequent penetration of the next one, or in fact virtually any step in replication and cell-to-cell spread of the virus.

2) In the same starting cell or cells, provide the missing viral genes in trans.

This could be by any convenient means, the only critical point being that these complementing genes must be restricted to the starting population of interest that is targeted by the initial infection.

With all the viral genes present in the starting cell population, the virus can spread from there to monosynaptically connected cells. Because those genes are not in those secondarily infected cells, however, the virus can't spread beyond them and therefore stops.

The virus and the deleted gene

The system presented here uses rabies virus, a nonsegmented negative-strand RNA virus specialized in infecting mammalian nervous systems[27].

Rabies virus has been used with great success as a transsynaptic tracer, crossing synapses, according to all available evidence, exclusively in the retrograde direction [16-19]. It has two major advantages over the more widely

used family of tracing viruses, the alpha-herpesviruses (such as pseudorabies virus and herpes simplex virus type 1). First, although obviously lethal, infected animals survive for much longer than those infected centrally by herpesviruses, and it is far less cytotoxic, with no widespread cell death even following death from rabies[17]. Second, it is far more efficient at infection: whereas tens of thousands of herpesvirus particles are required to begin an infection following intracerebral injection[20-22], only a single rabies viral particle injected into the brain will lead to a full case of rabies[23, 24]. Together these two advantages make it an ideal candidate for our complementation system, and the second one in particular, with the corollary that a single infected cell by itself is enough to start off an infection, bodes well for tracing connections to a single cell.

The rabies virion is depicted schematically in figure 1. The viral core, consisting of the RNA genome and associated proteins, is surrounded by the host-cell derived phospholipid bilayer envelope or membrane, into which is embedded the trimeric envelope glycoprotein (G). The glycoprotein is responsible for causing the virus to bud out through the host cell membrane, picking up its envelope and glycoprotein as it does so, for binding to receptors on the presynaptic membrane of the next cell to be infected, and, following endocytosis of the bound virus, for release of the viral core into the cytosol to begin a new cycle of infection.

The glycoprotein, therefore, is absolutely required for transsynaptic spread of the virus, as evidenced by *in vitro* and *in vivo* examination of recombinant virus with the glycoprotein gene deleted from its genome [28, 29] (see also chapter I).

However, it is not required for transcription of the viral genes or for replication of the genome [28]. As described in chapter I, a recombinant version with its glycoprotein gene replaced with the coding sequence for enhanced green fluorescent protein (EGFP) produces so much EGFP as to brightly fill even the fine dendritic and axonal details of infected neurons, yet it cannot spread beyond these initially infected cells. Because it leaves the transcription and replication machinery intact so as to permit extremely high-level expression of a transgene, but represents an apparently absolute block to transsynaptic spread, the glycoprotein gene is an ideal candidate for a gene to be deleted and provided *in trans* in our system. With the EGFP gene inserted instead, this self-amplifying virus should be highly effective at vividly illuminating the detailed morphology of infected cells.

Means of selective initiation of infection

The system presented here requires that both the missing viral gene and the initial viral infection be restricted to the postsynaptic cell or cell type of interest. Selective introduction of the complementing gene could be by any means, most obviously by generation of a genetically modified animal. A means of selectively initiating the infection in the same cells, however, is less apparent. Because rabies virus has an RNA genome with no DNA intermediate form at any point in its replication cycle[27], and one that moreover is tightly encapsidated and untouchable by host cell RNA processing enzymes, it is not possible to rely on intracellular modification of the genome by selective expression of a

recombinase (as has been used in another context for the alpha-herpesvirus pseudorabies virus[50, 57]). Universal infection followed by selective recombination, then, cannot work with rabies virus. There is, however, another way of achieving the same goal: selective targeting of the infection itself.

In general, for an enveloped virus whose glycoprotein gene has been deleted from its genome, viral particles can be made that incorporate some other virus' glycoprotein in their envelope instead of their own. Such "pseudotyping", perhaps requiring modification of the foreign glycoprotein so as to permit its effective interaction with the other viral proteins, typically confers the infectious properties of the foreign virus on the one that has been pseudotyped.

With the glycoprotein gene deleted from the viral genome, viral particles can be made that incorporate some other virus' glycoprotein in their envelope, provided that it is suitably modified so as to permit this incorporation. This requires only growing the glycoprotein-deleted virus in cells that express the foreign envelope gene, and confers the infectious properties of the foreign virus on the modified (or "pseudotyped") rabies virus. If the foreign virus in question does not infect neurons, or mammalian cells, then neither will the pseudotyped rabies virus. Such a virus, therefore, when injected into the brain should infect no neurons at all, except any that, by whatever means, have been induced to express the *receptor* for the foreign virus.

Targeting the initial infection to the cells of interest, then, can be achieved by pseudotyping the rabies virus with the glycoprotein from a foreign virus and selective expression of that virus' receptor in the target cells (figure 12).

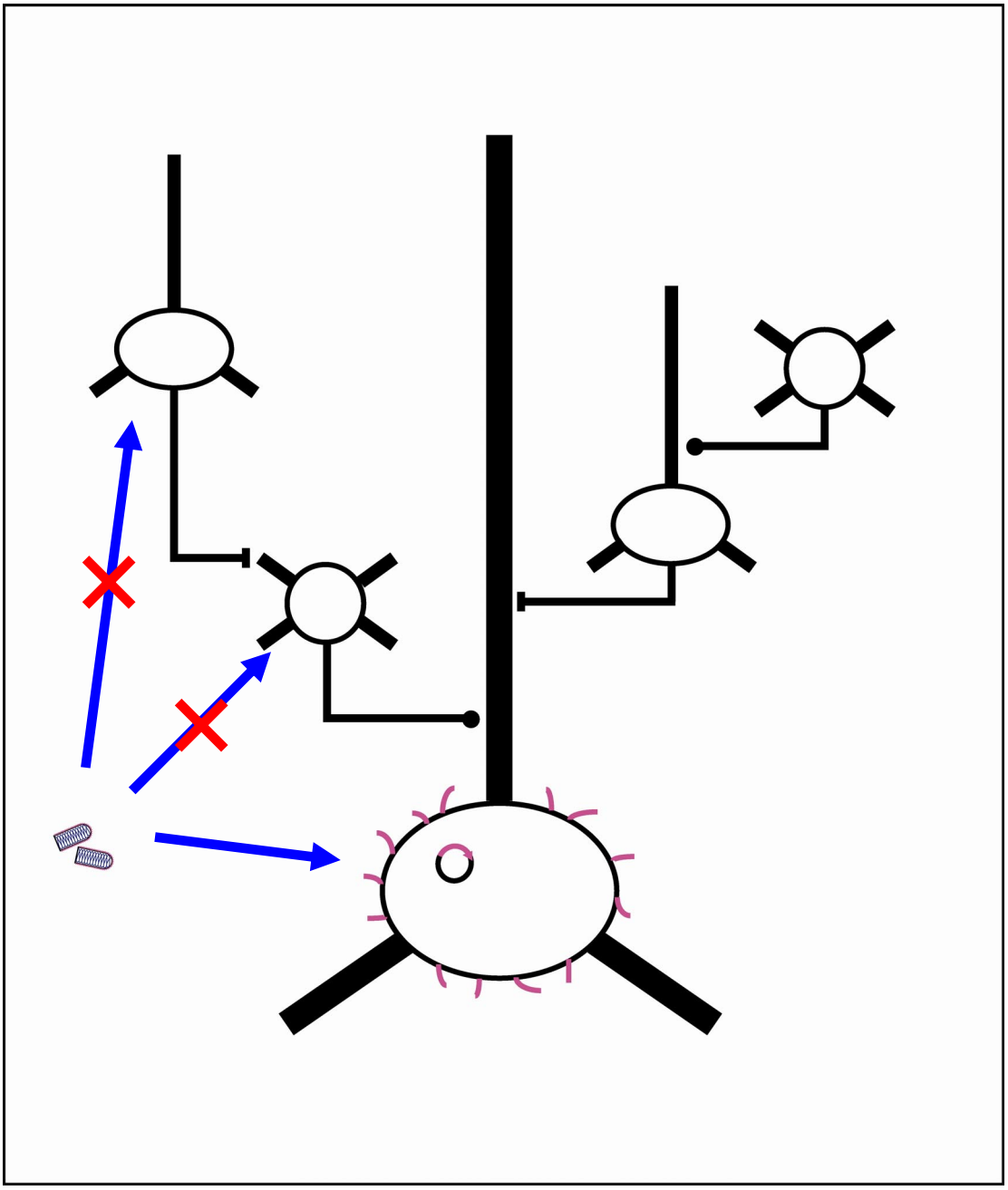

Fig. 12. Targeting infection. ASLV-A-pseudotyped rabies virus can't infect neurons, unless the gene for ASLV-A's receptor has been introduced into them. This causes the receptor to be expressed on the cells' surface, allowing infection by the pseudotyped virus.

We have done this with the envelope protein from a retrovirus endemic to birds, avian sarcoma and leucosis virus subgroup A (ASLV-A), which is highly effective at infecting avian cells but almost completely unable to infect mammalian ones[58]. Because most previous attempts to pseudotype rabies virus have worked only when the native cytoplasmic domain of rabies virus' own glycoprotein is substituted for that of the foreign one[59-61], we pseudotyped our virus with a chimeric protein consisting of the extracellular and transmembrane domains of the ASLV-A glycoprotein and the cytoplasmic domain of the rabies glycoprotein. The resulting virus, like ASLV-A, is ineffective at infecting a variety of cell lines (data not shown) except those that express the receptor for ASLV-A, a low-density lipoprotein (LDL) receptor-related cell surface molecule known as TVA[58, 62, 63].

We therefore constructed an ASLV-A-pseudotyped version of the glycoprotein-deleted, EGFP-encoding rabies virus SADΔG-EGFP described in chapter II. The resulting virus, SADΔG-EGFP(EnvA), should have little or no ability to infect neurons ordinarily, but any that express TVA should be readily infected. The intracellular behavior of this virus will be as seen in chapter II: with the viral core able to replicate, infected cells will fill with high levels of EGFP. If the glycoprotein gene is supplied *in trans* within these cells, the virus should spread to cells presynaptic to them and label them with similarly high levels of EGFP. If the glycoprotein gene is not present in these transsynaptically infected cells, the virus will be unable to spread beyond them. Monosynaptically connected cells will therefore be identified unambiguously.

The basic requirements of the system are depicted in figure 13. Two genes must be selectively introduced into the target cells prior to application of the pseudotyped virus: the TVA gene, so the ASLV-A-pseudotyped virus can enter the cells, and the rabies glycoprotein gene, so the virus can spread from these cells to those that are presynaptic to them.

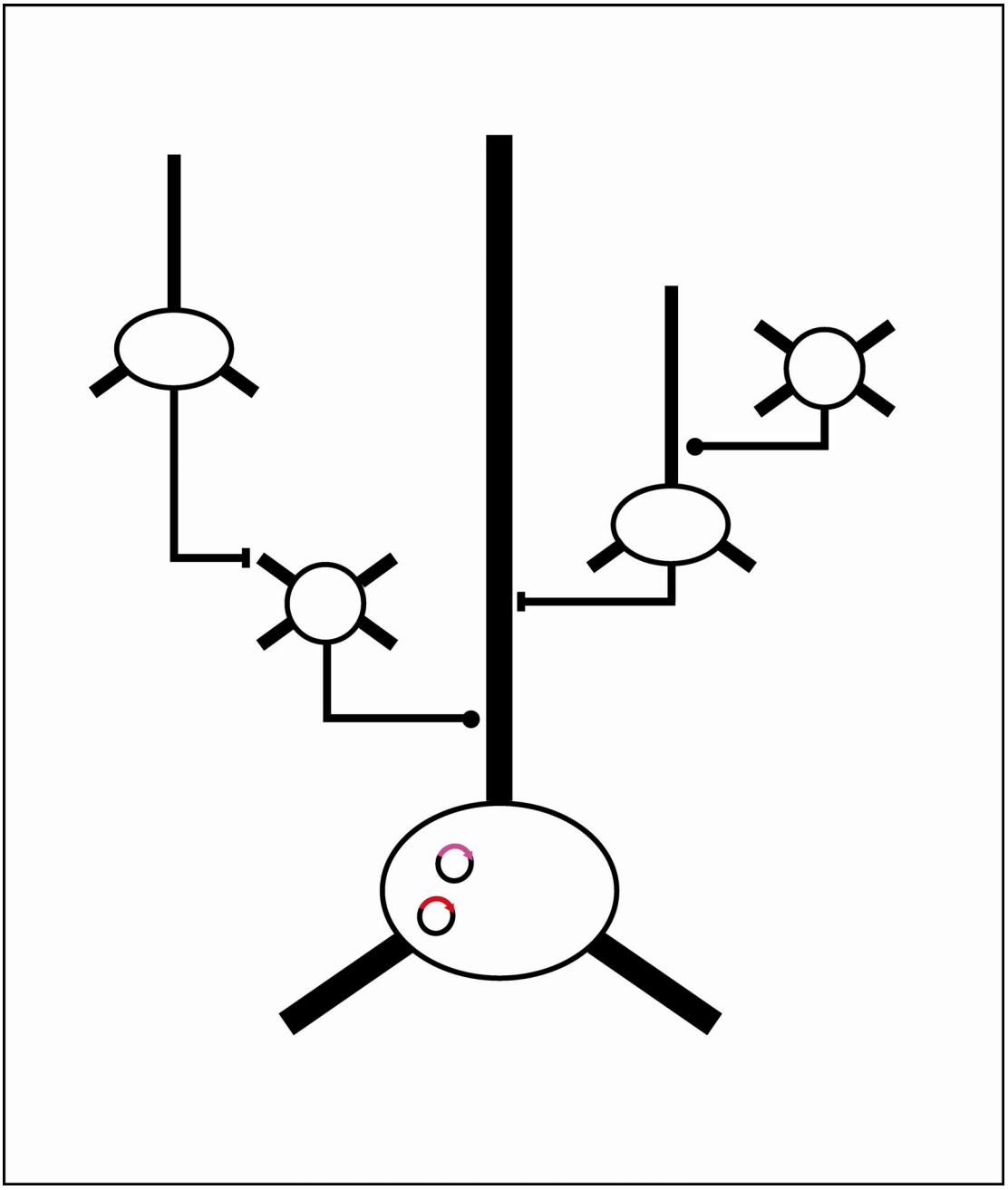

Fig. 13. Basic requirements of the system. First insert 2 genes into the cell or cell type of interest: the gene for the ASLV-A receptor, so the virus can enter, and the gene for the viral glycoprotein, so the virus can spread to synaptically coupled cells. Then apply ASLV-A-pseudotyped virus.

Testing in slice culture

We have tested the system in cultured slices of neonatal rat brain, using the “gene gun” (Bio-rad, Hercules, CA), as depicted in fig. 14, to transfect small numbers of neurons within each slices with the necessary genes.

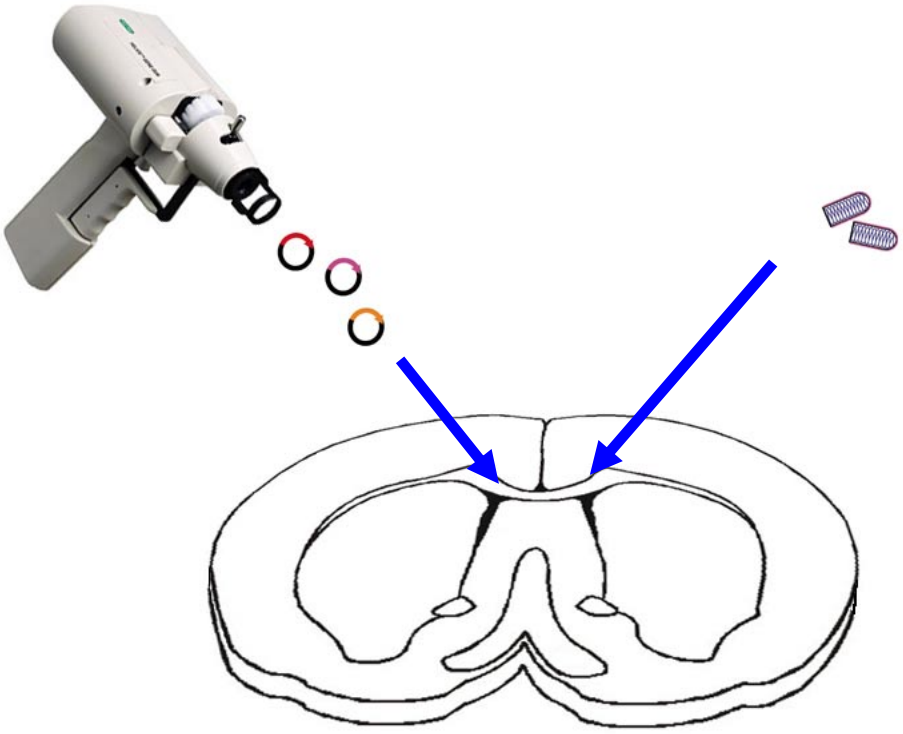

Fig. 14. Testing in slice culture. Isolated neurons in cultured slices of young rat brain were transfected using the gene gun to shoot in gold particles coated in DNA containing 3 genes: 1) The ASLV-A receptor, 2) The rabies virus glycoprotein, and 3) DsRed2 to mark transfected cells. ASLV-A-pseudotyped rabies virus was applied the following day.

Gold particles coated in plasmid DNA are shot by compressed air into the slice, resulting in isolated transfected neurons that are a small minority of the total population. A day after transfection, SADΔG-EGFP(EnvA) was added to the culture wells, and the tissue was examined several days later as described below.

As an initial control, however, we applied the virus to slices that had not been shot at all. As expected, and consistent with the very low tropism of ASLV-A for mammalian cells, this resulted in almost no infection: out of 20 slices to which virus was added and that were examined six days following infection, we found green fluorescence – the indicator of infection by the virus – in only a single cell.

As a second control, we transfected the slices using the gene gune but omitted the glycoprotein gene, including instead only the genes for TVA and, as a marker for transfected cells, DsRed2. This results in scattered strongly red fluorescent cells the following day. Application of the pseudotyped virus results in selective infection of the TVA-expressing cells (figure 15).

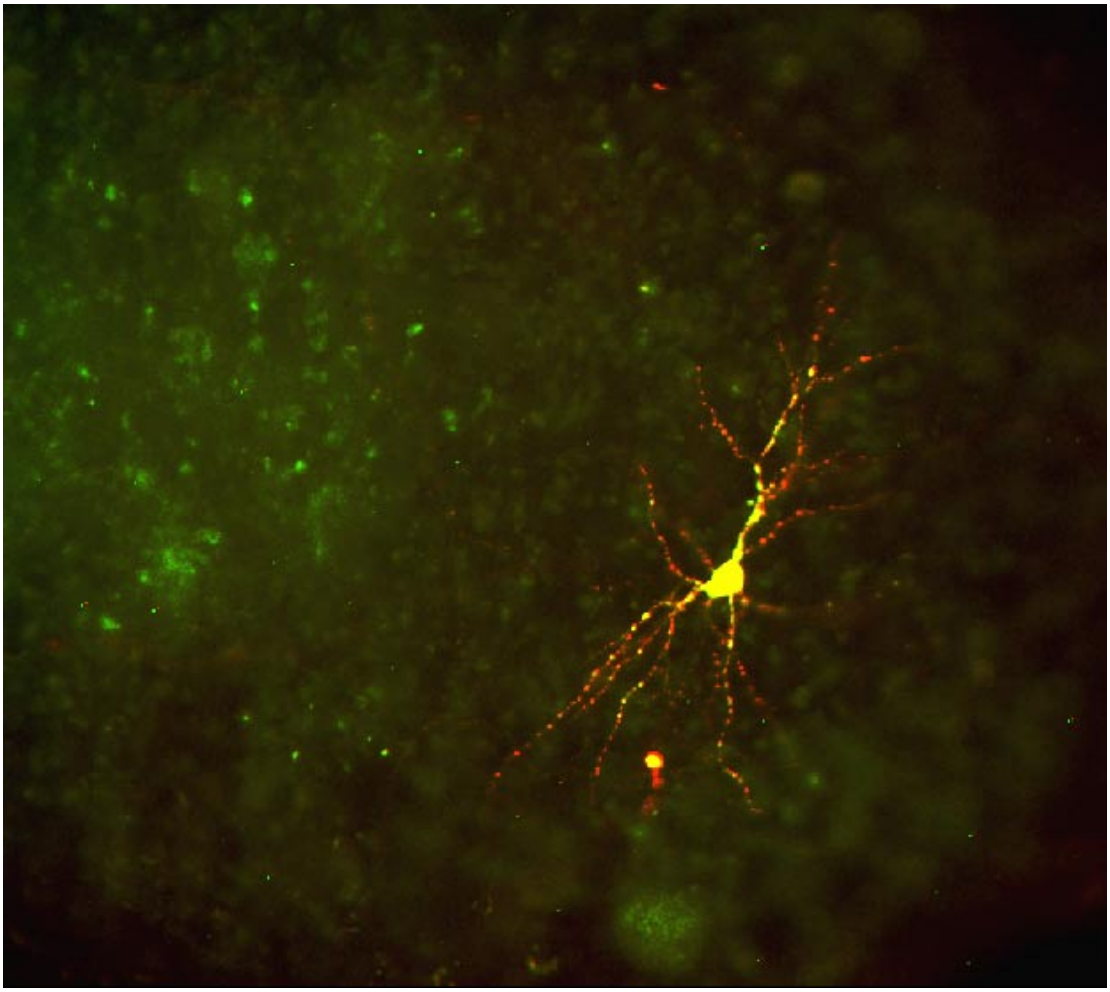

Fig. 15. Control slice in which neurons were transfected with DsRed2 and the ASLV-A receptor. One day later SADΔG-EGFP(EnvA) was applied to the slice. Viral infection (EGFP expression) is restricted to cells expressing the ASLV-A receptor (DsRed2 expression).

In 12 slices, again examined six days postinfection, there were a total of 43 cells expressing DsRed2, mostly clumped in 3 slices due to the vagaries of the gene gun technique. Of these, 23 also expressed EGFP, indicating that about hald of TVA-expressing neurons are infected with our methods. In these slices, again, only one cell that was not red expressed EGFP. These results indicate that pseudotyping ASLV-A is an effective way of selectively targeting rabies virus infection to TVA-expressing cells.

To test the ability of transcomplementing glycoprotein expression to allow viral spread from single neurons, we included the gene for the rabies glycoprotein as well as those for TVA and DsRed2 on the particles shot into slices. This results, again, in a subset of red cells expressing EGFP, but, spectacularly, these are now surrounded by large clusters of cells that are only green (figure 16).

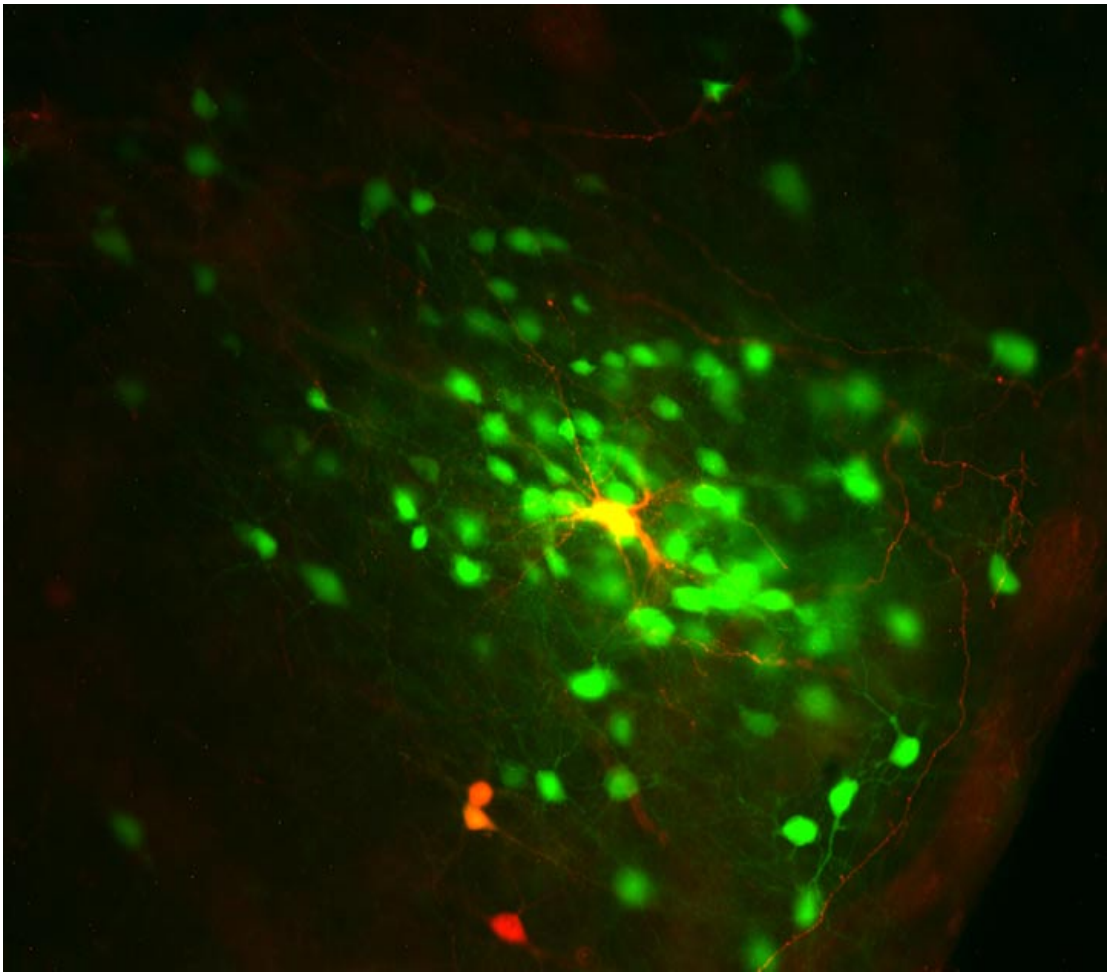

Fig. 16. Slice in which cells were transfected with the genes for DsRed2, the ASLV-A receptor, and the rabies glycoprotein. *In situ* complementation by the glycoprotein plasmid permits transsynaptic spread from the initially infected cells to clusters of cells surrounding them.

In a cohort of 6 slices shot with the three plasmids, infected the following day and examined six days postinfection (as before), we found a total of 242 red cells, of which 62 coexpressed EGFP. Cells expressing EGFP without DsRed2, in these slices, numbered 5424.

From these results we concluded that *in situ* complementation of the deletion mutant rabies virus worked extremely effectively, with initially infected TVA+ cells hosting a viral infection that spread to other neurons in the slice, vividly illuminating them with EGFP.

Due to the limitations of the gene gun technique, cells are generally shot in clumps when they are shot at all, resulting typically in multiple red cells per slice; we were not able to adjust parameters so as to transfect one neuron or fewer per slice. Despite this, in many cases, clusters of secondarily infected neurons clearly seemed to be centered on a single DsRed2-expressing cell, as seen in the fairly striking example shown in figure 17.

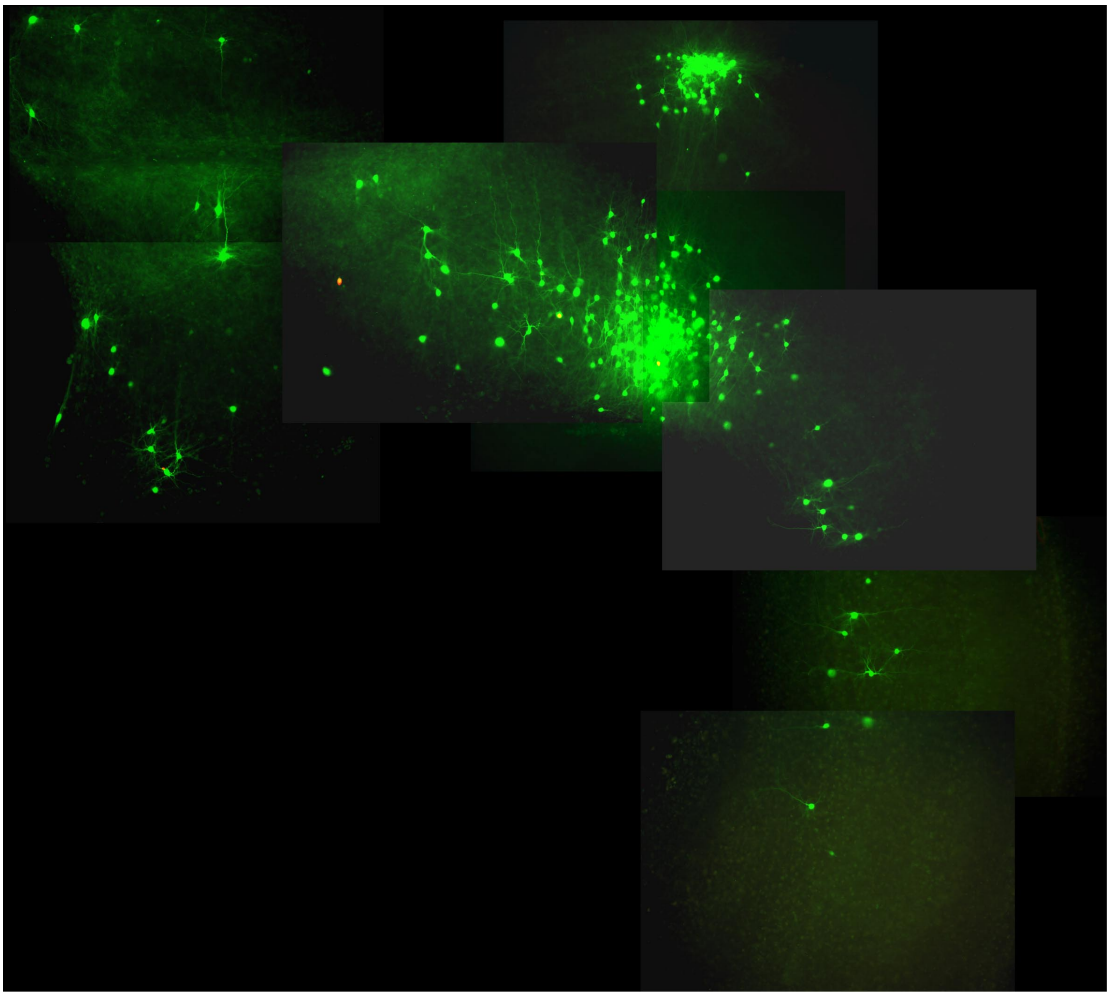

Fig. 17. Large-scale montage of a coronal cortical slice, 8 days postinfection. A huge cluster of green cells surrounds a single deep-layer pyramidal cell. Another dense cluster of cells is also infected in the superficial cortical layers immediately above it, consistent with known cortical connection patterns; distant deep-layer pyramidal cells are also infected, again consistent with known patterns of long-range intralaminar connectivity. To the left of the putatively initially infected cell, a second yellow cell, apparently secondarily - and recently – infected because of the lack of green cells surrounding it.

In this coronal slice of cortex viewed eight days post infection, a layer 5 pyramidal cell sits at the center of an enormous constellation of brilliant green. Consistent with the known significant projection from layers 2 and 3 to layer 5 cells, a second large cluster of green cells is present in superficial layers, with clearly visible axons descending to the deeper cluster. Again consistent with known connection patterns, numerous distant cells within the same layer as the putatively postsynaptic neuron are also labeled. To the far left of the main cluster, there is a red cell that does not express EGFP; closer in is a second cell that is both green and red. Because this cell is not itself at the center of its own cluster, it seems most likely to have been secondarily infected and would presumably have spawned its own cluster had the experiment been continued.

These clusters of green neurons with transfected neurons sitting in their epicenters strongly suggested that virus was spreading transsynaptically from single cells to cells presynaptic to them. It remained to be seen, however, whether this spread was specific to neurons in direct synaptic contact with the initially infected cell.

Testing synaptic specificity by paired recording

To test whether the viral spread was truly transsynaptic instead of nonspecific infection of neighboring cells, we conducted paired patch-clamp recordings from putatively pre- and postsynaptic cells (fig. 18).

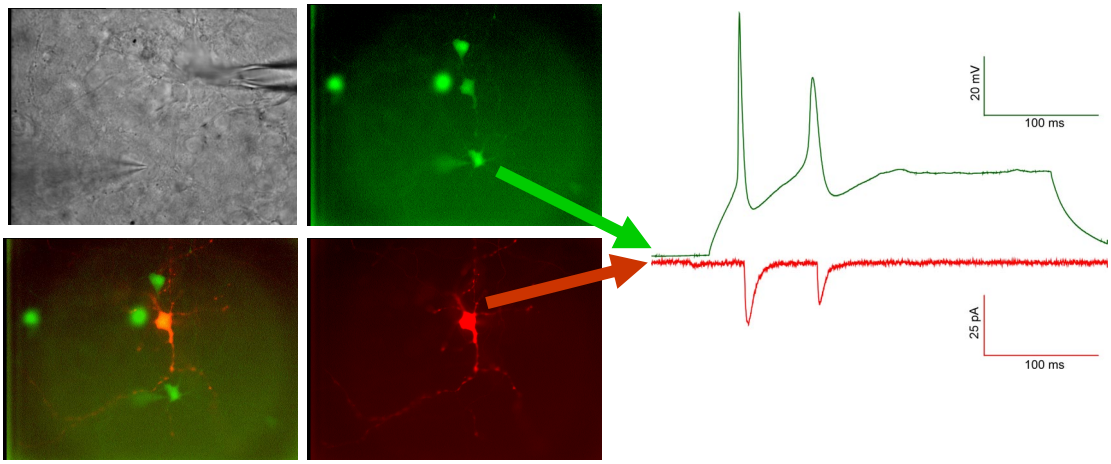

Fig. 18. Testing specificity of spread. a) DIC image of slice and recording pipettes targeting putatively pre- and postsynaptic neurons. b) green fluorescent, c) red fluorescent, and d) combined fluorescent image. e) Traces recorded simultaneously from presynaptic cells, in current clamp mode (green, top), and postsynaptic cell in voltage clamp (red, bottom): coincidence of presynaptic action potentials and postsynaptic currents demonstrates synaptic connection between the cells.

Cells that were fluorescent in both red and green channels (red/green cells) were recorded under voltage clamp while nearby green-only cells, held in current clamp, were depolarized to fire action potentials. Synaptic currents in the voltage-clamped red/green cell that were simultaneous with the green cell's action potentials indicated a monosynaptic connection as seen in the example traces in fig. 18. Nonfluorescent cells at similar distances from the red/green cells were also stimulated as controls.

The tendency of the gene gun, as mentioned above, to produce clumps of transfected neurons rather than evenly distributed ones complicated these experiments. Since there were no ideal cases of only a single transfected cell per slice, we relied on the imperfect criterion of only recording from red/green cells with no other red/green cells present within 250 microns. This made the numbers of recorded pairs low, since most slices were excluded by our criterion. Furthermore, because, as seen in figure 17, a single postsynaptic cell can apparently label cells across hundreds of microns, interpretation of situations in which green cells are not found to be connected to the nearest red/green cell is difficult.

Notwithstanding these caveats, the results were as follows. Nine control pairs, each consisting of a non-fluorescent neuron near a red/green cell, were recorded. None of these pairs were found to be connected. Of eleven green cells, nine were found to be directly connected to their nearby red/green putatively postsynaptic partners.

DISCUSSION

Limitations of the testing

Our paired recordings indicate, first, that the virus can spread effectively from individual cells, and, second, that this spread is to directly presynaptic cells in the strong majority of cases. We cannot speak to the issue of whether the virus would eventually label *every* cell connected to a given starting neuron, except to point to our limited paired recording data finding that none of the cells that were not green were connected to nearby red/green cells. The answers to this question will depend on further testing and experience with the system. Futhermore, given that two out of eleven recorded green cells were not found to be connected to the nearest transfected cell, the most begrudging interpretation of the data might be that viral spread in this system is to directly connected presynaptic cells most of the time.

However, there is reason to have more confidence in the technique than that, because of the following limitations of the strategy used here for testing it. First, as stated above, the gene gun typically transfects large numbers of cells within a slice rather than the single cell that would be required to ideally test the system. This means that, with multiple possible postsynaptic cells within each slice, the infected cells that were not found to be connected to the recorded transfected cells could easily have been, and we suspect were, connected to some other transfected cell elsewhere in the slice. A second drawback of our testing technique was the use of three separate plasmids to coat the particles

used in the gene gun. Although this results in coexpression most of the time [64], in some cases green cells may have been presynaptic to cells transfected with TVA and the rabies glycoprotein but that simply failed to express the red fluorescent marker. Third, because the gene gun transfected a random and unknown number of neurons in each slice, there is no way of knowing whether any transfected and initially infected cells died after transmitting virus to presynaptic cells, which would then appear to be unconnected to any initially infected ones. This latter possibility should be taken seriously because the rabies glycoprotein is cytotoxic when expressed at high levels, and our transfection technique gave us little control over the expression levels of any of the genes.

A much better way to test the system would be to transfect only one cell per slice using microinjection or single-cell electroporation, and to do so with a single plasmid containing all three genes (TVA, G, and fluorophore). This would solve the problem of having multiple possible sources of virus. A further improvement would be to use optical stimulation of putatively presynaptic cells, as well as controls, for a much higher throughput than we have managed here. Such rigorous testing is needed and would be welcomed.

Fundamentally, though, except for the possibility of the postsynaptic cell's death due to overexpression of the glycoprotein, there is no *a priori* reason to believe that the spread of the *in situ* complemented virus would be any less synaptically specific than that of replication-competent rabies virus, which all available evidence indicates to be highly specific[17, 18].

Limitations of the system

There are several suboptimal aspects of the system in the form presented here.

First, as mentioned above, the rabies glycoprotein, like many viral envelope proteins, is cytotoxic when expressed at high levels[65-67]. On the other hand, it is evidently not so toxic that the complementation system doesn't work remarkably well, even with the uncontrolled expression levels afforded by the gene gun. Work with the closely related rhabdovirus VSV furthermore indicates that naturally occurring virions vary over an order of magnitude in the number of glycoprotein molecules incorporated into their membranes[68]. Therefore implementations of our system are likely to have wide latitude to express the glycoprotein at levels that maintain cell viability but result in effective viral spread. That said, we expect that inducible expression will work best.

A second drawback of the system in its current form is that the deletion mutant virus retains some toxicity despite the absence of the glycoprotein gene (see chapter I). A significant component of this residual toxicity is likely to be due to the matrix protein, which, among its other roles, may itself induce apoptosis as has been found for other rhabdoviruses[35, 36] [69, 70]. Since deletion of both M and G genes does not appreciably reduce expression of other genes in the viral genome [34], use of a such a double deletion complemented *in situ* may be an improvement on the system in the form presented here.

A third suboptimal aspect of the system is that the ALSV-A-pseudotyped virus stocks retained some small residual ability to infect neurons in the absence of TVA expression. This is most likely due to contamination of the viral stock by remnants of the starting stock of virus, containing its own glycoprotein, that was used to make the pseudotyped version (see Methods). More sophisticated technique for generating the pseudotyped virus should eliminate this problem.

Lastly, our system is limited to identifying cells presynaptic to a cell or cell type of interest. There is no immediate prospect of modifying the system presented here to identify *post*synaptic cells, because there are no known strains or mutants of rabies virus that travel anterogradely. The alpha-herpesviruses, by contrast, travel bidirectionally in their wild form, and the strains used for tracing have various defects in this capacity so as to render them unidirectional. The HSV-1 strain H129, in particular, appears to travel solely anterogradely[71, 72]. *In situ* complementation of a deletion-mutant H129, therefore, might provide an anterograde counterpart to the current system. Unfortunately, we tried to implement the *in situ* complementation idea using a widely used alpha-herpesvirus, pseudorabies virus, with little success before turning to rabies virus (data not shown). We attributed this failure to the high cytopathicity and low efficiency of the herpesviruses relative to rabies virus as described in the Introduction. However, since postsynaptic cells are as interesting as presynaptic ones, it may be worth revisiting these efforts.

Implementation in vivo

It should be possible to implement our system *in vivo* in a variety of ways. The basic requirements are simply to introduce the genes for TVA and the rabies glycoprotein into the cell or cells of interest, then to apply the pseudotyped virus after TVA has been expressed. An obvious way of introducing the two genes into a particular cell type would be simply to genetically modify mice. A caveat to this approach is that, since the rabies virus will spread through any connected cells that express its glycoprotein, expression should be as restricted as possible to avoid labeling all inputs to a heterogeneous population of cells. An alternative means of gene delivery could be a helper virus, perhaps with cell type specificity determined by its enhancer sequence[73-75]. Single-cell electroporation represents yet another approach[76, 77] that suggests the possibility of identifying the cells presynaptic to a neuron whose response properties have been characterized. All of these techniques for gene delivery would need to be followed by stereotaxic injection of the pseudotyped virus after TVA has been expressed.

Finally, the recombinant rabies virus could of course be modified to contain essentially any gene of interest besides that of EGFP. This opens the door to a wide variety of possibilities including optical monitoring, or control, of the activity of the labeled cells.

Conclusion

The system we have presented here is a genetically targetable method of directing gene expression in cells that are immediately presynaptic to a given

neuron or neuron type. In our view it is likely to be highly useful in a variety of areas of neuroscience.

METHODS

Production of packaging cell line

The extracellular and transmembrane domains of the ASLV-A envelope protein were amplified from the plasmid pAB6 [78] using the following primers: TTTCAGCGGCCGCATGGAAGCCGTCATAAAGGC forward (NotI site underlined) and AGGTTCTGATCGATTGACTCTTCTGCAAGGCAGGCACACTACTAGC reverse (homology with rabies virus glycoprotein gene underlined). Accuprime Pfx (Invitrogen, Carlsbad, CA) was used for all PCR reactions. The cytoplasmic domain region of the SAD B19 glycoprotein gene was amplified from pHCMV-RabiesG[40] using primers GCTAGTAGTGTGCCTGCCTTGCAGAAGAGTCAATCGATCAGAACCT forward (homology with TVA800 gene underlined) and GACGGCGGATCCTCACAGTCTGGTCTCACCCCCAC reverse (NotI site underlined). The resulting products were then combined in a third reaction using the first and last primers listed above, producing a chimeric gene was cloned into the NotI and BamHI sites of the murine leukemia virus (MLV) transfer vector pCMMP-IRES-GFP [79]. The insert region of the final cloning product, termed pCMMP-EnvARGCD-IRES-GFP, was verified by sequencing.

VSV-pseudotyped MLV was then produced as described[79]. Briefly, 293T cells (ATCC, Manassas, VA) were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol using the following DNA quantities in a 10 cm plate: the transfer vector described above, 2.5 µg; the MLV packaging construct pMD.old.gagpol, 2.5 µg; and the VSV glycoprotein expression vector pMD.G, 3 µg. Supernatants were collected 48 and 72 h posttransfection, pooled, filter sterilized and pelleted by ultracentrifugation for 1 hours at 111,000 g. Pellets were resuspended in 1 ml medium and applied after titering to BHK-21 cells (ATCC) at an MOI of ~4 overnight. After four passages, cells were sorted for high EGFP fluorescence with a FACSDiva (BD Biosciences, San Jose, CA). The resulting cell line was termed BHK-EnvARGCD.

Production of pseudotyped rabies virus

BHK-EnvARGCD cells were plated in 12 well plates at 2E5 cells/well. The following day, the glycoprotein-deleted rabies virus SADΔG-EGFP (see chapter I) was added at an MOI of 1.5. One day later, the cells in each well were trypsinized and replated into a 10 cm plate. Supernatants were harvested two days later, filter sterilized and frozen at -80^{o}C in 1 ml aliquots.

Determination of virus titers and fluorescence analysis

Virus titers were determined by serial dilution and overnight infection of 293T-TVA800 cells [80] and 293T cells followed by fluorescence activated cell

sorting on a FACscan (BD Biosciences) 3 days later. Data were subsequently analyzed with CellQuest software (BD Biosciences) to determine the fraction of fluorescent cells and the titer taken as $-\ln(1-p)*N_o/v$, where p is the fraction of fluorescent cells, N_o is the number of cells at infection, and v is the volume of applied virus.

Preparation and maintenance of cultured brain slices

Brain slices were prepared from the cortex of 3-7-day-old rats as described previously for ferrets[81, 82]. Briefly, animals were deeply anesthetized with sodium pentobarbitol (100 mg/kg, i.p.) and decapitated. Brains were extracted under sterile conditions and submerged in 4°C HEPES-buffered artificial CSF (ACSF) (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 24 dextrose, 10 HEPES, and 1 $CaCl_2$, pH 7.4. The cortex was cut into 400 µm parasagittal slices using a tissue slicer [11]. Slices were then transferred onto cell-culture inserts (0.4 µm pore size; Falcon, Franklin Lakes, NJ) in six well culture dishes and fed with medium from below. The medium was composed of 50% basal Eagle's medium without glutamine, 25% HBSS, 330 mM dextrose, 10 mM HEPES, 200 mM L-glutamine, 10 U/ml penicillin-streptomycin (all from Invitrogen, San Diego, CA), and 25% horse serum (Hyclone, Logan, UT).

Biolistics and virus application

One day following slice preparation, slices were transfected using the Helios Gene Gun (Bio-Rad, Hercules, CA). Gold microcarriers (1.6 µm diameter;

Bio-Rad) were coated with vector DNA at a concentration of 60 µg of DNA per 12.5 mg of gold, according to the supplier's instructions and shot into the slices at 90-100 psi. The following plasmids were used. Controls: pCAG-DsRed2, 5 µg; pCMMP-TVA800[80], 30 µg. Experimental: pCAG-DsRed2, 5 µg; pCMMP-TVA800, 30 µg, pHCMV-RabiesG[40], 15 µg. All transgenes were expressed under the control of the human cytomegalovirus (CMV) immediate-early promoter except for DsRed2, which was driven by the CAG hybrid promoter[83, 84]. One day following transfection, 50-100 ul of virus stock solution (7.8E4 pfu/ml) was applied to the surface of each slice.

Electrophysiological recordings

3-9 days following application of virus, slices were transferred to recording chambers perfused with room temperature artificial cerebral spinal fluid (ACSF), composition in mM: 124 NaCl, 5 KCl, 1.25 KH_2PO_4, 1.3 $MgSO_4$, 3.2 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose. Glass recording electrodes (7-10 MΩ resistance) filled with an intracellular solution consisting of 130 mM potassium gluconate, 6 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 10 mM HEPES, 2.5 mM Na_2ATP, 0.5 mM Na_2GTP, 10 mM potassium phosphocreatine and 0.3% biocytin, adjusted to 7.25 pH with KOH, were used for whole-cell current-clamp recordings. Cells were targeted using fluorescence and DIC optics.

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IV. CONCLUSION

This dissertation has described two new techniques for use in neuroscience. The first is the use of a recombinant rabies virus expressing enhanced green fluorescent protein to identify and illuminate the details of neurons that project to an injection site. This allows either detailed reconstruction of these cells or targeting them for physiological study. The second is the use of a modified form of the same virus, in conjunction with selective gene expression, to identify, in a comprehensive way, cells that are directly presynaptic to either a particular cell type or to a single cell.

These techniques, particularly the second one, will enable the acquisition of large amounts of highly detailed data about the interconnections between neurons in the brain. Such information alone may not, however, reveal much about the neural bases of perception, cognition, and behavior. Instead, we anticipate that it will suggest ideas and hypotheses that can then be tested using these and many other tools at a variety of levels of analysis from molecular to behavioral, with the goal of understanding how the operations of the brain produce the mind. These efforts will be helped by the fact that exponential advances in computing technology, coincident with those in the biological sciences, allow testing of increasingly detailed models.

This of course is likely to provide great benefit in enabling sophisticated approaches to preventing and treating neurological disorders. However, these advances in computers, and in electronics in general, also offer the possibility of

technology transfer of any insight about the nature of intelligence that might be gained. This will eventually and inevitably result in the implementation of the mechanisms of mind in synthetic hardware. This development will probably be of quite considerable impact.

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