Transneuronal Circuit Analysis With Pseudorabies Viruses

Over the past decade there has been a dramatic increase in the use of viruses as transneuronal tracers of neuronal circuitry. The method exploits the propensity of neurotropic viruses to invade neurons and then produce infectious progeny that cross synapses to infect other neurons within a circuit. In essence, the virus provides a self-amplifying tracer that moves progressively through the neurons that compose a circuit. Thus, through temporal analysis of the distribution of viral antigens, the synaptology of a polysynaptic circuit can be defined. Presented in this unit is the basic methodology for application of this experimental approach. Since viral invasiveness is determined by properties of the virus *and* the host neurons, there are no "generic" methods for applying the method. Consequently, knowledge of the life cycle of the virus and the basic organization of the system of interest should be carefully considered in the design, execution and interpretation of experiments. Figures 1.5.1 and 1.5.2 summarize fundamental information relevant to both of these issues.

The protocols and commentaries included in this unit focus upon the use of the swine alpha herpesvirus known as pseudorabies virus (PRV) for polysynaptic analysis. This DNA virus has been widely applied in viral transneuronal studies of rodents. However, it is important to note that the human pathogen, herpes simplex virus (HSV), and neurotropic RNA viruses, such as rabies, have also been successfully employed for transneuronal analysis in a variety of species. The focus upon PRV is related to the wide host range of the virus and the availability of well characterized attenuated strains exhibiting reduced cytopathogenicity. Importantly, PRV has also benefited from a number of mechanistic studies that have defined the role of virally encoded proteins in invasiveness, transynaptic passage, and virulence. These studies have identified attenuated strains useful for transneuronal analysis, and the use of these viruses for transneuronal analysis has defined model systems that are of great value for more directed studies of the viral life cycle. This interdependent multidisciplinary approach has proven integral to establishing both the specificity and usefulness of PRV as a transneuronal tracer (for reviews see Card and Enquist, 1995; Card, 1998a; Enquist et al., 1999).

The viral transneuronal method incorporates many procedures that are fundamental to tract tracing in the nervous system with classical tracers. In this unit, those aspects of experimental design that have the greatest import for successful use of viruses in circuit definition are presented. Accordingly, the protocols included in this unit can be applied in concert with methods in which the use of classical tract tracers has been detailed. The following protocols review the methods directly related to the use of PRV for transneuronal tracing in the rat CNS. A procedure for retrograde infection of CNS circuits by peripheral injection of virus is detailed (see Basic Protocol 1), while transneuronal analysis by intracerebral injection is also described (see Basic Protocol 2). A variant of these procedures is transneuronal analysis with multiple recombinant strains, the considerations for which are described in the Alternate Protocol. Support Protocol 1 details the growth and titering of viral stocks, while Support Protocols 2 and 3 present procedures for single and dual immunohistochemical localization of viral antigens in fixed brain tissue.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Contributed by J. Patrick Card and Lynn W. Enquist
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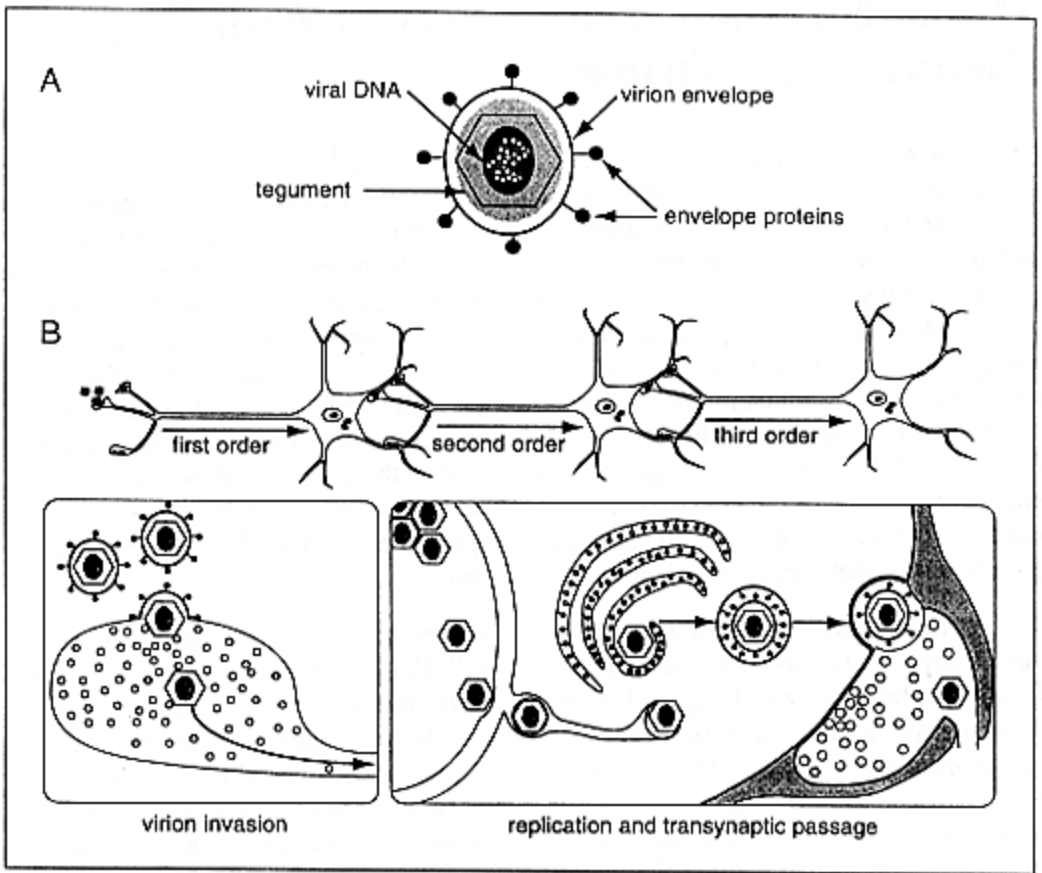

Figure 1.5.1 (**A**) The structural characteristics of alpha hepesviruses. Viral DNA is sequestered within a capsid composed of virally encoded proteins. The capsid and a surrounding tegument of each virion is contained within a viral envelope acquired from the host cell. The envelope contains a second set of virally encoded proteins that are important for target cell recognition, attachment, and the receptor-mediated fusion event that leads to the release of the capsid into a permissive cell. (**B**) A model of virion assembly postulated for pseudorabies virus (Card and Enquist, 1995). Assembly of virions is a multistep process that leads to retrograde transynaptic passage of virions through polysynaptic circuits. Adapted with permission from Card (1998b).

BASIC PROTOCOL 1

RETROGRADE INFECTION OF CNS CIRCUITS BY PERIPHERAL INJECTION OF VIRUS

PRV-Bartha has been used in numerous investigations to define the organization of CNS circuits that control the activity of autonomic or somatic motor neurons. These studies exploit the high affinity of PRV for axon terminals and the tendency of this virus to move retrogradely through a multisynaptic pathway. However, variation in the synaptology of a circuit or the cytoarchitecture of a target organ can have profound effects upon the onset of replication and the progression of infection within a circuit. In this protocol, retrograde infection of CNS circuitry is achieved through inoculation of the peripheral targets of autonomic or somatic motor neurons. Emphasis is placed upon aspects of the method that must be addressed to ensure reproducible and reliable patterns of infection. The surgical procedure is dependent upon the target selected for injection, and injection procedures will also differ according to the target. Where appropriate, reference is made to sections of the commentary that provide a more comprehensive treatment of a particular topic.

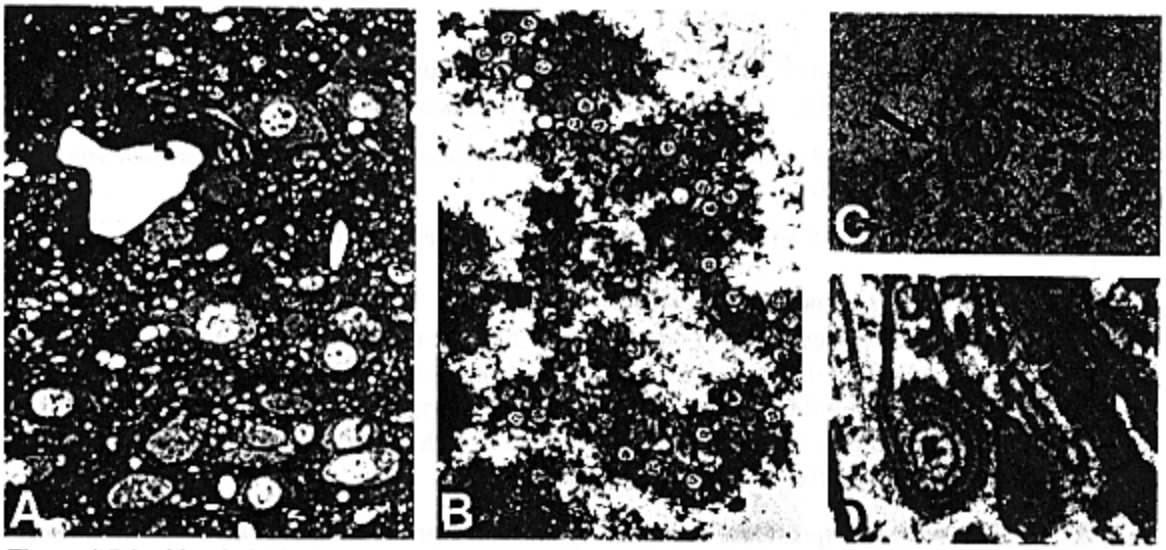

Figure 1.5.2 Morphological correlates of the events shown in Figure 1.5.1, panel B. **(A)** Formation and aggregation of capsids in the cell nuclei produces the classical nuclear inclusions characteristic of herpesvirus-infected neurons (arrows). **(B)** In electron micrographs, individual capsids appear as spherical structures containing electron-dense viral DNA. **(C)** In the model illustrated in Figure 1.5.1, capsids are enveloped in a two-stage process involving sequential passage of capsids through the nuclear membrane (arrow) and endoplasmic reticulum (ER). **(D)** Release of capsids from the ER is followed by final envelopment at the trans face of the Golgi complex. Envelopment at the trans face of the Golgi provides the two membranes that are necessary for progeny virus to leave the parent cell and gain access to the next permissive cell. Reprinted with permission from Card (1998b).

Materials

Experimental animal
Ketamine
Xylazine
Sterile physiological saline (0.9% w/v NaCl)
Titered PRV-Bartha virus (see Support Protocol 1)
PLP fixative (see recipe)

Appropriate syringes and needles for injection of anesthetic into experimental animal
Surgical instruments (will vary depending on required surgery)
Sutures and/or wound clips
Heating pad or heat lamp
10-µl Hamilton syringe equipped with a 26-G needle that has a sharpened beveled tip (sterilize by autoclaving; do not use cold sterilization as the solution will compromise the titer of the inoculum)

Additional reagents and equipment for perfusion fixation (*UNIT 1.1*)

NOTE: PRV and other neurotropic viruses are class 2 infectious agents that require a laboratory which meets Biosafety Level 2 (BSL-2) regulations as defined in Health and Human Services Publication 88-8395 (see Critical Parameters). Among these regulations is the requirement that infected animals be confined to the BSL-2 laboratory throughout the experiment and that the laboratory be dedicated to the viral studies. Thus, one must ensure that the laboratory contains all of the reagents and equipment necessary for the experiment. Aseptic surgical procedures should be used, but it is important to include precautions that protect against compromising the concentration of the inoculum (see Critical Parameters).

Perform surgery

1. Deeply anesthetize animal by injecting a mixture of ketamine (60 mg/kg) and xylazine (7 mg/kg) intraperitoneally or intramuscularly.

   Animals should be deeply anesthetized so that they are unresponsive to sensory stimuli (e.g., tail pinch).

2. Perform the surgical procedure appropriate for the target organ.

Inject virus and allow post-inoculation recovery

The principal features that will determine the method of injection are the density of the axonal innervation and the cytoarchitecture of the peripheral target. Here, the general procedures that should be employed in making a peripheral inoculation are presented. However, it is important to review the discussion in this unit on designing the injection strategy for an experiment (see Critical Parameters).

3. Draw up and express sterile physiological saline prior to loading the 10-µl Hamilton syringe with virus. Perform the same draw-and-express procedure with three or four aliquots of virus (to prevent dilution of the virus with saline), and then load the syringe for injection.

   The saline draw ensures a tight seal of the plunger with the glass barrel of the syringe and increases the likelihood of accurately delivering the desired amount of virus to the injection target. However, it is imperative that residual saline be removed from the needle to prevent dilution of the inoculum. The importance of purging the syringe of saline cannot be overemphasized, since the microliter syringes are loaded with small volumes of virus and residual saline can dilute the inoculum by several log units. Such alterations of titer will certainly introduce variability in the extent of infection between animals and, in the worst circumstances, may prevent productive replication of virus altogether (see Anticipated Results).

4. Inject the inoculum into multiple areas of the target organ (but see Critical Parameters).

   IMPORTANT NOTE: *Do not remove or disrupt connective tissue or fascial sheaths associated with the injection target prior to injection. Envelope glycoproteins of PRV and other alpha herpesviruses have high affinity for extracellular matrix proteins and these sheaths serve to limit diffusion of the virus from the injection site.*

5. Close incisions with sutures and/or wound clips and place the animal on a heating pad or under a heat lamp until it recovers consciousness, and then return it to its home cage. House infected animals individually. Provide food and water ad libitum and standardize the photoperiod to 10- to 12-hr light per 24-hr cycle. Periodically weigh the animals to monitor the onset of infection symptoms.

   In general, use sutures to close invasive incisions of the body cavity (such as the abdomen) or use wound clips to close incisions through the skin.

   As noted earlier, BSL-2 regulations stipulate that infected animals be confined to the BSL-2 laboratory. Thus, the laboratory should contain a HEPA-filtered unit for housing the animals.

   Injected animals should be carefully monitored during the post-inoculation period to ensure that the viral infection is not compromising their ability to attend to their bodily needs.

   When attenuated strains of virus are used, essentially all elements of a circuit can be characterized within a post-inoculation interval during which the animals are free of symptoms. Nevertheless, it should be emphasized that even attenuated strains of virus will ultimately cause death at long survival intervals and animals usually exhibit signs of infection at these late stages of infection. Monitoring the weight of an animal provides a

reliable means of predicting the onset of debilitating consequences of infection. In unpublished observations, the authors have noted that animals exhibit a precipitous drop of ~20% body weight during the day preceding imminent death. Thus, it is probable that any animal that becomes lethargic and experiences a large weight loss is at risk. These animals should be anesthetized and perfused.

Perform perfusion fixation of tissues

6. At the appropriate post-inoculation interval, anesthetize (see step 1) and sacrifice animals by transcardiac perfusion fixation of the entire animal with PBS and then with PLP fixative (see *UNIT 1.1* for perfusion fixation protocols; see Reagents and Solutions for fixative recipe). Remove the brain and any other tissues to be included in the analysis. Post-fix this tissue in PLP fixative for 1 to 2 hr at 4°C.

The methods employed are those that are routinely applied in morphological studies, with the following important exceptions. First, the entire animal should be perfused. Studies of the CNS classically clamp the descending aorta and perfuse only the head, since preservation of brain structure and antigenicity is the preeminent goal. Transcardiac perfusion in viral tracing studies has the added goal of inactivating virus throughout the animal, which is particularly important in paradigms involving peripheral inoculation. PRV and other alpha herpesviruses are inactivated by aldehydes, and the vasculature offers the most efficient means of delivering fixative to the entire animal.

Dispose of wastes properly

7. Treat everything that comes in contact with an infected animal during the course of an experiment as contaminated biohazardous material, including the perfused carcass, instruments, gauze, and syringes used for injections, as well as animal bedding, food, water, and caging. Place disposable materials, including animal tissues, in a biohazard bag and incinerate according to institutional regulations. Decontaminate instruments and equipment with ethanol and then wash them with standard detergents.

Instruments can also be sterilized by autoclaving in preparation for the next surgery, although cold sterilization procedures are more than adequate. Perfused carcasses are considered contaminated even though the perfusion fixation has presumably inactivated the virus.

Analyze tissues

8. Perform immunohistochemical analysis of tissues (see Support Protocols 2 and 3).

TRANSNEURONAL ANALYSIS BY INTRACEREBRAL INJECTION

BASIC PROTOCOL 2

Strick and colleagues championed intracerebral injection of HSV as a means of defining multisynaptic circuits in primate brain (Zemanick et al., 1991; Hoover and Strick, 1993, 1999; Middleton and Strick, 1994, 1996). Recently, this method has been applied successfully using PRV in the rodent CNS. Studies of HSV have identified strains that move selectively in either the anterograde or retrograde direction through a circuit. Studies of PRV have shown that wild-type virus will move in both directions through a circuit after injection into the brain, and that PRV-Bartha only moves retrogradely through a circuit following intracerebral injection (Card et al., 1998). It is therefore of preeminent importance to select a strain of virus in which the direction of transport has been rigorously defined. This protocol provides guidance for the use of PRV-Bartha in studies involving retrograde transynaptic passage.

Many of the procedures and issues detailed in Basic Protocol 1 are also relevant to experiments involving intracerebral injection. Since the fundamental methods outlined in this protocol are quite similar to those applied in classical tract tracing, it is advisable to

consult other investigators and publications to obtain detailed guidance for intracerebral injection of classical tracers.

Materials

Experimental animal
Ketamine
Xylazine
Sterile physiological saline (0.9% w/v NaCl)
Titered PRV-Bartha virus (see Support Protocol 1)
PLP fixative (see recipe)

Stereotaxic apparatus
Surgical instruments
Syringe with needle (see note below)
Bone wax or gel foam
Wound clips
Heating pad or heat lamp

Additional materials for perfusion fixation (*UNIT 1.1*) and immunohistochemical localization of neurochemicals (see Support Protocol 2 and *UNITS 1.1 & 1.2*)

NOTE: The needle or cannula used for injection will influence the zone of viral diffusion. Hamilton microliter syringes equipped with fixed needles of 26- to 32-G are generally adequate for injection of large cell groups. The needles affixed to these syringes are either blunt or have a sharpened tip with the opening on the beveled surface. If a beveled needle is used, the opening should be directed towards the cell group of interest. This is quite important, since the affinities of virions for extracellular matrix molecules restrict virus diffusion to the immediate vicinity of the injection. More restricted injections can be made with glass pipets. The pipets are pulled using standard procedures (see Chapter 6) and the tip broken back so that the internal diameter is 15 to 20 µm. The shaft of the pipet is placed over the needle of a Hamilton syringe preloaded with virus, and the interface is sealed with beeswax. It is important to keep the length of the glass sleeve as short as possible, since the space between the glass and the needle becomes a reservoir for virus that must be filled before virus can be ejected from the pipet tip.

Perform surgery

1. Deeply anesthetize animal by injecting a mixture of ketamine (60 mg/kg) and xylazine (7 mg/kg) intraperitoneally or intramuscularly.

   Animals should be deeply anesthetized so that they are unresponsive to sensory stimuli (e.g., tail pinch).

2. Secure the head in a stereotaxic frame. Make an incision to expose the cranium and use coordinates determined using a stereotaxic atlas (e.g., Paxinos and Watson, 1986; Swanson, 1992) to position the syringe on the skull over the desired area of injection. Drill a hole in the cranium at this location and pierce the dura mater in preparation for lowering the cannula/needle into the brain parenchyma.

Inject virus and allow post-inoculation recovery

3. Affix the syringe to the stereotaxic holder and lower the tip of the needle or glass pipet to the desired dorsoventral coordinate through the hole in the cranium. Inject the virus at 10 nl/min and leave the needle/pipet in situ for a minimum of 5 min after completion of the injection, to reduce reflux of virus along the injection tract. Slowly withdraw the needle/pipet from the brain, fill the hole in the cranium with bone wax or gel foam, and close the scalp with wound clips.

Generally, an injection of 50 to 100 nl of virus will produce a restricted zone of viral uptake (see Critical Parameters for more detail). Detailed information regarding the extent of viral diffusion following injection alone or in combination with classical tracers can be found in Jasmin et al. (1997), O'Donnell et al. (1997), and Card et al. (1999).

4. Allow the animal to recover on a heating pad or under a heat lamp and then return it to its home cage in the animal housing unit.

Perform perfusion fixation of tissues

5. At the desired post-inoculation interval, anesthetize (see step 1) and sacrifice animals by transcardiac perfusion of the entire animal with PBS and then with PLP fixative (see *UNIT 1.1* for perfusion fixation protocols; see Reagents and Solutions for fixative recipe). Prepare the tissue for immunohistochemical localization of infected neurons (see Support Protocol 2 and *UNITS 1.1 & 1.2*).

For guidance in determining appropriate survival intervals, see Critical Parameters.

TRANSNEURONAL ANALYSIS WITH MULTIPLE RECOMBINANT STRAINS

ALTERNATE PROTOCOL

One of the most recent advances in the viral transneuronal method is the use of multiple recombinant strains of virus to examine issues of collateralization. The unique reporters expressed by these recombinants can be discriminated with monospecific antibodies using dual-labeling immunofluorescence methods (*UNIT 2.1*). There are a variety of factors that should be considered in both the design of these studies and the interpretation of data. Thus, the issues discussed in Critical Parameters and illustrated in Figures 1.5.3 and 1.5.4, should be carefully considered before embarking upon this type of experimental analysis. The following protocol is based upon the use of two PRV-Bartha derivatives in which the bacterial *lacZ* gene and jellyfish enhanced green fluorescent protein (EGFP) gene have been inserted into the same region of the viral genome.

This method is compatible with either peripheral (Basic Protocol 1) or intracerebral (Basic Protocol 2) injection paradigms. In each case, the single most important aspect of the experimental design is determining the invasiveness of each of the recombinant strains in single-injection paradigms. That information will be crucial in determining whether the two viruses should be injected simultaneously or in two temporally separated surgical procedures. Figure 1.5.3 illustrates this issue schematically.

The goal of experiments of this type is to determine if a single population of neurons provides a common influence upon multisynaptic projections to two different sites. To address this issue, two recombinant viruses are injected into separate targets and a temporal analysis is conducted to determine if the retrograde transynaptic passage of the two viruses ultimately leads to infection of a single population of neurons. However, the number of neurons that contribute to each circuit are often different (see Figs. 1.5.3 and 1.5.4). Thus, different amounts of time will be required for each virus to reach the collateralized neurons, and injection of the two viruses will have to be temporally separated to accommodate this difference. For discussion of other factors that may influence the design of these studies, see Critical Parameters.

Selection of Recombinant Viruses

A preeminent concern in this type of experiment is that one virus will interfere with the replication of the second strain. One way of reducing this possible confounding factor is to use recombinant viruses that are *isogenic*. Other factors that are important to the success of the experiment are (1) selection of viruses that are attenuated for virulence, but maintain their invasiveness phenotype, (2) demonstration that the reporter proteins of each virus

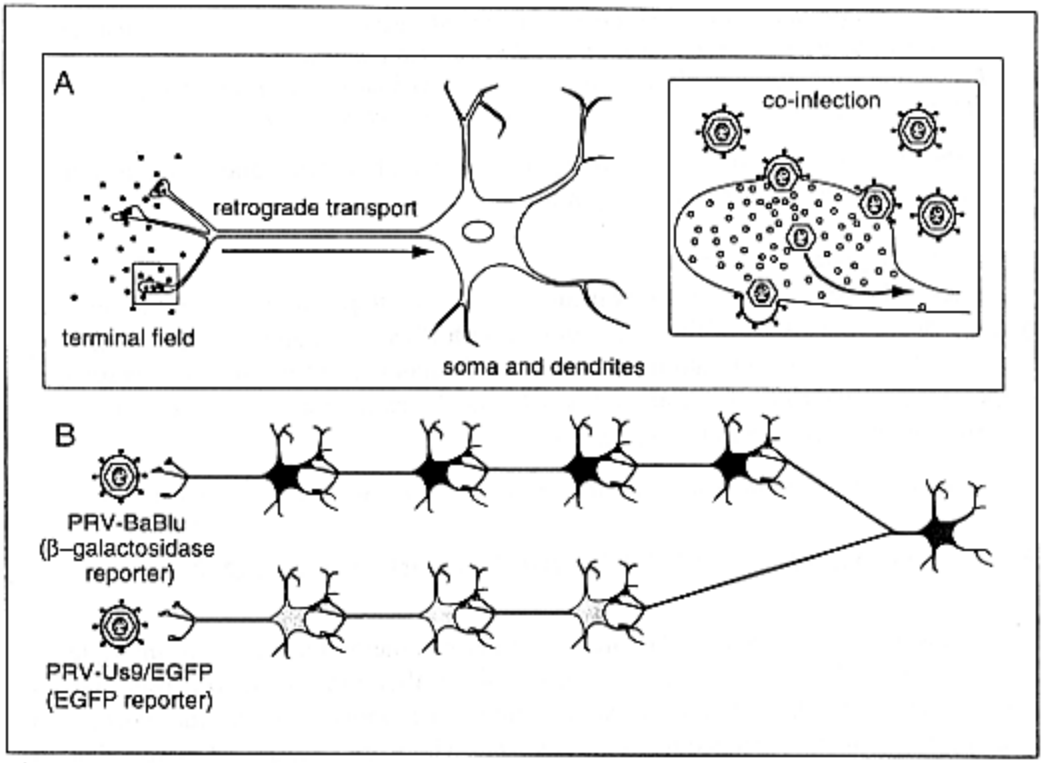

Figure 1.5.3 Use of recombinant viruses for definition of complex circuitry. The method is based upon the ability to detect unique reporter proteins produced by the two recombinant viruses. **(A)** In this case, either bacterial β-galactosidase or jellyfish enhanced green fluorescent protein (EGFP) genes have been engineered into the same locus of the PRV-Bartha genome. Importantly, EGFP is fused to a viral gene (*Us9*) that is lacking in PRV-Bartha and which has been shown to differentially traffic to the Golgi complex (Brideau et al., 1999). **(B)** Injection of the two recombinants into different peripheral targets leads to retrograde transynaptic passage of virus through parallel circuits, and, ultimately, to coinfection to neurons that collateralize to innervate both circuits.

are efficiently expressed in quantities that can be detected immunohistochemically, and (3) availability of specific antisera generated in different species that will produce reliable localizations of each reporter protein.

Injection of the Recombinant Viruses

The single most important aspect of the injections is to ensure that syringes are not contaminated with both viruses. The best way to ensure this is to dedicate separate syringes to the different viruses. Otherwise, the procedures established in the single injection analysis of each recombinant should be reproduced in the dual-injection studies.

Post-Inoculation Survival and Tissue Processing

As noted above, the timing of the two injections should be adjusted to optimize the possibility that both viruses will reach the collateralized population of neurons at the same approximate post-inoculation interval. Nevertheless, temporal analysis should still be conducted to ensure that both of the recombinant viruses are moving through circuitry according to the temporal profiles established in the single-injection analyses. Tissue sections should be processed using the dual-labeling immunofluorescence protocol detailed in Support Protocol 3.

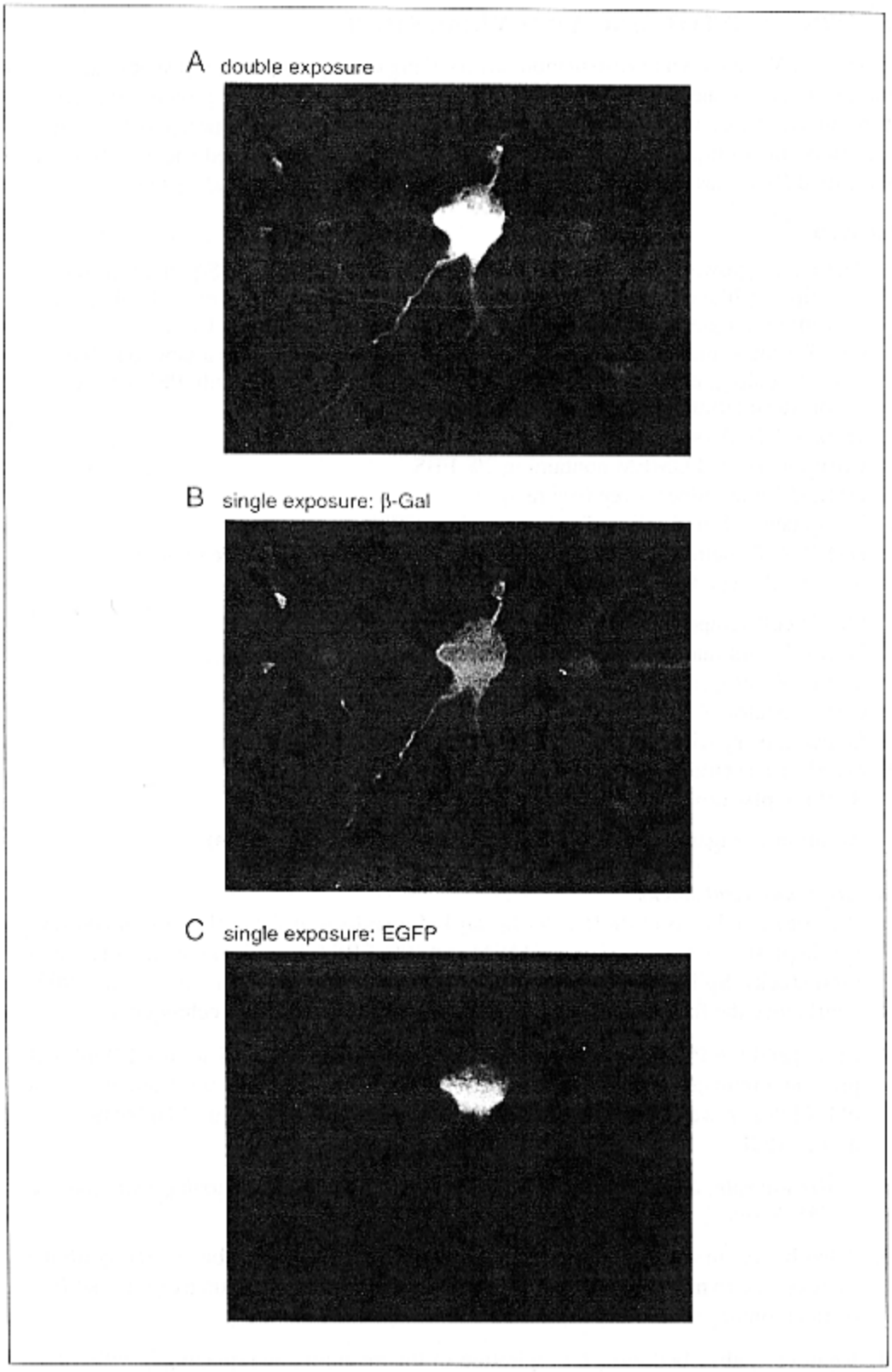

Figure 1.5.4 An example of a neuron coinfected with both of the viruses described in Figure 1.5.3. Dual-labeling immunofluorescence was used to localize the two transgenes. Through the use of appropriate filters, the fluorophors can be visualized either simultaneously (**A**) or individually (**B**,**C**). Yellow fluorescence marks the colocalization of the red (Cy3) and green (Cy2) fluorescence. Note that β-galactosidase immunoreactivity extends throughout the soma and dendrites of the neuron (panel B) whereas EGFP, which is fused to *Us9*, is differentially concentrated in paranuclear membranes of the cell soma (panel C). **See color figure.**

SUPPORT PROTOCOL 1

GROWING AND TITERING A PRV VIRAL STOCK

Stocks of PRV are grown in vitro in monolayers of pig kidney (PK15) cells using relatively straightforward tissue culture procedures. Some of the methods for growing the virus differ among strains (e.g., multiplicity of infection and incubation time). The following protocol produces high-titer (10^8 to 10^9 pfu/ml) stocks of an attenuated vaccine strain of PRV called PRV-Bartha and its derivatives (Bartha, 1961; Enquist et al., 1999).

Materials

PK15 cells grown in 100-mm dishes containing DMEM/10% FBS/pen-strep (see recipe). Although not commercially available, these cells can be easily obtained from investigators who routinely work with PRV (e.g., Patrick Card)
PRV-Bartha—and other strains of PRV—are not commercially available but, like PK15 cells, can be readily obtained from investigators who study PRV or use it for transneuronal analysis (e.g., Patrick Card)
Trypsin-EDTA (see recipe)
Unsupplemented DMEM containing 2% FBS
DMEM/2% FBS/pen-strep (see recipe)
Phosphate-buffered saline (PBS; see recipe), 37°C
DMEM/1% methocel/sodium bicarbonate/2% FBS/pen-strep (see recipe)
0.5% methylene blue in 70% methanol

Plastic cell scraper
Sterile 1.7-ml microcentrifuge tubes, snap cap
Sterile 50-ml screw-cap plastic tubes
Cup sonicator
Screw-cap cryovials vials
6-well tissue culture plates
Rocking platform

Additional reagents and equipment for tissue culture (*APPENDIX 3B*)

Prepare crude viral stocks

1. Prepare monolayers of PK15 cells the day before infection. Grow the cells in 100-mm sterile plastic dishes containing DMEM and 10% FBS (typically three plates for each viral stock). Split the cells using trypsin-EDTA so that the monolayers reach 90% confluence the following day (see *APPENDIX 3B* for tissue culture techniques).

2. Resuspend the PRV-Bartha virus in DMEM containing 2% FBS so that 1.0 ml will provide a multiplicity of infection (MOI) of 0.01. Aspirate medium from each dish of PK15 cells, wash once with PBS and aspirate, and add the virus in 1.0 ml of medium at that MOI.

   For example, infect 3×10^6 PK15 cells with 3×10^4 plaque forming units (pfu) of PRV-Bartha.

3. Adsorb virus to cells for 1 hr in a humidified 37°C, 5% CO_2 incubator. Gently tilt the plates every 15 min to ensure that the medium is uniformly distributed over the surface of the monolayer.

4. Remove unabsorbed virus by aspiration of the medium and replacing it with 10 ml of fresh 37°C DMEM/2% FBS/pen-strep.

5. Incubate monolayers for 2 to 3 days in a humidified 37°C, 5% CO_2 incubator. When the monolayer shows extensive cytopathic effects, harvest the cells and medium with a plastic scraper. Combine the contents of all infected plates in one 50-ml, screwcap conical plastic tube. Mix the contents carefully. From this tube, transfer 1 ml to each

of ten microcentrifuge tubes. Store the remaining crude stock in the 50-ml screw cap tube to be used as backup. Freeze all tubes at –70°C.

The length of time to incubate infected monolayers will depend upon the rate at which virus spreads through the monolayer. This is monitored by the appearance of cytopathic changes in infected cells which can be determined by periodically examining the plate of cells with an inverted microscope. Visible cytopathic effects are the distinct rounding of cells and margination of the chromatin.

These crude stocks can be stored frozen at –70°C for long periods of time or can be used immediately for the preparation of purified "neurostocks" according to steps 6 to 10.

Prepare neurostocks

6. If the crude stocks from step 5 were stored frozen, rapidly thaw the required amount in a 37°C water bath and gently mix the samples every 5 min until all ice disappears. Place the individual samples on wet ice and then sonicate each sealed tube using a cup sonicator (10 pulses for a total of 10 sec at an amplitude of 80%) to disperse aggregated virus and separate virions from cellular debris. Invert the samples to mix contents and store on ice in preparation for division into aliquots.

   Keep the tubes sealed during the sonication to avoid creation of aerosols.

7. Divide the viral stock into 1-ml aliquots in sterile 1.7-ml microcentrifuge tubes or screw-cap cryovials. Either store the vials at –70°C or continue with the separation of cellular debris to prepare the neurostock.

   Use one of these vials to determine the titer of the stock (see steps 11 to 18).

8. If the virus has been stored frozen, thaw and sonicate each sample as described in step 6 before proceeding with the rest of the purification. Remove the cellular debris from each sample by centrifuging for 5 min at 2000 × *g* (5000 rpm in the Fotodyne 24 place rotor) at room temperature.

9. Pipet off the supernatant, being careful to exclude the cell debris in the loose pellet at the bottom of each tube.

10. Divide the supernatant containing purified virus into 100- to 200-µl aliquots in screw-cap cryovials, snap-freeze on dry ice, and store at –70°C.

    Do not aliquot in volumes less than 50 µl since this may produce freezing artifacts that reduce the titer.

    Virus can be stored frozen in aliquots at –70°C for extended periods of time without consequence as long as the temperature remains ≤–70°C. Under no circumstances should these samples be thawed and refrozen, as this will reduce the titer of the sample. To help detect freezer failures or unexpected increases in temperature, the samples should be stored with Cryoguard M-40 thermal exposure indicators (Controlled Chemicals). These indicators remain green at temperatures below –50°C but turn irreversibly red when the temperature rises above –40°C. Samples in a box containing red thermal indicators should be discarded.

Determine virus concentration or titer in plaque-forming units (pfu)

11. Prepare monolayers of PK15 cells the day before the assay is to be performed by splitting a 100-mm dish of cells that has reached confluence and plating the cells in to three 6-well tissue culture plates.

    Cells should reach about 90% confluence at the time of use in step 14.

12. Rapidly thaw and sonicate a 1-ml tube of crude stock (from step 7) using the procedures in step 6. If the titer is being determined from a neurostock (step 10), it is not necessary to sonicate the sample.

13. Prepare a series of 10-fold dilutions of virus as follows.

    a. Place 0.9 ml of DMEM/2% FBS/pen-strep in each of seven sterile microcentrifuge tubes.

    b. Add 0.1 ml of the viral stock to the first tube, mix well and add 0.1 ml of that mixture to the second tube.

    c. Repeat this procedure using new tubes for the desired number of dilutions.

14. Aspirate the medium from each well of the 6-well plates and wash the cells with 37°C PBS. Add 0.2 ml each of the 10^{-5}, 10^{-6}, and 10^{-7} virus dilution to duplicate wells and return the plates to the incubator.

15. Adsorb the virus to the monolayers in each well for 1 hr in a humidified 37°C, 5% CO_2 incubator, taking care to rock the plate every 15 min to ensure that there is an even distribution of virus over the monolayer.

16. Remove the plates from the incubator and aspirate the inoculum from each well. Next, add 3 ml of DMEM/1% methocel/sodium bicarbonate/2% FBS/pen-strep. Return the plates to the incubator and leave them undisturbed for 2 to 3 days.

    This viscous solution facilitates the formation of foci of virus spread (plaque formation) by restricting the diffusion of virus from infected cells.

    Leaving the plates undisturbed is essential to avoid interfering with plaque formation on the monolayer, thereby ensuring accurate determination of the titer of the viral stock. PRV-Bartha titers can be reliably determined by 2 days of incubation, but other strains may require a longer incubation period.

17. Remove the plates from the incubator and aspirate the methocel solution from each plate. Wash each well once with 37°C PBS and then add 1 ml of 0.5% methylene blue in 70% methanol. Place the plates on a rocking platform for 10 min at room temperature to ensure even staining of the monolayer. Lastly, rinse off the excess stain under a gentle stream of tap water to visualize the viral plaques.

    Plaques result from virus-induced lysis of cells in the monolayer and appear as clear holes in the monolayer surrounded by a halo of rounded cells. The staining procedure kills both virus and cells.

18. To calculate the titer in plaque-forming units per ml (pfu/ml), count the total number of plaques on all countable plates, divide by the total volume plated on these plates based on the lowest dilution giving countable numbers of plaques, and multiply by the reciprocal of the lowest dilution that gave countable plates.

    As an example, if there are 12 plaques in the well containing 0.2 ml of the 10^{-7} dilution and 175 plaques in the well containing 0.2 ml of the 10^{-6} dilution, the total number of plaques would be 187 plaques. The volume plated would be 0.22 ml of the 10^{-6} dilution. Thus, 187 plaques divided by 0.22 ml times 10^6 gives you a titer of 8.5×10^8 pfu/ml. This method is more accurate than determining the titer based on 12 plaques, or a titer based on the average of titers determined for each dilution.

IMMUNOHISTOCHEMICAL PROCESSING AND DETECTION

SUPPORT PROTOCOL 2

These experiments are labor intensive, and it is obviously beneficial to obtain the maximum amount of information from each case. For example, it may become desirable to establish the phenotype of neurons that constitute a polysynaptic circuit defined by viral transport. Since one cannot predict all possible components of a circuit that may be defined by viral transport, there is a necessary delay in the ability to perform the phenotypic analysis. This delay could be problematic if measures were not taken to protect the antigenicity of the tissue. The following methods of tissue processing and storage are designed to protect antigenicity and thereby maximize the amount of information that can be obtained from an experiment. Central to this approach is the storage of sectioned tissue in a glycol-based cryoprotectant that preserves tissue antigenicity for extended periods.

Localization of viral antigens with the immunoperoxidase method provides a reliable first step in defining and documenting the extent of viral transport through neuronal circuitry in each case. One can then further dissect the organization and phenotype of the circuit using dual-labeling methods. The avidin-biotin modification (Hsu et al., 1981) of the peroxidase-antiperoxidase method is presented for this purpose. It produces excellent signal against a low background with high dilutions of primary antibody.

Materials

Perfused tissue from experimental animal (see Basic Protocol 1 or 2)
PLP fixative (see recipe; McLean and Nakane, 1974)
0.1 M and 10 mM sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
20% to 30% (w/v) sucrose in 0.1 M sodium phosphate buffer, pH 7.4
Glycol-based cryoprotectant (see recipe; Watson et al., 1986)
0.5% (w/v) sodium borohydride in PBS (prepare fresh; optional)
0.5% (v/v) H_2O_2/30% (v/v) methanol in PBS (prepare fresh; optional)
Primary antibody solution (see recipe)
Biotinylated, affinity-purified secondary antibody against IgG of species used to raise primary antibody
Normal serum generated in same species as secondary antibody
10% (v/v) Triton X-100
Vectastain Elite kit (Vector Laboratories)
Diaminobenzidine (DAB)
Tris-buffered saline (TBS), pH 7.6 (*APPENDIX 2A*)
30% H_2O_2
50%, 70%, 95%, and 100% ethanol
Xylene
Bleach
Resin for mounting coverslips (Cytoseal 60; Stephens Scientific)
Freezing microtome and chucks
Rocking platform
Plexiglas compartments with porous nets on the bottom (Brain Research Laboratories)
Gelatin-coated (subbed) microscope slides (*UNIT 1.1*)
Coverslips

Prepare tissue

1. Remove perfused tissue and post-fix it in PLP fixative for 2 to 4 hr at 4°C.

   This is sufficient to preserve tissue structure without compromising antigenicity.

2. Rinse the tissue in 0.1 M sodium phosphate buffer, pH 7.4, and place it in a vial containing 20% to 30% sucrose in 0.1 M sodium phosphate buffer at 4°C, until the tissue sinks to the bottom of the vial, which indicates that satisfactory cryoprotection has been achieved.

This generally occurs within 12 to 24 hr at 4°C.

Section tissue

3. Bisect the brain with a cut at the level of the midbrain and place the two halves on the chuck of a freezing microtome (also see *UNIT 1.1* for sectioning procedures). Freeze the tissue to −50°C and section it in the coronal plane at 35 µm per section throughout the rostrocaudal extent of the forebrain and brainstem. Place sections sequentially into six 20-ml vials of glycol-based cryoprotectant. Repeat this sequential collection of sections until all of the tissue has been sectioned. Each vial with then contain a representative sample of sections through the brain at a frequency of 210 µm.

This frequency provides an accurate sampling of all regions of the neuraxis.

4. Store the tissue in the glycol-based cryoprotectant at −20°C.

This solution preserves antigenicity of tissue for periods in excess of 6 years. This has the obvious benefit of permitting a systematic analysis of the synaptology and phenotype of neurons in a polysynaptic circuit defined by viral transport.

Remove cryoprotectant from tissue

5. Place the tissue in Plexiglas compartments with porous nets on the bottom and remove the cryoprotectant with four 15-min washes of 0.1 M sodium phosphate buffer, pH 7.4 (100 ml per wash), with agitation on a rocker platform.

These chambers are available in a number of different sizes and allow easy movement of tissue through multiple buffer changes.

The cryoprotectant is fairly viscous and has a tendency to adhere to tissue. As a result, if it is not completely removed, it will prevent antibody penetration and produce patchy, unreliable staining. If patchy staining is routinely encountered, increase the number of washes.

6. *Optional:* Reduce problems with high background by pretreating the tissue at this point as follows.

   a. Wash for 15 min in 0.5% sodium borohydride in PBS.
   b. Wash four times as described in step 5, each time for 15 min in 0.1 M sodium phosphate buffer.
   c. Wash for 15 min in 0.5% H_2O_2/30% methanol in PBS.
   d. Wash four times, each time for 15 min in 0.1 M sodium phosphate buffer.

This pretreatment eliminates background without compromising antigenicity.

7. Transfer the tissue to the primary antibody solution (Table 1.5.1) for localization of infected neurons. Dilute the primary with PBS containing Triton X-100 and normal serum according to Table 1.5.1. Incubate tissue 24 to 48 hr in the primary antiserum at 4°C, and then allow it to warm to room temperature over 30 min.

If necessary, the length of this step can be extended to 3 hr without compromising viral antigenicity.

8. Wash the tissue four times with agitation at room temperature, each time for 15 min in 10 mM sodium phosphate buffer, pH 7.4.

9. Dilute the biotinylated affinity-purified secondary antibody 1:200 by mixing the following:

   5 µl of biotinylated secondary antibody
   20 µl of normal serum generated in same species as secondary antibody
   30 µl of 10% (v/v) Triton X-100
   945 µl PBS.

10. Place the tissue in the diluted secondary antibody solution and incubate 60 to 90 min at room temperature.

11. Wash tissue as in step 8.

12. Combine 5 µl each of Vectastain Elite kit components A and B 90 min prior to use. Just prior to use, bring to a final volume of 1 ml with 960 µl PBS and 30 µl of 10% Triton X-100. Incubate tissue in this solution for 90 to 120 min at room temperature with agitation.

    This solution will generate the avidin-biotin complex, which in this kit is conjugated to horseradish peroxidase, and may be detected with DAB and hydrogen peroxide as in the following steps.

13. Wash with buffer as in step 8.

14. Prepare a "saturated" solution of diaminobenzidine (DAB) in TBS, pH 7.6. Filter the solution prior to use.

    Saturated in this context is defined as the amount of DAB that will go into a solution during 5 min of vigorous stirring at room temperature (do this in a hood). This eliminates the necessity of weighing out this carcinogen.

15. Preincubate the tissue in the saturated DAB solution for 10 min at room temperature with occasional agitation.

16. Add 35 µl of H_2O_2 per 100 ml of DAB solution and monitor the reaction visually by occasionally examining a section under the microscope. To accomplish this use a paintbrush with fine bristles to transfer a section to a Tris buffer bath and then use

Table 1.5.1 Preparation of a Dilution Series for Primary Antibody Incubation

Final dilution	Stock (µl)	Serum (µl)	TX-100 (µl)[a]	PBS (µl)
Using a 1:10 stock of primary antibody				
1:1,000	10	10	30	950
1:2,000	5	10	30	955
1:5,000	2	10	30	958
Using a 1:100 stock of primary antibody				
1:1,000	100	10	30	860
1:10,000	10	10	30	950
1:20,000	5	10	30	955
Using a 1:500 stock of primary antibody				
1:10,000	50	10	30	910
1:20,000	25	10	30	935
1:50,000	10	10	30	950

[a]The volumes of Triton X-100 are of a 10% solution. This diluted solution can be more reliably pipetted than the viscous concentrated solution sold by the manufacturer.

the brush to manipulate the section onto the portion of a microscope slide that has been submerged in the buffer. (Make sure that the slide is not gelatin coated, since the tissue section will be returned to the DAB solution following examination.) Briefly examine the wet section under the microscope to determine the density of the immunoperoxidase reaction product relative to background staining.

The best staining is achieved when the specific signal is prominent and the background is low. Of course, this can only be determined in tissue sections that contain infected neurons, so it is important to make an informed selection of the section that will be analyzed.

The goal of this exercise is to optimize specific staining (i.e., produce the most intense signal) while keeping the background staining low. This is typically achieved within 3 min when the reagents are used at the recommended dilutions.

17. Terminate the reaction by washing the tissue in multiple changes of fresh sodium phosphate buffer. Inactivate the DAB solution, and all washes, with bleach, and then dispose of the solutions according to the biohazard regulations of your institution.

18. Organize the sections from rostral to caudal in a dish of buffer and then mount the sections on gelatin-coated slides and let them dry on the slides overnight at room temperature.

19. Dehydrate the slides using an ethanol series (ethanol solutions of 50%, 70%, 95%, 95% again, 100%, and then 100% again, 10 min each). Clear the slides in three changes of xylene (15 min each), and coverslip with resin for microscopic observation.

SUPPORT PROTOCOL 3

DUAL IMMUNOFLUORESCENCE LOCALIZATION

Dual localizations of multiple antigens provide a powerful method for further functional dissection of a polysynaptic circuit. Different-color fluorophores conjugated to species-specific secondary antibodies can be used to determine the phenotype of neurons that contribute to a multisynaptic projection, or for simultaneous localization of recombinant viruses that express unique reporters (Figs. 1.5.3 and 1.5.4). This approach has been greatly aided by the recent development of fluorophores that are resistant to fading and that can be dehydrated, cleared, and coverslipped.

Materials

Sectioned tissue in cryoprotectant (Support Protocol 2)
Primary antibodies generated against PRV or reporter proteins (see note below)
Primary antibodies generated against phenotypic markers of neurons that contribute to the circuit of interest. These antibodies should be generated in a species different from those raised against PRV (see recipe).
Secondary antibodies generated against the IgG of the two species used for the primary antibodies, conjugated, respectively to Cy2 and Cy3 (Jackson ImmunoResearch Laboratories)

Light-proof vials
50%, 70%, 95%, 100% ethanol
Xylene
Mounting media (Cytoseal 60; Stephens Scientific)
Coverslips
Fluorescence microscope (*UNIT 2.1*)

Additional reagents and equipment fir immunohistochemical processing and detection of tissues (see Support Protocol 2) and fluorescence microscopy (*UNIT 2.1*)

NOTE: Antibodies against reporter proteins should be generated in different species. The reporters most commonly used in the construction of recombinant strains of PRV are β-galactosidase and the enhanced green fluorescent protein (EGFP). Antibodies generated against both of these reporters are commercially available from a variety of vendors.

1. Wash cryoprotectant from tissue using the same method described in the immunoperoxidase procedure (see Support Protocol 2, step 5).
2. Place the tissue in a mixture of the two primary antibodies diluted with PBS containing Triton X-100 and normal serum according to Table 1.5.1. Incubate 24 to 48 hr at 4°C.

   The antibodies must be generated in different species to prevent cross-reactivity and maintain the specificity of immunolabeling.

   The ideal dilution for immunofluorescence labeling using the following procedures is 1/10th the dilution that produces ideal labeling with the immunoperoxidase method described in Support Protocol 2. Thus, if an antibody is used at a 1:20,000 dilution for immunoperoxidase localizations, one can assume that it will work well at 1:2000 for immunofluorescence. However, this should be determined directly in separate parametric studies with each antiserum before initiating the dual labeling investigations.
3. Wash the tissue with multiple changes of 10 mM sodium phosphate buffer, pH 7.4, over 30 min at room temperature with agitation.
4. In light-proof vials, dilute the Cy2- and Cy3-conjugated, species-specific secondary antibodies to a final concentration of 1:500 with PBS and incubate the tissue for 2 hr in this solution at room temperature with agitation.

   These fluorophores produce green (Cy2) or red (Cy3) fluorescence and, unlike FITC and rhodamine, are resistant to fading. Cy2 and Cy3 are not as sensitive to light as other fluorophores; nevertheless, the incubations should be performed in light-proof vials and the processed slides stored in light-tight boxes to produce and preserve the optimal signal.
5. Wash the tissue as in step 3.
6. Mount the sections on gelatin-coated slides and air dry overnight. Dehydrate and coverslip the sections as described in step 19 of Support Protocol 2.
7. Examine slides using a fluorescence microscope (*UNIT 2.1*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DMEM/1% methocel/sodium bicarbonate/2% FBS/pen-strep

Weigh out a 2% (w/v) solution of methocel (methylcellulose) in distilled water. Add a stir-bar and shake to disperse. Autoclave 30 min using a liquid cycle; be sure to keep the stir-bar in the solution during autoclaving. Resuspend the autoclaved methocel by stirring overnight at 4°C. Prepare the final solution in a sterile bottle by mixing equal volumes of 2× DMEM and the methocel suspension. Add 2.5 ml of 100× pen-strep (Life Technologies) to 500 ml of the final solution. Add 25 ml of 7.5% (w/v) sodium bicarbonate per 500 ml medium to provide optimal buffering capacity. Lastly, add heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*) for a final concentration of 2%. Store up to 3 months at 4°C

Table 1.5.2 Components of Fixative Solution

	100 ml	250 ml	500 ml	1000 ml
Paraformaldehyde	4.00 g	10.00 g	20.00 g	40.00 g
Sodium metaperiodate	0.22 g	0.54 g	1.10 g	2.14 g
Lysine	1.37 g	3.43 g	6.85 g	13.70 g

DMEM/2% (or 10%) FBS/pen-strep

Modify Dulbecco's modified Eagles medium (DMEM; available commercially from a variety of vendors; store 100-ml and 500-ml aliquots in sterile screw-cap bottles) as follows:

Just prior to use, add:
100 U/ml penicillin G
100 µg/ml streptomycin
Sodium bicarbonate to 7.5% (w/v) final
Heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*) to 2% or 10%
Store prepared medium up to 3 months at 4°C

Use only qualified, performance-tested, mycoplasma-, virus-, and endotoxin-tested FBS for tissue culture. It is best to use the same lot number as much as possible.

Glycol-based cryoprotectant

300 g sucrose
10 g polyvinyl-pyrrolidone (PVP-40)
500 ml 0.1 M sodium phosphate buffer, pH 7.2
300 ml ethylene glycol

Mix 200 g of sucrose and 10 g of PVP-40 in a 1500-ml beaker. Add 500 ml 0.1 M phosphate buffer slowly and stir until the sucrose and PVP-40 dissolve. Add the remaining (100 g) sucrose with stirring. Stir in the ethylene glycol. Add distilled water to bring the final volume to 1000 ml. Store indefinitely at –20°C.

Phosphate-buffered saline

0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*)
2.7 mM KCl
1.5 mM KH_2PO_4
137 mM NaCl
Store up to 6 months at room temperature

PLP fixative

Heat the desired volume of 0.1 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*), to 70°C in a hood on a hot plate with stirring. Add the appropriate amount of paraformaldehyde and stir vigorously until the solution clears. Chill the solution to room temperature on ice and then add the appropriate amounts of periodate and lysine (see Table 1.5.2) while stirring. Filter the solution and store at 4°C until it is used for perfusion.

It is best to use this solution the same day it is made.

Primary antibody solution

The best signal is achieved with a polyclonal antiserum generated against inactivated virus. The advantage of this approach is that the immunoreactivity will identify all virally encoded proteins and thereby permit identification of neurons at all stages of productive infection (see Anticipated Results). Although not commercially available, numerous laboratories have generated antisera specific for PRV and are

continued

willing to provide aliquots to those interested in conducting tracing studies with PRV (e.g., Patrick Card). However, if this experimental approach is integral to the research focus, these specialized reagents should be generated in-house. For PRV, the authors have raised rabbit polyclonal antibodies against acetone-inactivated wild-type virus. These antibodies provide very reliable staining at dilutions of 1:10,000. Dilute antibodies with 10 mM sodium phosphate buffer, pH 7.4 (*APPENDIX 2A*), containing 0.3% (v/v) Triton X-100 to increase antibody penetration and 1% normal serum to reduce background. The normal serum should be of the same species used to generate the secondary antibodies. A final volume of 1 ml is adequate for incubation of one vial of tissue. Table 1.5.1 provides a convenient means of preparing various dilutions of antiserum.

Trypsin-EDTA

Obtain the stock solution containing 0.5% trypsin and 5.3 mM EDTA (Life Technologies) and store this stock at –20°C in 2-ml aliquots. To prepare the final working concentrations for tissue culture, dilute in PBS (see recipe) to 0.1% trypsin and 1.06 mM EDTA.

COMMENTARY

Background Information

Viral transneuronal analysis shares many of the methodological considerations important for the successful use of classical anterograde and retrograde monosynaptic tracers. Nevertheless, the viral transneuronal method differs considerably from classical methods in that it is dependent upon the tropism, invasiveness, and life cycle of live pathogens. The fact that these pathogens exploit the polarized architecture and synaptic associations of neurons provides the fundamental basis for the method and emphasizes the importance of understanding the biological mechanisms through which these pathogens parasitize the nervous system. This mechanistic foundation has provided much of the support for the specificity of transynaptic passage of virus and is central to successful application of the method. The following sections review issues fundamental to the successful use of PRV for transneuronal analysis. However, for those who wish to apply the method to seek a more detailed understanding of the life cycle of PRV and other alpha herpesviruses, Rixon (1993), Card (1995), and Enquist et al. (1999) provide good reviews of the literature in this area.

Critical Parameters

Safe handling of viruses

PRV and other alpha herpesviruses are designated class 2 infectious agents on the basis of CDC/NIH criteria. Although PRV is not a human pathogen, the biosafety level 2 (BSL-2) classification mandates that all experiments be conducted in a laboratory that meets the criteria defined in Health and Human Services Publication #88-8395 (*Biosafety in Microbiological and Biomedical Laboratories*). The regulations in that publication stipulate that BSL-2 laboratories (1) be dedicated to the viral studies and have restricted access, (2) contain a biosafety cabinet for handling of virus, (3) contain a HEPA-filtered housing unit for infected animals, and (4) have containers for disposal of infectious waste. Procedures mandated for experimental procedures in BSL-2 laboratories and for disposal of waste are also detailed. Some institutions interpret these regulations differently, so investigators should check with their respective institutional biosafety officers regarding the regulations, to obtain approval for conducting the studies. Importantly, action plans for dealing with accidents should be approved by the institutional biosafety official and discussed with all personnel participating in the study.

Maintaining viable virus stocks

Several features are central to the successful application of the transneuronal tracing method. However, none are more important than the generation and maintenance of high-titer viral stocks. Infection of an animal requires delivery of a minimum concentration of biologically active virus. Thus, it is absolutely essential to maintain high-titer stocks of virus in order to ensure consistent and reproducible results. In parametric analyses, a minimum titer of 10^5 pfu is necessary to achieve productive replication of PRV in 100% of injected animals

(Card et al., 1995). When viral titer is reduced by a single log unit, the percentage of animals that become infected drops to ~20%. Additionally, studies involving intracerebral injection have shown that the onset and progression of viral infection within a circuit is dependent, at least in part, upon the concentration of the inoculum (Card et al., 1999). Thus, in general, the higher the concentration of virus, the earlier the onset of viral replication within a multisynaptic circuit. Of course, the number of cells susceptible to infection at the site of virus injection is also a critical parameter.

The lipid envelope of alpha herpesviruses is essential for infection of permissive cells, so anything that damages this envelope and its constituent proteins will reduce the titer of the stock, often dramatically. Ultraviolet light, bleach, detergents, alcohols, aldehydes, and organic solvents all reduce viral titer in this fashion. Ice-crystal formation resulting from freeze-thaw cycles also reduces titer through damage to the viral envelope. As a general rule, the small volumes of virus (100 to 200 µl) that result from the division of the neurostocks into aliquots should not be thawed and refrozen. Rather they should be thawed immediately prior to use and the unused portion should be inactivated and discarded. Similarly, neurostocks should be transferred immediately to a low-temperature freezer and stored at less than or equal to −70°C until use. Storing these aliquots at temperatures higher than −70°C will reduce their titer. Stocks should never be frozen at −20°C under any circumstances, as titer drops four to five log units due to ice formation, with resulting virion damage. Also, it is important to avoid creation of large surface areas exposed to air during frozen storage (e.g., placing small volumes in large tubes) as this practice invariably results in reduced titers upon thawing.

It is often desirable to use a single aliquot of virus to inject more than one animal during the same sitting. If this is the case, the virus should be keep cool by storing vials on wet ice during the period throughout the multiple surgeries. In unpublished studies, the authors have determined that the titer of PRV-Bartha or its derivatives will not exhibit a detectable reduction over a period of 3 hr when stored in this manner.

Selecting a strain of virus

Although directionally selective strains of HSV have been identified (Zemanick et al., 1991), there is no known strain of PRV that is transported only in the anterograde direction through CNS circuitry. PRV-Bartha and related strains are well known for their selective retrograde transport through the brain following inoculation of peripheral targets innervated by autonomic or somatic motor neurons. These strains also move selectively in the retrograde direction after intracerebral injection (Kaufman et al., 1996; Jasmin et al., 1997; O'Donnell et al., 1997; Card et al., 1998, 1999; Chen et al., 1999). However, PRV-Bartha has the capacity to infect CNS neurons by anterograde transport of virus through visual circuitry after injection into retina (Card and Enquist, 1995). This emphasizes the importance of rigorously characterizing the invasiveness of each strain of virus, particularly if it has not been examined in prior studies.

The use of recombinant viruses expressing reporter proteins, such as β-galactosidase or EGFP, in dual-injection paradigms imposes additional demands upon selecting and characterizing the strains of virus that will be used in the analysis. Ideally, the two recombinants will only differ in the transgenes that they contain. In other words, the genome of the two strains should be identical with the exception of the reporter genes that provide the unique identifier of each virus. The goal is to have two strains that are attenuated for virulence, equivalent in their invasive characteristics, and which have the ability to simultaneously replicate in the same neurons. Prior studies have shown that it is possible to coinfect neurons with strains of virus that are not isogenic (Jansen et al., 1995; Levatte et al., 1998; Kim et al., 1999). However, the use of nonisogenic viruses that differ in invasiveness and virulence can produce false negatives that result from one strain of virus interfering with the ability of the second strain to replicate in the same neurons (Kim et al., 1999). This interference is more prevalent when the virulence of one strain exceeds that of the other. Construction of isogenic strains is designed to reduce the variables that can lead to this type of interference.

Recombinant viruses must be evaluated for the efficiency of transgene expression in the paradigm under investigation, especially when transgene expression is the sole indicator of viral invasiveness. It is therefore important to define both the magnitude and temporal expression of transgenes in single-injection studies, examining the invasiveness of the recombinants in the same circuitry that will be the subject of the dual-injection experiments. Dual-immunofluorescence localizations of transgenes and viral immunoreactivity pro-

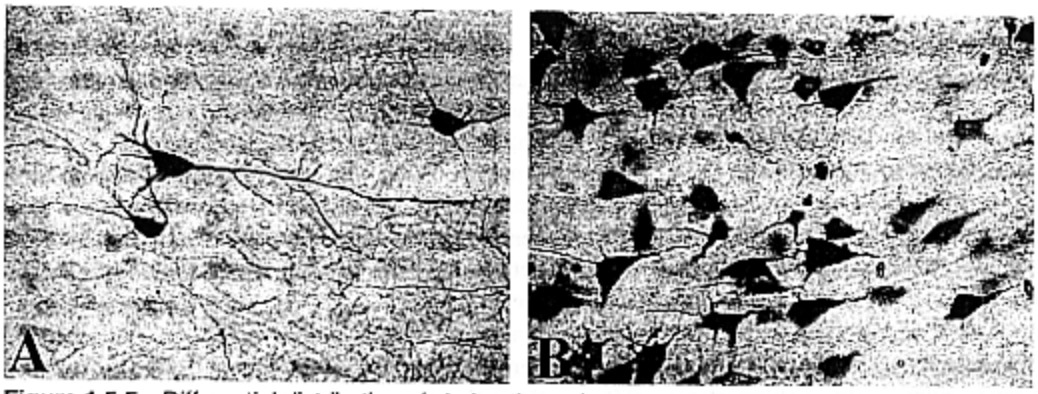

Figure 1.5.5 Differential distribution of viral antigens in pyramidal neurons infected with different strains of PRV. In both instances, the neurons were infected by retrograde transport of virus injected into the medial prefrontal cortex (see Card et al., 1998, for more detail). **(A)** Dense and extensive staining of the somatodendritic compartment of a pyramidal neuron 36 hr following injection of PRV-Bartha into the prefrontal cortex. **(B)** In contrast, viral immunoreactivity is sequestered within the somata and proximal dendrites of pyramidal neurons infected with a wild-type strain of PRV, even though the postinoculation interval is >10 hr longer.

vides the most rigorous determination of the effectiveness of the transgene as an identifier of the invasiveness of recombinants. The two fluorophores identifying the location of the transgenes and viral immunoreactivity should be entirely colocalized in these single-injection experiments. If neurons exhibit viral antigen—but no transgene immunoreactivity—then the transgene is not being expressed by all infected neurons. If this is the case, one should either seek out another recombinant strain or determine if the measures outlined below (see Troubleshooting) can solve the problem.

The cellular staining resulting from viral replication is another important feature that should be evaluated when selecting a strain of virus. PRV-Bartha and other strains produce a robust staining of the somatodendritic compartments of infected neurons that often produces "Golgi-like" labeling after prolonged replication. This extensive staining is quite different from the more restricted cellular staining that results from replication of the wild-type strain of PRV known as Becker (Fig. 1.5.5; Card et al., 1998). The intracellular distribution of reporter proteins expressed by recombinant viruses can also vary substantially from one strain to another. This differential localization has utility in dual-labeling strategies. For example, a strain of PRV in which the *lacZ* gene was inserted into the *gG* locus of the unique short region of the viral genome produces the reporter β-galactosidase, which is widely distributed throughout the infected cell and its dendrites (Fig. 1.5.4; Standish et al., 1995). In contrast, another recombinant virus containing a different transgene at the same locus of PRV-Bartha characteristically exhibits a much more restricted distribution of the transgene product. The latter transgene consists of the jellyfish enhanced green fluorescent protein (*EGFP*) fused to a PRV gene (*Us9*) that is absent from PRV-Bartha. In this case, *Us9* and the fused *EGFP* reporter differentially accumulate within the trans-Golgi network of infected neurons, producing a localized, but dense, paranuclear label (Fig. 1.5.4; Brideau et al., 1999). This restricted distribution is not ideal for single-infection studies, since it does not effectively define cellular morphology. However, it is quite useful in double-infection studies where the EGFP immunofluorescence produces a distinct paranuclear fluorescence within neurons that are more extensively labeled with the transgene product (β-galactosidase) of the second virus (Fig. 1.5.4).

Designing an experiment

Aside from selection of the virus that will be used, there are four primary considerations that should be evaluated when designing a transneuronal tracing study. These include (1) the amount of virus that will be injected, 2) the cytoarchitecture of the area of injection, (3) the distance that the virus must travel and the estimated number of neurons that may contribute to the circuit, and (4) the number of post-inoculation survival time points that should be examined to document the sequential movement of virus through the elements of the polysynaptic circuit. Collectively, these factors exert impor-

tant influences upon the outcome and interpretation of an experiment.

Concentration of injected virus and cytoarchitecture of injection site

The concentration of virus in pfu per injected volume necessary to define a circuit is heavily dependent upon the cytoarchitecture of the area of inoculation. This is in large part due to the variable affinities of alpha herpesviruses for different cellular receptors found on cells of the tissue under study (Vahlne et al., 1978, 1980; Marchand and Schwab, 1987), and the demonstration that infectivity and the onset of viral replication in a circuit is concentration dependent (Card et al., 1995, 1999). PRV and HSV have very high affinities for axon terminals, astroglia, and extracellular matrix proteins; diffusion of virus from the injection site is quite restricted in areas where these elements are present in high concentration. In contrast, when they are present in low density, or when the area of injection has a high extracellular fluid volume, virus diffusion will be greater and the concentration of virus accumulated by permissive profiles within the region of injection will be lower. As a result, larger amounts of virus are necessary to elicit a productive replication of virus in these circumstances. The importance of considering these factors in planning and executing an experiment is illustrated by the following examples.

A comparison of the invasiveness of PRV following injection of the kidney and spleen effectively illustrates the dramatic influences that tissue architecture exerts upon the outcome of infection. The spleen is a highly vascular organ in which an "open" circulation substantially increases the fluid content of its parenchyma relative to other organs. In contrast, the kidney contains a "closed" circulation network in which capillary beds shunt blood directly from the arterioles to venioles. This difference in architecture dramatically influences the amount of virus that must be injected to elicit a productive replication of virus in the neurons innervating each of these organs (Cano et al., 1997, 1999). The increased fluid content of the spleen parenchyma causes an immediate dilution of virus injected into this organ that is not observed after injection of the same concentration of virus into the kidney. As a result, 1 µl of virus (1×10^8 pfu/ml) produces a robust infection of neurons innervating the kidney, while 6 µl of the same viral stock must be injected into the spleen to elicit an infection of the neurons giving rise to the spleenic innervation.

Intracerebral injection of PRV requires the same consideration of tissue architecture. The high affinity of PRV for axon terminals has an important influence on the outcome of infection. The availability of virus that can invade neuronal profiles is directly related to the concentration of the injected virus, terminal field density, and the concentration of astroglia in the area of viral injection. These factors combine to provide an advantage for neurons that densely innervate the region of injection over those that provide a moderate or sparse innervation of the same region. This was demonstrated directly in an analysis of the uptake of different concentrations of PRV in the striatum (Card et al., 1999). Decreasing concentrations of PRV did not compromise the onset of viral replication in the dense nigrostriatal dopaminergic projection system, but caused a temporal delay or entirely blocked the onset of viral replication in other neurons giving rise to striatal afferents of moderate or sparse density (Card et al., 1999). Furthermore, the magnitude of the delay correlated with the density of innervation. When one considers the demonstrated affinity of alpha herpesviruses for astroglia and extracellular matrix molecules, it becomes apparent that several aspects of tissue architecture will influence the amount of virus available to neuronal elements in the region. Thus, knowledge of the tissue architecture of a region is essential for planing intracerebral studies, particularly when afferents of moderate or sparse density are central to the circuit of interest.

Length of transport and number of neurons in a circuit

The distance that the virus must travel through the brain and the number of neurons that must replicate the virus as it moves through a circuit have an important influence upon experimental design. It is probable that other variables (e.g., neuronal activity and cellular metabolism) also influence the progression of infection through a circuit, but these influences are more difficult to measure. Since the ultimate goal of a transneuronal study is to define the sequential passage of virus through a circuit, a temporal analysis including multiple postinoculation intervals should be an integral component of a transneuronal tracing study (Fig. 1.5.5). The timing and frequency of the postinoculation intervals will have to be determined empirically for each circuit, but a good strategy is to define the longest postinoculation timepoint that contains infected neurons in all of the

cell groups of interest, and then to work backwards toward the first-order neuron. Since ~6 hr is necessary for the production of infectious progeny by an infected cell, one can estimate this time point on the basis of the number of neurons that are thought to contribute to a circuit and the distance that separates them. Nevertheless, the unexpected inclusion of interneurons within a circuit, and other factors, may render these initial estimates inaccurate. Therefore, one should not be surprised if the initial results to not correspond with these estimates.

Verifying the route of viral transport

With advancing postinoculation survival, it becomes increasingly possible that a cell group became infected by a route other than that initially hypothesized. Similarly, it is often difficult to distinguish projection neurons, infected by retrograde transport of virus, from interneurons in the same area that were infected by transynaptic passage of virus from the infected projection neurons. The most direct way of addressing these issues is to verify the postulated route of viral transport by incorporating lesions or knife cuts in the experimental design and to use classical monosynaptic tracers in combination with the viral transneuronal analysis.

Lesions have proven to be crucial in eliminating alternative routes of viral transport in studies involving intracerebral injection of virus. This approach was used in two recent studies involving the intracerebral injection of PRV-Bartha. O'Donnell et al. (1997) used electrolytic lesions of the ventral pallidum or globus pallidus to confirm that these areas were involved in disynaptic circuits connecting the nucleus accumbens with the mediodorsal thalamic nucleus. Similarly, Jasmin et al. (1997) used lesions produced by ibotenic acid injection to demonstrate that a polysynaptic nociceptive pathway included the parabrachial nucleus in the midbrain. In both cases, these control studies were essential to establishing the postulated routes of viral transport, since the postinoculation intervals were long and the temporal analysis could not exclude alternative routes of viral transport. Other circumstances where the postinoculation interval is shorter may be less dependent upon such verification, but in most cases it will be desirable to use lesions or knife cuts to validate the contribution of one or more cell groups to the postulated circuit. In these cases, the lesions should be made ~1 week prior to the injection of the virus, and all other aspects of the analysis should reproduce those employed in the initial viral tracing experiments. Importantly, the postinoculation survival should be well in excess of the time necessary to infect all elements of the circuit in cases not involving lesions.

The use of viral tracing in combination with conventional monosynaptic tracers can be quite effectively applied to discriminate first-order projection neurons from local circuit neurons that synapse upon those neurons and reside in the same vicinity. This is effectively illustrated by experiments in which PRV-Bartha and the β subunit of cholera toxin (βCT) were simultaneously injected into the wall of the stomach. Both tracers invaded the peripherally projecting process of preganglionic parasympathetic neurons and were then retrogradely transported to accumulate in the parent neurons in the dorsal motor vagal nucleus (DMN) of the caudal brainstem. However, PRV replicated and passed transynaptically to infect neurons in the immediately adjacent nucleus of the solitary tract, while βCT remained trapped within the projection neurons of the DMN. Similar approaches could be used to discriminate interneurons from projection neurons in motor nuclei that innervate peripheral targets, or in intracerebral-injection paradigms. However, it is important to recognize that PRV is not compatible with all classical tracers. LaVail et al. (1993) have shown that FluoroGold interferes with the ability of HSV to invade and replicate within trigeminal neurons when the two are simultaneously applied to scarified cornea. Thus, it is very important to conduct parametric studies in well characterized systems to ensure that the classical tracer does not interfere with viral replication.

Troubleshooting

Low rates of infection

If animals show no signs of infection, or if labeling of a new neuronal circuit gives unpredictable results, one must immediately suspect that the concentration of biologically active virus is too low. The best strategy in these cases is to determine the invasiveness of the same stock in another circuit in which viral transport is well characterized. If variable results are observed in that analysis, it is probable that the titer of the virus has dropped during storage and handling. This can occur after prolonged storage of viral aliquots, even in the absence of a rise in temperature. Thus, the titer of the stock

should be reexamined, and, if the titer is low, the stock should be discarded.

Variability in the progression of infection

Many of the reasons for variability in the progression of infection were discussed in the protocols. Variations in the temporal course of viral transport through the CNS following peripheral injection is probably related to variability in the diffusion of virus from the injection site. This is not unusual in peripheral-injection paradigms, since the innervating axons often arborize diffusely and the cytoarchitecture of the injection target makes it difficult to precisely determine the diffusion of the virus from the injection site. The best approach under these circumstances is to measure the progression of viral infection from the first-order neuron in the CNS. For example, the first CNS infection after injection of an autonomic target will occur in preganglionic neurons. In the case of a stomach injection, these neurons will be found in the intermediolateral cell column of thoracic spinal cord and the dorsal motor vagal nucleus of caudal brainstem. Once these neurons become infected, the progression of infection through the balance of the polysynaptic CNS circuit synapsing upon them should occur in a predictable manner. If the number of neurons in the sites of first-order CNS infection vary dramatically from one animal to another, and one is sure that equivalent amounts of virus are being injected in a consistent manner, it should be determined if the titer of the viral stock has become compromised.

One has greater control over the diffusion of virus in experiments involving intracerebral injection, and the progression of infection is, therefore, more predictable than in experiments involving peripheral injection. If variability does occur in intracerebral paradigms, one should investigate the following potential confounding variables. First, one should make sure that the inoculum is not being drawn up the cannula tract as the cannula/pipet is being withdrawn from the brain parenchyma. This is sometimes difficult to determine, since virions have a preferential affinity for axon terminals and are transported to a distant site following uptake. The best approach to resolving problems of this nature is to extend the post-injection interval following completion of injection and removal of the pipet/cannula. Second, if one is injecting a cell group in close proximity to the ventricles, it is quite probable that some of the inoculum is leaching into the cerebrospinal fluid (CSF). This reduces the effective concentration of virus at the injection site and will produce substantial variability in the uptake of virus from one animal to another. It is generally quite easy to determine if this is a problem, since leakage of virus into the CSF will lead to viral replication by the ependymal cells lining the ventricles (Chen et al., 1999). To address this problem: (1) pulled glass pipets should be used, since they cause less tissue damage; (2) the virus should be injected at a slower rate, so that it is more easily accommodated by the parenchyma; and (3) the pipet should be kept in place for a longer post-injection interval, so that the inoculum becomes more firmly seated in the injection site. Each of these will have to be determined empirically according to the cell group that is being injected.

False negatives

There are two circumstances where false negatives can be an important consideration. The first is the failure to detect viral immunoreactivity in a cell group known to project to the area of injection. One should always be aware of the possibility that not every infected neuron will produce infectious virus. Such a phenomenon was directly demonstrated in the work of Rotto-Percelay et al. (1992), who demonstrated that dorsal root ganglion neurons showed no signs of viral replication after injection of the gastrocnemius muscle, but contained viral DNA. This is a difficult issue to deal with experimentally and emphasizes the importance of being conservative in interpreting viral tracing data. In essence, the presence of viral immunoreactivity can be confidently interpreted as evidence that a neuron is part of a circuit, but negative data should not be used to exclude a neuron from a circuit.

False negatives related to inefficient expression or lack of expression of transgenes by recombinant viruses can also confound the interpretation of dual-injection experiments. The potential interference of one strain of virus with the replication of a second strain was discussed earlier (see Critical Parameters). That data emphasizes the importance of selecting isogenic strains that are attenuated for virulence. However, even attenuated strains of PRV have been shown to ultimately evoke an immune response. Mabon et al. (1999) have recently demonstrated that treatment with the immunosuppressant cyclosporin A improves the efficiency of transgene expression in sympathetic preganglionic neurons infected with replication deficient strains of HSV type 1. This finding suggests that a similar treatment may be effective

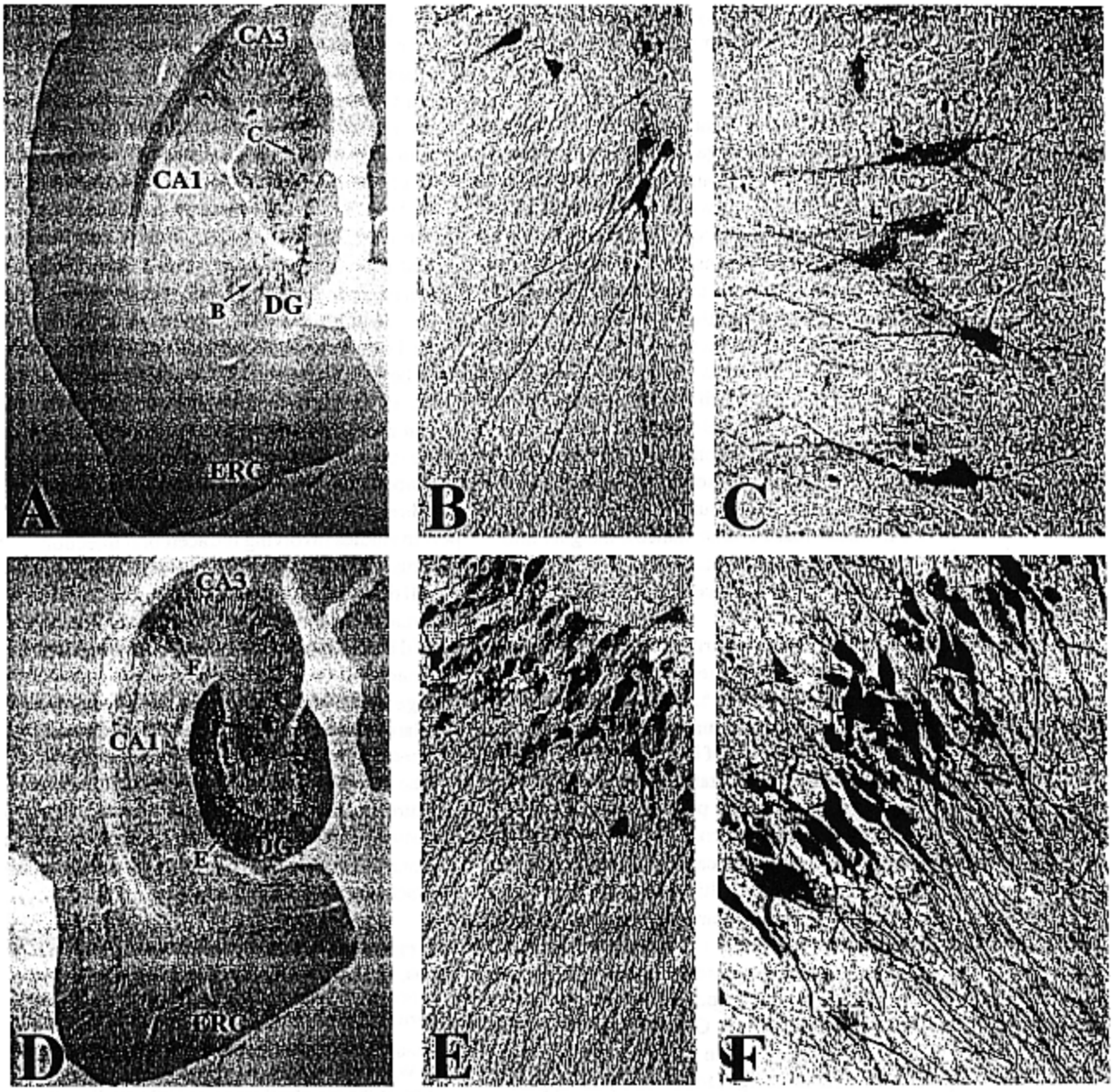

Figure 1.5.6 The sequential retrograde transynaptic passage of PRV-Bartha through hippocampal circuitry of the rat. The figures illustrate the distribution of infected neurons 45 hr (Panels **A** to **C**) and 50 hr (Panels **D** to **F**) following injection of 100 nl of virus into the junction of the CA3 and CA1 subdivisions of Ammon's Horn. In both cases, the brains were sectioned in the horizontal plane. At 45 hr, there is first-order infection of CA3 pyramidal cells (panels A and C) as well as transynaptic infection of neurons in the dentate gyrus (DG) and entorhinal cortex (ERC) as illustrated in panels A and B. The distribution of viral antigen in these neurons provides further insight into the extent of infection of individual neurons. For example, in panel B, neurons are in the early stages of infection, where viral immunoreactivity is confined to cell nuclei and perikarya and other cells, where viral antigen fills the polarized dendrites of individual granule cells. Sites at which virus leaves neurons can often be discriminated by the dense punctate staining of the somata and proximal dendrites of neurons at early stages of infection (panel C). With longer post-inoculation survival, the number of infected neurons in all three areas is substantially increased, but staining is confined to this well-established circuitry.

in dual-injection paradigms involving recombinant viruses, but this remains to be tested.

Anticipated Results

The temporal sequence of viral replication and transynaptic passage will lead to a sequential passage of virus through synaptically linked neurons. Thus, antigen will appear sequentially within these neurons according to the hierarchy of synaptic connections. When the neurons contributing to a circuit are spatially separated, it is a relatively straightforward process to follow progressive movement of virus through the circuit by conducting a temporal analysis in which animals are sacrificed at multiple post-inoculation intervals. However, there will be instances when projection neurons and interneurons are intermixed, making it difficult to determine first- versus second-order infection. Under those circumstances, insights into temporal progression can be derived from examination of the intracellular distribution of viral antigens.

The intracellular distribution of viral antigen typical of different stages of cellular infection is illustrated in Figure 1.5.6. At the earliest stage of infection, viral immunoreactivity is largely confined to the nuclei of infected neurons (Fig. 1.5.6B,C). This localization reflects the concentration of structural proteins assembling to form viral capsids that contain viral DNA. With advancing replication, capsids bud through the nuclear envelope and ultimately enter the cytoplasm, where they are enveloped by membrane from the parent cell which contains virally encoded membrane proteins (Fig. 1.5.1). These envelope proteins are particularly enriched in the membranes of the Golgi complex, which has been implicated in capsid envelopment. Paranuclear labeling is followed by the appearance of punctate staining on cell soma and proximal dendrites. As replication proceeds, the punctate staining extends further into the dendritic arbor and the staining proximally coalesces to produce uniform staining (Fig. 1.5.6C). Ultimately, the entire dendritic arbor contains dense, uniform staining (Fig. 1.5.6B,F). Thus, attention to the intracellular distribution of viral antigens is effective in providing insights into the temporal sequence of viral transport through a circuit.

Time Considerations

The length of time necessary to complete an experiment is directly dependent upon the route of injection, the extent of the circuit that one is investigating, and the virulence of the virus used. All PRV strains used for tracing ultimately kill the animal under study. It is useful to determine the average time to death (or terminal symptoms) for each virus in each paradigm. For most experiments using peripheral injection of PRV-Bartha strains, infected animals can be expected to live no longer than 5 or 6 days. For experiments involving central injection of PRV-Bartha, animals can be expected to live no longer than 3 days. Experiments involving peripheral inoculation of CNS targets (e.g., muscles, organs, or ganglia) require the longest periods of time, since the virus must be transported from the periphery into the CNS. In general, four days are adequate to obtain extensive transneuronal labeling of the neuraxis in most experiments involving peripheral inoculation, but it is necessary to determine this directly in each paradigm. Adequate transport in studies involving intracerebral injection of virus can generally be achieved within 72 hr.

All tissue must be processed quickly following transcardiac perfusion fixation. The tissue should be post-fixed, cryoprotected in sucrose, sectioned, and placed into cryopreservative for storage at −20°C, without delay. This approach standardizes the preservation of all tissue and reduces the possibility that tissue antigenicity will be compromised by storage at lower temperature. It also permits a systematic characterization of the organization and phenotype of neurons labeled by viral transport over a more protracted period of time.

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This chapter describes the use of PRV in a variety of systems.

Contributed by J. Patrick Card
University of Pittsburgh
Pittsburgh, Pennsylvania

Lynn W. Enquist
Princeton University
Princeton, New Jersey