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Viral Tracers for the Analysis of Neural Circuits

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Abstract: Viral transneuronal tracing can be used to analyze neural circuits in the central nervous system (CNS). In particular, the pseudorabies virus (PRV) strain Bartha, an attenuated form of a pig alphaherpesvirus, is an excellent retrograde transneuronal tracer for labeling neural networks. This virus is transported from the axon terminal to the cell body of an infected neuron and enters the nucleus. There, it replicates, producing progeny virions that are distributed throughout the cytoplasm. These new viruses are then transferred into the axon terminals of second-order neurons that innervate the infected neuron, and the process is repeated. This technique has been used to analyze CNS networks involving chains of two or more functionally connected neurons. Due to the high sensitivity of viral transneuronal labeling, false-positive data can be generated, leading to potential pitfalls of interpretation—examples are discussed in this chapter. Protocols for growing PRV and viral tracing methodology are included.

Keywords: Bartha pseudorabies virus, herpes simplex virus, HSV1, PRV, rabies, transneuronal, transsynaptic

I. INTRODUCTION

The identification of neural networks is fundamental to understanding brain functions. In fact, the input and output connections of every part of the mammalian brain have been studied by neuroanatomical tracing methods that depend on the axonal transport of either proteins or fluorescent chemical markers. This approach, while providing important data, is limited to the analysis of single neurons, not circuits of functionally connected neurons. A clear operational guide regarding the mammalian brain is dependent on the knowledge of these circuits and the genetic expression patterns of individual neurons that form them.

Even though conventional tracing techniques label only single neurons, they can be used in combination to analyze multisynaptic circuits. A two-neuron circuit, for example, can be visualized by the combination of two tracer injections into the brain of the same animal. First, a group of neurons is retrogradely labeled following injection of a protein tracer, such as cholera toxin β-subunit (CTb), into its axon terminal field. Then, a

second injection of an anterograde axonal tracer, such as the plant lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L), is made at a central site that is known or predicted to project to the retrogradely labeled neurons. After several days, the brains from these animals are processed by a double immunohistochemical procedure that allows for the light microscopic identification of the so-called close contacts~~PH~~A-L-labeled axon terminals abutting on CTb retrogradely labeled neurons. Definitive evidence is dependent on immunoelectron microscopy (see chapter by Sesack *et al.* in this volume). Since this technique is tedious and time-consuming, few reports have used this methodology, prompting a search for other approaches for the anatomical identification of functionally connected sets of neurons.

One of the first transneuronal tracers was identified in the early 1980s with the discovery that when the protein conjugate wheat germ agglutinin–horseradish peroxidase (WGA-HRP) was injected into the eye, it produced transneuronal labeling in second- and third-order visual relay neurons (Gerfen *et al.*, 1982; Itaya and van Hoesen, 1982). However, when this protein was injected into the brain, the volume of WGA-HRP injections had to be more limited~~un~~der these conditions, only vanishingly small amounts of WGA-HRP were transferred transsynaptically (Fig. 9.1). Other agents, such as the nontoxic fragment C of tetanus toxin, were also found to have limited utility as transneuronal tracers (Manning *et al.*, 1990). The approach was abandoned for more than a decade, but transgenic mice have been developed with neurons expressing plant lectin genes, such as WGA, and produce transneuronal labeling in defined neural circuits (Braz *et al.*, 2002; Horowitz *et al.*, 1999; Zou *et al.*, 2001).

Other attempts to develop anterograde transneuronal tracers included the use of radioactive amino acids (Wiesel *et al.*, 1974). These experiments required injections of large amounts of isotope into the target site, such as the eye. Then, after several days or weeks, the circuit could be identified in histological sections prepared for autoradiography. Since these studies require highly concentrated injections of expensive isotopes, this technique did not gain wide appeal. Even with these setbacks, the search continued for transneuronal tracers. In 1983, Martin and Dolivo made a key discovery when they demonstrated that viruses could be used to map central pathways (Martin and Dolivo, 1983).

Neurotropic herpesviruses (Fig. 9.2) have now proven extremely useful for detailed analysis of brain circuits. Briefly, viral retrograde transneuronal tracing occurs in the following manner. Live viruses are injected into a peripheral or central nervous system (CNS) target in a laboratory animal. After several hours, the viruses enter axon terminals innervating this structure. From here, the viruses are transported retrogradely to the parent cell bodies, undergo replication, and produce progeny virions, which become dispersed throughout the cytoplasm of each infected cell. These progeny virions are then transmitted to the incoming axon terminals that innervate the infected neurons, and the infectious process is repeated. Multiple

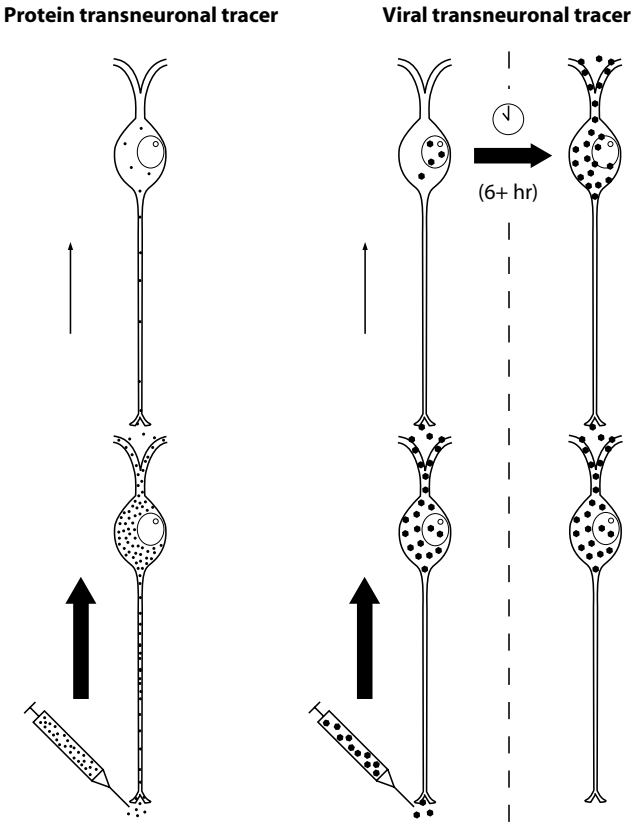

Figure 9.1. Comparison of protein and viral retrograde transneuronal tracers. Protein tracers, such as WGA-HRP (at left), can be used in transneuronal labeling studies, but only a small amount of tracer is transferred to second-order neurons. On the right, viral tracers, such as PRV, undergo replication within the second-order neurons and become self-amplifying transneuronal markers (Kuypers and Ugolini, 1990).

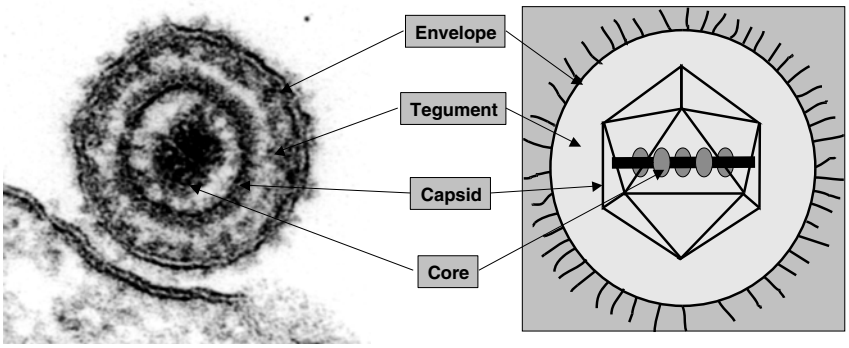

Figure 9.2. Morphology of a herpes virion. Herpesviruses, such as HSV1 and PRV, are composed of four structures. The core, a linear double-stranded DNA genome, is enclosed in an icosahedral capsid shell. The tegument, a layer of more than 15 different proteins, surrounds the capsid. The virus particle is enclosed in a lipid envelope in which viral glycoproteins are inserted (Mettenleiter, 2003).

rounds of replication and spread produce robust transneuronal labeling within neural circuits (Fig. 9.1).

II. HISTORICAL BACKGROUND OF VIRAL TRACING

One hundred years ago, the route of viral entry into the CNS was a matter of debate. The conventional view held that viruses spread locally, breaching epithelial boundaries, spaces, and fluids to enter the brain after establishing a foothold in the periphery. Another view proposed that viruses traveled the axonal processes of neurons as conduits into the CNS.

For herpesviruses, evidence subsequently accumulated in support of the concept of axonal transport. Goodpasture and Teague provided the earliest support for anterograde and retrograde axonal transport of viruses (Goodpasture, 1925; Goodpasture and Teague, 1923). In 1938, Albert Sabin, later famous for the development of polio vaccine, made the important observation that viruses enter the brain via preferential neural pathways (Sabin, 1938). For example, vesicular stomatitis and eastern equine encephalitis viruses ravaged the olfactory pathway to produce a lethal CNS infection, while pseudorabies virus (PRV), a herpes family virus, traveled in the sympathetic and trigeminal pathways without apparent olfactory infection.

The observations of Sabin were followed in the 1970s by clear demonstrations of specific axonal transport of herpesviruses to neuronal cell bodies and transneuronal spread in the CNS (Bak *et al.*, 1977; Cook and Stevens, 1973; Kristensson *et al.*, 1971, 1982). However, not until Martin and Dolivo (1983) published their study using PRV was it recognized that herpesviruses could be used as transneuronal tracers for defining neural circuits. In particular, they drew attention to the greatest advantage of using a virus as a transneuronal tracer—it replicates in each infected neuron. Thus, viruses can be viewed as self-amplifying markers, robustly labeling each hierarchical level of a neural circuit, in contrast to the diminishing transsynaptic diffusion of chemical tracers (Fig. 9.1). PRV tracing was used in two additional studies in the 1980s but, unfortunately, the investigators did not indicate the specific viral strain, source, and dose used in their experiments (Rouiller *et al.*, 1986, 1989). Attempted transsynaptic tracing with a wild-type form of PRV (Becker strain) was found to result in uncontrolled, nonspecific, and rapidly lethal infections, preventing its use as a specific transneuronal tracer (Strack *et al.*, 1989b).

Ugolini and colleagues injected herpes simplex virus type 1 (HSV1) into peripheral nerves and showed that it produced a transneuronal infection in rat brain (Kuypers and Ugolini, 1990; Ugolini *et al.*, 1987, 1989). However, the virus also spreads locally and nonspecifically to adjacent glial cells and neurons (Ugolini *et al.*, 1987). Subsequently, false-positive transneuronal labeling occurred in neurons connected to sites of nonspecific infection. This latter finding raised concern over whether viral infections could be

contained within specific neural circuits, undermining enthusiasm for this method.

Because of these nonspecific infections, viral tracing was not widely exploited until the discovery that a less virulent derivative of PRV, Bartha PRV, produces highly specific retrograde transneuronal infections (Jansen *et al.*, 1993; Strack *et al.*, 1989b; Strack and Loewy, 1990). In contrast to the fulminant infections produced by wild-type PRV, Bartha PRV infections remain restricted mainly to synaptically linked chains of neurons and move only in the retrograde direction. Moreover, rats survive more than twice as long—up to 7 daysfollowing an injection of Bartha PRV into a peripheral target (Westerhaus and Loewy, 2001), whereas wild-type PRV kills rats within 3 days (Strack *et al.*, 1989b). This attribute allows transneuronal propagation to higher order neurons of a neural circuit (Enquist, 2002). Ensuing studies revealed specific genetic alterations responsible for the retrograde specificity and reduced infectivity of Bartha PRV (see below).

Bartha PRV is an effective and well-characterized retrograde transneuronal tracer that produces infections in most laboratory species (mouse, rat, gerbil, hamster, ferret, sheep, and chicken). Unlike other transneuronal viral tracers, such as HSV1 and rabies, PRV does not infect humans (Gustafson, 1975). Thus, Bartha PRV was quickly recognized as a safe and accessible tool for retrograde transneuronal tracing experiments. However, PRV does not cause infections in primates. Other neurotropic virusesHSV1 and rabieshave been tested and used for circuit analysis in monkeys. For further details, see section "Other Viruses Used for Transneuronal Tracing Studies."

Bartha PRV has been genetically modified in various ways to create new viral tools for neural circuit analysis. Advances in herpesvirus biology, including the availability of the complete PRV genome sequence (Klupp *et al.*, 2004), provide the opportunity to construct selective viral tracers, which should allow neuroscientists to unravel specific multisynaptic pathways in unprecedented detail.

III. BARTHA PRV AS A NEUROANATOMICAL TRACER

While Bartha PRV has primarily been used to identify chains of central neurons innervating peripheral targets, it has also been utilized for tracing circuits within the brain. In addition, viral tracing has been combined with various other well-established neuroanatomical techniques. Innovative methodologies continue to appear, making PRV an even more useful neurobiological tool. Genetically engineered PRV strains allow double transneuronal tracing experiments and the detection of transneuronally labeled neurons in living tissue, by fluorescent protein expression, for electrophysiological recording. Recombinant PRV has even been used in transgenic mice in an attempt to selectively label the inputs to specific neuronal phenotypes.

A. Phenotypic Characterization of PRV-Labeled Neurons

Bartha PRV was used to provide the first direct neuroanatomical identification of brainstem and hypothalamic neurons that regulate sympathetic outflow systems (Strack *et al.*, 1989a, b). Beginning with this work, it was demonstrated that peptide antigens could still be detected in infected neurons. This was a fortuitous discovery, since herpesviruses terminate protein synthesis in other cell types, via expression of the viral host shutoff protein gene, UL41 (Smiley, 2004). However, this viral host shutoff process does not occur in neurons (Nichol *et al.*, 1994).

Therefore, identification of the phenotype of infected neurons, by double-immunohistochemical labeling, can add important information about transneuronally labeled neurons (Fig. 9.3). In addition, investigators have successfully used in situ hybridization to demonstrate various mRNA transcripts in PRV-labeled cells (Boldogkoi *et al.*, 2002; Broussard *et al.*, 1996; Giles *et al.*, 2001; Song and Bartness, 2001; Stornetta *et al.*, 2004).

One caveat should be noted regarding the detection of marker molecules within PRV-infected neurons~~ex~~pression of various peptides and enzymes within neurons infected with Bartha PRV tends to be reduced, often dramatically, relative to uninfected neighboring neurons of the same phenotype. Consequently, central injection of colchicine 24 h prior to killing an infected rat is sometimes used to boost the labeling of axonally transported peptides.

B. Use of PRV in Conjunction with Conventional Neural Tracers

The PRV transneuronal tracing technique can also be combined with conventional neural tracing methods. In one example, anterograde PHA-L tracing was combined with the PRV method to map the specific central regions targeted by the periaqueductal gray matter (PAG) to modulate sympathetic functions. In two series of rats, PHA-L injections were made into either the lateral PAG column, implicated in the fight-or-flight reactions, or the ventral PAG column, which mediates the opposite behavioral responses. Two days later, Bartha PRV was injected into the stellate sympathetic ganglion. After an additional 4 days, select regions of the hypothalamus and brainstem contained PHA-L terminals contacting PRV-labeled neurons. Specific sites, particularly the raphe magnus nucleus, through which PAG could modulate sympathetic activity, were thus identified (Farkas *et al.*, 1998). In this situation, PRV tracing alone was capable of demonstrating only that PAG neurons have multineuronal connections to the stellate ganglion~~th~~e specific presympathetic groups through which retrograde transneuronal PAG labeling had occurred were revealed by simultaneous anterograde tracing.

In a second example, a key hypothalamic relay nucleus was identified in a circuit implicated in circadian arousal functions~~th~~e pathway from the suprachiasmatic nucleus (SCN) to the locus coeruleus (Aston-Jones *et al.*,

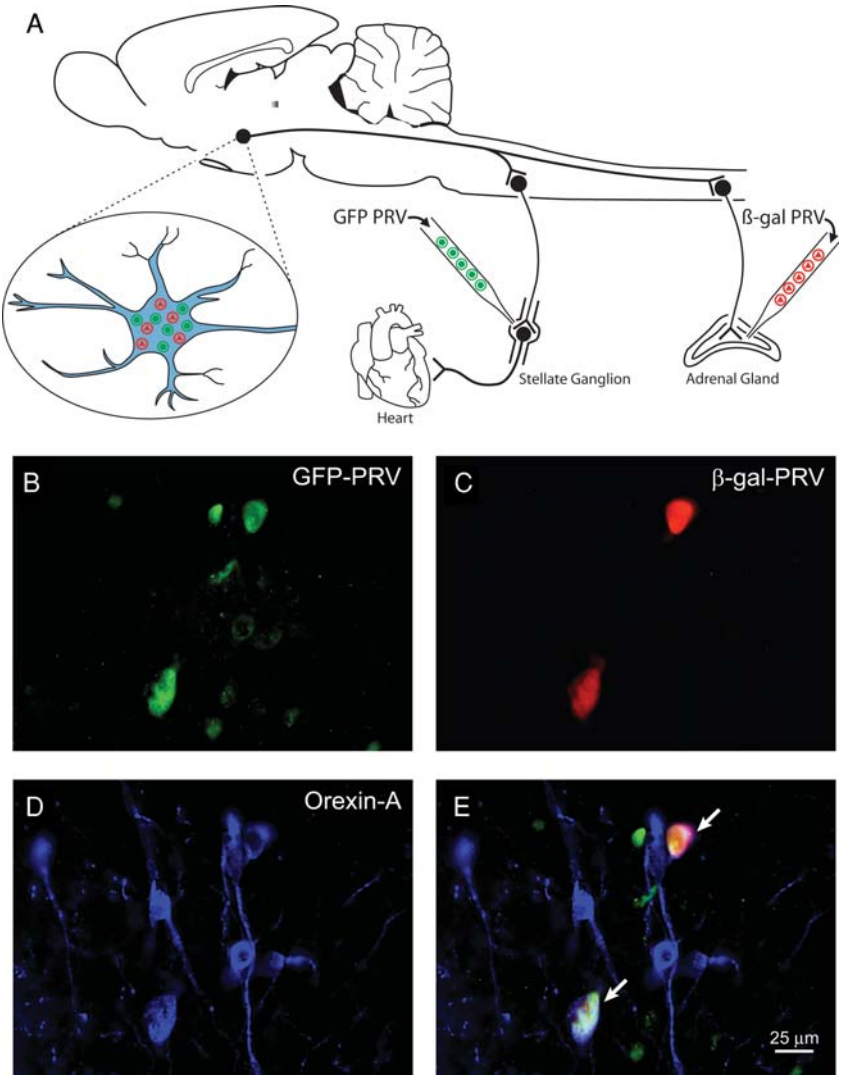

Figure 9.3. Double viral transneuronal tracing experiments can be used to identify specific neuronal phenotypes implicated as potential candidates regulating behavioral functions, such as the fight-or-flight response. In this example, orexin neurons (blue, panel D) in the lateral hypothalamus are transneuronally double labeled with Bartha PRV strains expressing two unique reporters: GFP-PRV from the stellate ganglion (green, B) and β-gal PRV from the adrenal gland (red, C). Triple-labeled cells appear white in panel E (Geerling *et al.*, 2003).

2001). The SCN was screened for neurons that project both transneuronally to the locus coeruleus and directly to the specific hypothalamic nuclei, such as the dorsomedial hypothalamic (DMH) nucleus. Bartha PRV was injected into locus coeruleus in animals with CTb injections into various

hypothalamic nuclei. The highest number of double-labeled SCN neurons occurred in animals with CTb injections into the DMH, as opposed to other hypothalamic regions such as the paraventricular and lateral hypothalamic nuclei and the preoptic region. Since the DMH provides a direct input to locus coeruleus and DMH lesions blocked the circadian changes in locus coeruleus neural activity, a strong case was made for the existence of an SCN → DMH → locus coeruleus circuit (Aston-Jones *et al.*, 2001).

C. Double Retrograde Transneuronal Tracing with Two Isoforms of PRV

Neurons with branched axonal projections can be identified by double retrograde tracing techniques. For example, when two different retrograde tracers (e.g., Fluorogold and CTb) are injected into two central regions, it is possible to identify single neurons innervating both sites by the colocalization of the two tracers within the same cell.

Jansen *et al.* (1995a) demonstrated that this approach could be extended to transneuronal retrograde tracing by using two unique viruses. Two Bartha-derived PRV strains were separately injected into different sympathetic target tissuesadrenal medulla and stellate ganglion. The two recombinant viruses were uniquely identifiable. One contained a *lacZ* gene insertion within the nonessential gG gene locus. This virus could be identified by immunohistochemical staining for β-galactosidase (the protein product of the *lacZ* gene). In the other virus, the wild-type gC gene, which is mutated in Bartha PRV, was restored. Hence, this viral strain could be uniquely identified by its expression of the wild-type gC membrane protein, which is absent in the *lacZ* recombinant.

Double virus tracing can be a powerful method for detailing the exact central sites that regulate complex behavioral activities. The double-viral tracing technique was used to identify groups of putative central command neurons of the sympathetic nervous system, positioned to coordinate the activation of multiple different sympathetic target organs (Jansen *et al.*, 1995a). This study demonstrated the feasibility of double viral tracing. Later, other investigators applied this technique to demonstrate central neurons positioned to synchronize both somatomotor and sympathetic activations (Kerman *et al.*, 2003; Krout *et al.*, 2003), as well as brainstem neurons that could coactivate inspiratory and expiratory respiratory muscles that could discharge in parallel, which occurs during vomiting (Billig *et al.*, 2000). Double virus transneuronal tracing was used to demonstrate that vasopressin-containing SCN neurons (Ueyama *et al.*, 1999) and individual orexin neurons in the lateral hypothalamus (Geerling *et al.*, 2003) can coordinately regulate multiple sympathetic outflow systems. Double transneuronal labeling in orexin neurons is shown in Fig. 9.3. A similar strategy, using two uniquely identifiable strains of HSV1, has been used to compare the origins of sympathetic outflows within the spinal cord (Levatte *et al.*, 1998).

Since the introduction of the double virus labeling method, additional technical refinements have greatly increased its usefulness. The original study by Jansen and colleagues was extremely inefficient, requiring 256 rats in order to obtain eight animals with well-matched double infections. This 3% yield was partly due to the conservative viral dose used in this study, resulting in only a 20% success rate for single-virus studies (Strack *et al.*, 1989b), but also to unmatched infectivity between the two viral tracing strains (Sams *et al.*, 1995). These difficulties have been overcome with the addition of several new genetically engineered viral strains (see below).

One surprising aspect of this technique is that simultaneous infections can frequently be established by two separate viral strains, arriving from separate sites of origin, within the same neurons. This occurrence was not completely predictable, based on in vitro work demonstrating a cellular phenomenon called superinfection resistance. The herpesvirus membrane protein gD can inhibit the subsequent infection of the cell by additional virus (Campadelli-Fiume *et al.*, 1988). Whether or not a similar effect is relevant for infections with Bartha PRV derivatives in vivo, a growing body of double viral tracing data unequivocally demonstrates that, in viral tracing paradigms, robust double infections can occur in many neurons.

Concern over superinfection resistance in double viral tracing was first raised when it was reported that infection by a Bartha PRV-derived strain with enhanced virulence, due to restoration of the virulence-enhancing wild-type gI gene (Whealy *et al.*, 1993), was shown to greatly inhibit a second transneuronal infection in the same pathway by a β-galactosidase-expressing virus injected 24 h later (Kim *et al.*, 1999). However, when the order was reversed, with the β-galactosidase strain injected first, it was unable to reciprocate this strong inhibition. Whether the first effect was due to a direct superinfection resistance mechanism in PRV-infected neurons, as opposed to indirect consequences of the greatly increased virulence of the first strain, was not resolved. For example, strong glial reaction at the injection site or rapid injury to the infected first-order neurons could have simply resulted in decreased entry and retrograde transport of virus injected a day later.

Banfield *et al.* (2003) found that when two isogenic Bartha recombinants were injected at the same time and site, they produced a double transneuronal infection in a large number of higher order neurons. More than 75% of the infected third-order paraventricular hypothalamic neurons expressed reporter genes from both viruses (green and red fluorescent proteins) after injection of a mixture of the two strains into one eye (Cano *et al.*, 2003). Parallel experiments were performed on cultured dorsal root ganglion cells. These data showed that infection with one strain of PRV greatly reduced the susceptibility to infection by another PRV strain within 2 h and completely prevented superinfection after 4 h. Whether this in vitro time limit represents a similar constraint in vivo remains unknown. The robust double infections observed when the viral exposure times were matched exactly (by coinjecting them) combined with the indication that a small time window may exist for superinfection suggests that optimization of the

double-virus technique requires matching viral rates of progression as closely as possible.

For this reason, an important aspect of double-viral tracing studies is the choice of viruses with similar transneuronal infection kinetics (Ter Horst, 2000). Optimal yields of double-infected neurons may be obtained when viral rates of transit are most closely matched, such that the two viruses arrive at an afferent site with minimum delay between strains. The first two double-viral tracing studies used two viruses with significantly different virulence characteristics (Jansen *et al.*, 1995a; Kim *et al.*, 1999). In each case, the infectivity of the *lacZ*/β-galactosidase strain (a minimally altered version of Bartha PRV) was significantly reduced relative to the second strain (a version of Bartha to which a virulence-endowing membrane glycoprotein gene had been restored) (Kim *et al.*, 1999; Mettenleiter *et al.*, 1987, 1988; Sams *et al.*, 1995).

Following these original studies, double transneuronal tracing has benefited from the genetic engineering of isogenic viruses in which insertions of reporter genes have been targeted to the same genetic locus. For example, inserting the green fluorescent protein (GFP) gene within the same gG locus used for *lacZ* (Jons and Mettenleiter, 1997) has allowed more comparable double viral infections at equivalent doses and times (Geerling *et al.*, 2003; Krout *et al.*, 2003; Ueyama *et al.*, 1999). However, discordant rates of expression between the two reporter genes, within double-infected cells, can result in unequal detectability of the two strains, whether or not infectivity is equal. In some studies, two different promoters have been used to drive the expression of reporter genes~~th~~e intrinsic gG promoter and the human cytomegalovirus (CMV) immediate-early promoter. Different promoters could necessitate the use of two different viral doses to match the timing of reporter expression, even when the rates of viral spread may be comparable (Cano *et al.*, 2003). Differences in relative expression levels of reporter proteins can be largely overcome by the use of the CMV promoter in both strains, driving high levels of gene expression as early as possible (Banfield *et al.*, 2003). Nonetheless, any pair of viruses used in double tracing experiments should be compared to establish similar rates of transneuronal progression.

D. PRV Fills Neuronal Dendritic Trees

Card *et al.* (1993) noted that pseudorabies virions fill the entire dendritic tree of an infected neuron, out to the distal branches. This property can be useful for ultrastructural analysis (Carr *et al.*, 1999; Carr and Sesack, 2000). More interestingly, dendritic filling by PRV has been exploited to solve a long-standing problem in neuroanatomical tracing~~id~~entifying the synaptic afferents to the distal dendrites of a group of neurons.

When the dendrites of a particular group of neurons extend into adjacent cytoarchitectonic regions~~ou~~tside the boundaries of its parent cell

group as defined in Nissl-stained sections~~it~~ can be difficult to determine, simply by retrograde tracer injections, which are true neural inputs to the particular cell group (Bourgeais *et al.*, 2003; Luppi *et al.*, 1995). In some instances, this problem can be overcome by injecting PRV within the center of a group~~aft~~er replication in the soma, viral progeny spread throughout the neuron and can spread transsynaptically into even the most distal synaptic afferent terminals (Aston-Jones and Card, 2000). This approach has been used to demonstrate spinal lamina I afferents to the distal dendrites of amygdala-projecting neurons in the external lateral parabrachial nucleus (Jasmin *et al.*, 1997). More recently, Aston-Jones *et al.* (2004) injected PRV within the core of the locus coeruleus and found that the virus replicated and spread throughout these noradrenergic neurons, producing transneuronal infections in input neurons that contact the most distal dendritic branches.

E. Electrophysiological Recordings from Transneuronally Labeled Neurons

Various methods have been utilized to identify specific neurons for electrophysiological study in brain slices. In particular, fluorescent dyes such as Fluorogold have been used to identify retrogradely labeled neurons in living tissue (Kangrga and Loewy, 1995).

In 2000, Smith *et al.* first demonstrated that electrophysiological recordings could be targeted to identified neurons with multisynaptic connections to a specific target. These investigators created the Bartha-derivative PRV 152, designed to produce high, early expression of enhanced green fluorescent protein (EGFP, driven by a CMV promoter). They demonstrated that EGFP-expressing, PRV-infected neurons were easily identifiable in tissue slices. Most important, despite viral infection, the electrophysiological properties of these cells were comparable to uninfected neurons. This study was followed by a similar demonstration of electrophysiological recording from PRV-infected neurons, using a different GFP-expressing strain (Irnaten *et al.*, 2001).

These findings built confidence that recordings can be obtained from the neurons identified as multisynaptic afferents to a specific target. However, infected cells may show electrophysiological abnormalities (Fukuda *et al.*, 1983). Still, high-quality recordings have been made from visually identified GFP-PRV neurons and this method can be quite useful for studying neural circuits.

F. Genetically Engineered PRV for Highly Specific Tracing

Molecular biological tools have created opportunities for constructing viruses with improved properties as neural tracers (Boldogkoi *et al.*, 2004).

New viral tracers may allow more selective labeling within neuronal circuits. For example, the use of cell-specific conditional expression technology should allow the targeting of infection or viral reporter gene production to neurons of a particular phenotype. In this way, local CNS injections of specific viruses may produce transneuronal labeling restricted to the inputs of a functionally specific type of neuron.

The feasibility of this approach was demonstrated by DeFalco *et al.* (2001), who injected a genetically engineered Cre recombinase-dependent strain of PRV into a transgenic mouse that expresses Cre recombinase in only one neuronal phenotype. They began with Bartha PRV and removed its thymidine kinase (tk) gene, which is necessary for viral replication in vivo. The tk sequence was then reinserted, along with an EGFP reporter gene, in the nonessential gG locus (the same site used for the *lacZ* and GFP reporters described above), driven by a CMV promoter. A STOP sequence, flanked by loxP sites, was inserted upstream from the tk and EGFP sequences. This STOP sequence was positioned to prevent expression of these genes and, therefore, both viral replication and cellular expression of EGFP reporter.

However, the Cre recombinase enzyme can join the loxP sites, removing the intervening STOP sequence. Therefore, this replication-deficient virus, termed Ba2001, was injected into the brains of transgenic mice expressing Cre under the control of specific genetic promoters (neuropeptide Y or the leptin receptor). In these mice, Ba2001 could enter many types of neurons, but could replicate only in Cre-expressing neurons. In these specific neurons, infection with replication-competent virus was reported by concurrent EGFP expression. With these tools, the specific neural networks regulating NPY- or leptin receptor–expressing neuronal subpopulations within the hypothalamic arcuate nucleus could be selectively labeled.

This important methodological advancement raises the possibility that designer herpesviruses could become important tools for mapping neural circuits with unprecedented specificity. Unfortunately, in the 4-year period since publication, these findings have not been detailed or extended. Since certain CNS sites reported to be infected in the DeFalco report (DeFalco *et al.*, 2001), such as somatosensory cortex, seem incompatible with known inputs to the hypothalamus, the potential for spontaneous viral genetic mutation causing spurious labeling in vivo must be addressed.

The promise of custom-made viral tracers remains alluring (Boldogkoi *et al.*, 2004), but the enthusiasm surrounding this technology should not cause investigators to overlook the necessity of detailed neuroanatomical characterization of all new viral tracers. Any new viral strain should be carefully compared with a well-characterized strain, such as Bartha PRV, with respect to transsynaptic specificity, kinetics of infectious spread, tropism, and critical viral doses (Banfield *et al.*, 2003). Even minor alterations in what may appear to be insignificant regions of the PRV genome, such as the nonessential gG gene locus, can result in significant differences in infectivity (Cano *et al.*, 2003; Demmin *et al.*, 2001; Sams *et al.*, 1995). Finally,

it is important that experimental results obtained with any viral strain be critically compared with existing neuroanatomical data.

IV. SPECIFIC RETROGRADE TRANSPORT OF BARTHA PRV

Since the first transneuronal tracing experiments with Bartha PRV, it was clear that this viral strain spreads preferentially, if not exclusively, in a retrograde direction (from the axon terminal to the cell body~~the~~ opposite direction of neural transmission). An early argument for retrograde specificity came from studies that showed that, when PRV was injected into skeletal muscle, it produced retrograde labeling in ventral horn motor neurons, but not in the dorsal root ganglia or central somatosensory sites (Rotto-Percelay *et al.*, 1992).

Patterns indicative of retrograde-only transport have also been observed after Bartha PRV injections within the CNS. After injection into the mediodorsal nucleus of the thalamus, O'Donnell *et al.* (1997) noted that infection within the cortex first occurred within deep layer neurons, consistent with retrograde transport from the thalamus. Card *et al.* (1998) showed that, unlike that of wild-type Becker PRV, injection of Bartha PRV in the prefrontal cortex did not produce an anterograde transneuronal infection in the striatum, a major efferent target. Chen *et al.* (1999) reported that, even when entering fibers of passage through an injection site, Bartha PRV did not produce anterograde transneuronal labeling.

Wild-type PRV clearly *does* spread anterogradely. This was known from Sabin's early observations of infection in the central trigeminal sensory pathways after olfactory instillation (Sabin, 1938). Later, it was observed that wild-type PRV injected into the eye produces a fulminant infection of all retinorecipient sites within the brain (Card *et al.*, 1991). Since there are relatively slight genetic differences between wild-type and Bartha PRVs, yet major differences in their transport properties, a search was initiated for the specific genes responsible for anterograde infectious spread.

At least three PRV genes appear necessary for anterograde spread: gE, gI, and Us9. All three of these genes are absent from Bartha PRV, due to a large deletion in the unique short (Us) region of the PRV genome (Lomniczi *et al.*, 1984). The deletion of any one of these genes from wild-type PRV eliminates anterograde viral transmission (Brideau *et al.*, 2000; Card *et al.*, 1992; Whealy *et al.*, 1993).

Both gE and gI are membrane glycoproteins and form a functional heterodimer (Mettenleiter *et al.*, 1988; Whealy *et al.*, 1993). These two genes had been previously characterized as encoding important PRV virulence-enhancing factors (Mettenleiter *et al.*, 1987, 1988). Deletion of either gene from wild-type PRV was shown to eliminate anterograde spread from the retina to the retinorecipient visual sites in the brain (Card *et al.*, 1992; Whealy *et al.*, 1993). Loss of anterograde spread in gE- and gI-null mutants has also been confirmed in the olfactory pathway (Babic *et al.*, 1996; Kritas *et al.*,

1994). Injection of a mixture of gE-null and gI-null PRVs within the eye, however, resulted in restoration of a wild-type anterograde infection pattern (Enquist *et al.*, 1994). This implies that both mutants infect retinal ganglion cells, but require the addition of their respective missing gene products—upon coinfection of the same cell~~for~~ productive anterograde spread. It is still unclear exactly how gE and gI allow anterograde transmission (Enquist *et al.*, 2002; Tomishima *et al.*, 2001).

In contrast, the mechanism by which the Us9 gene product influences anterograde transport is better characterized. As with gE and gI, absence of the Us9 gene inhibited the anterograde spread of wild-type PRV (Brideau *et al.*, 2000). Tomishima and Enquist (2001) further demonstrated in vitro that, without Us9, necessary membrane glycoproteins do not enter the axon of an infected neuron. While other viral proteins proceed normally into the axon, this lack of membrane protein trafficking prevents anterograde transmission of complete, infectious virions.

While the exact molecular mechanisms required for PRV anterograde infectious spread remain unknown, the studies cited above have highlighted some of the key factors. The identification of specific genetic mutations preventing anterograde transneuronal infections by Bartha PRV gradually cast doubt upon the only cited evidence that this strain could produce an anterograde infection~~the~~ delayed infection of SCN after injection into the eye (Brideau *et al.*, 2000; Card, 2000; Card *et al.*, 1991, 1992; Enquist *et al.*, 1994; Husak *et al.*, 2000; Moore *et al.*, 1995; Smith *et al.*, 2000; Whealy *et al.*, 1993). Careful neuroanatomical analysis, however, revealed that this purportedly anterograde infection was actually produced by retrograde spread via multisynaptic autonomic outflows to the eye (see discussion under "Practical Considerations and Pitfalls"; Pickard *et al.*, 2002; Smeraski *et al.*, 2004).

In summary, a great deal of collective neuroanatomical experience with Bartha PRV indicates that this virus moves selectively in a retrograde direction. Three key PRV genes have been individually shown to be necessary for anterograde viral spread~~B~~artha is deficient in each one. Together, these findings build a strong case that Bartha PRV is a retrograde neuronal tracer.

V. TRANSNEURONAL TRANSFER OF BARTHA PRV AT SYNAPTIC TERMINALS

The pattern of Bartha PRV transneuronal labeling is largely consistent with specific transfer at synapses without leakage to nearby neurons or local axons. For example, after PRV injection into any visceral tissue or autonomic ganglion, transneuronal labeling was consistently found in the parvocellular subdivision of the paraventricular hypothalamic nucleus~~an~~ area known from earlier work to be a key site regulating autonomic functions. Importantly, nearby neurons lying in the intermingled magnocellular subdivision

of this nucleus, which projects solely to the posterior pituitary, were not labeled (Strack *et al.*, 1989b). Such restricted labeling indicated that random cell-to-cell spread did not occur.

This consistent pattern led to the proposal that transneuronal spread of Bartha PRV occurred specifically through neuronal synapses (Strack and Loewy, 1990). However, beginning with the first use of Bartha PRV as a transneuronal tracer, it was observed that Bartha PRV can infect glia within infected neuronal sites (Rinaman *et al.*, 1993; Strack *et al.*, 1989b). Although only limited infections of astroglia occurred, this observation raised significant concern over the potential for false-positive labeling via not only local spread to unrelated neurons but also subsequent transneuronal propagation. Indeed, Ugolini *et al.* (1987) had reported that tracing with HSV1 resulted in formidable local spread to neurons within unrelated circuits. HSV1 injected into the mouse hypoglossal nerve spread from nerve roots in the ventral medulla to both glia and inferior olivary neurons and, via transneuronal transfer within only a few days, to neurons in the cerebellum. Although no such nonspecific infection had been reported for Bartha PRV, this potential roadblock was carefully examined in early experiments validating the virus as a transneuronal tracer.

In 1990, Strack and Loewy demonstrated that, after Bartha PRV was injected into the eye or the skin of the ear, retrograde labeling in the sympathetic superior cervical ganglion (SCG) was completely restricted to the subset of neurons afferent to the particular site of injection. Even after 4 days, infection did not spread locally within the SCG (Strack and Loewy, 1990). A similar result was reported for the CNS by Jansen *et al.* (1993). After injection of Bartha PRV into either the stellate ganglion or the adrenal medulla, coincident with CTb injection into the other sympathetic target, the percentage of spinal cord neurons labeled with both virus and CTb was not different from the double-labeled proportion found after injections of two conventional retrograde tracers (CTb and Fluorogold). This indicated that PRV infection within this first-order afferent site remains confined to specific sympathetic preganglionic neurons. When both experiments are considered together, a convincing case can be made against the likelihood that Bartha PRV produces lateral infections involving neighboring neurons (Scenario 3 in Fig. 9.4).

Consistent with these findings, diffusion of PRV through the neuropil may be hindered by the large size of infectious virions (200 nm) and by its binding to cell surface heparin sulfate moieties (Aston-Jones and Card, 2000). The spread of PRV to axons and local glia, but not to adjacent neuronal cell bodies, is also consistent with an earlier report indicating a greater herpesvirus-binding affinity for synaptic terminals and glial cells, relative to neuronal perikarya (Vahlne *et al.*, 1978).

However, besides local spread to adjacent neuronal cell bodies, one potential avenue of nonspecific PRV spread remains~~the~~ leakage of virions into adjacent nonsynaptic axons (Scenario 2 in Fig. 9.4). Whereas the experiments cited above (Jansen *et al.*, 1993; Strack and Loewy, 1990) dispelled

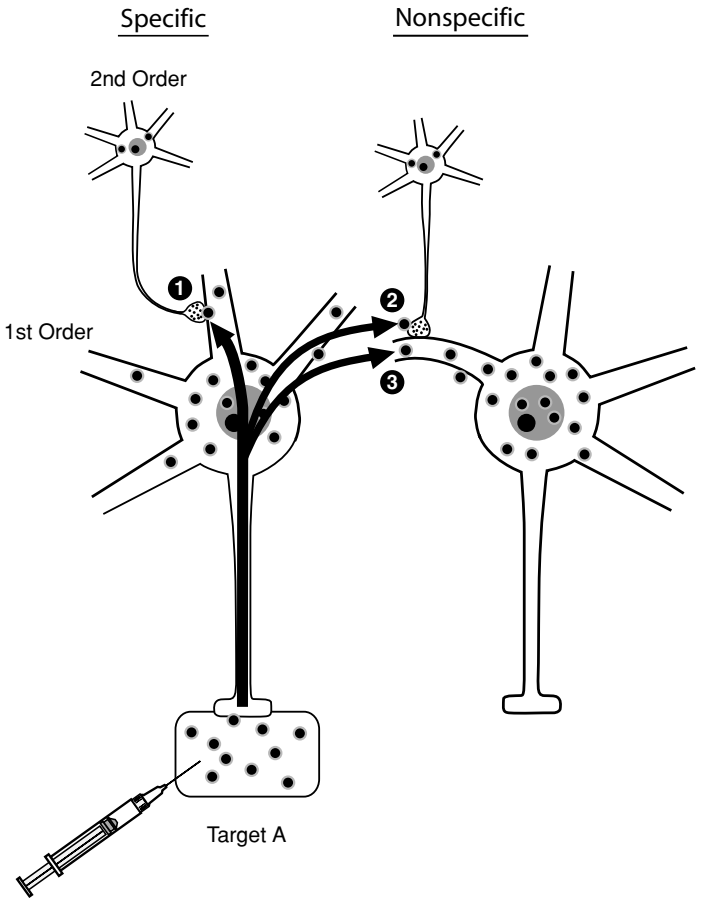

Figure 9.4. Viruses can produce both specific and nonspecific retrograde infections in the CNS. (1) The primary mode of transneuronal spread, for Bartha PRV and other viruses, is via direct transfer to the synaptic afferents of an infected neuron. (2) Spread of a viral tracer to adjacent axons and terminals that do not synapse upon the infected neuron may occur (see "Transneuronal Transfer of Bartha PRV at Synaptic Terminals"). (3) Lateral leakage of virus to neighboring neurons does not appear to occur with Bartha PRV (Jansen *et al.*, 1993), but may present a problem with other viral tracers, such as HSV1 (Ugolini *et al.*, 1987).

concern over the potential for local spread to neuronal cell bodies, only indirect tests dealt with the possibility that some of the PRV released from an infected neuron may infect adjacent axons or axon terminals.

First, when PRV was injected into the eye or into the skin of the ear, it produced second-order transneuronal labeling in the appropriate distribution of sympathetic preganglionic neurons in the spinal cord, as determined by prior electrophysiological data (Strack and Loewy, 1990). The eye- and ear-specific SCG neurons, through which transneuronal transport had occurred, are highly intermixed. Hence, this result indicated that transfer

of PRV from infected first-order SCG neurons took place in a preferentially transsynaptic manner, not simply by transmission of virions to all nearby axon terminals. However, because the distributions of preganglionic neurons infected after injections into eye and ear overlapped between spinal levels T2 and T4, the possibility remained that a small proportion of the labeled neurons in this zone was the result of nonsynaptic viral transfer in the SCG.

This is the only tracing experiment to directly address the issue of Bartha PRV spread to the nearby axons. Clearly, Bartha PRV is *preferentially* transferred to synaptic afferents, but only circumstantial evidence exists concerning whether or not a small proportion of virions is nonspecifically transferred to adjacent axons. This possibility is of potential significance, given the exponential amplification expected to occur after false-positive labeling.

An attractive theory was proposed that reconciled the observation of astroglial infections with a lack of spread to adjacent neurons, and that offered a mechanism by which transsynaptic specificity may be preserved (Rinaman *et al.*, 1993). Virions were observed, by electron microscopy, to be preferentially released from an infected neuron at sites of synaptic contact (Card *et al.*, 1993). These virions did not appear to breach the synapses themselves; they spread parasynaptically and equally infiltrated the afferent axon terminal and the astroglial processes that form a barrier around the synaptic region (Card *et al.*, 1993). Thus, it was proposed that astrocytic processes may absorb any PRV not incorporated into afferent terminals, preventing nonspecific spread to adjacent structures (Card, 1998). Furthermore, Card *et al.* (1993) noticed that, in contrast to neurons, the PRV produced within astrocytes did not acquire a viral envelope, which is a necessary component for infectious virions. These investigators proposed that, rather than serving as a source of PRV production and nonspecific local spread, astrocytes limit viral spread to nearby axons without producing normal infectious virions (Rinaman *et al.*, 1993).

This appealing theory may explain the transsynaptic pattern of PRV spread and the lack of PRV spread to adjacent neurons (Jansen *et al.*, 1993; Strack and Loewy, 1990), despite infection of adjacent glia (Rinaman *et al.*, 1993; Strack *et al.*, 1989b). However, the spread of some amount of virus to adjacent nonsynaptic axons remains an important possibility that cannot be addressed by circumstantial evidence or by inferential approaches. The issue of whether transneuronal PRV spread occurs exclusively via synaptic afferents remains unsettled. This possibility is an important consideration because the brain regions where nonspecific transfer presents the greatest obstacle to interpretation are those in which viral transneuronal tracing is most useful sites such as the brainstem and hypothalamus, which contain spatially intermixed, yet functionally diverse, populations of neurons.

In summary, Bartha PRV is a retrograde transneuronal tracer that is preferentially taken up by synaptic terminals. Concomitant astroglial infections do not appear to lead to local nonspecific neuronal labeling and may even

restrict PRV transfer to increase the probability that virus uptake occurs at synaptic sites. However, whether or not PRV is transferred *exclusively* to synaptic afferents remains unresolved.

VI. NEUROANATOMICAL TRACING WITH PRV—PRACTICAL CONSIDERATIONS AND PITFALLS

Viral tracing is a highly sensitive technique. When applied judiciously, the viral transneuronal labeling method can produce information regarding central neural circuits that is unattainable by other methods. Its high sensitivity is, however, inseparable from a significant potential for nonspecific labeling of unrelated neural circuits. The need for conservative interpretation of the patterns of central labeling is important because even attenuated viral tracers are capable of infecting many different cell types in the brain (neurons, astroglia, and ependymal cells) by various routes. An analysis of a series of sections throughout the brain should be performed for each PRV case in any given study to rule out the possibility that nonspecific viral labeling had occurred. For example, when PRV is injected into peripheral targets, such as an autonomic ganglion, inspection of the supraoptic and magnocellular paraventricular subnucleus can be used to determine whether a viremia had occurred, since labeling in these two sites would be the result of uptake from the vascular system. Similar screening scenarios are important for the evaluation of other types of experiments as well.

A. Viral Tracing in the CNS

The interpretation of data obtained from viral transneuronal infections within the CNS can be extremely difficult, compared with viral injections into peripheral structures. Viral entry into the CNS from the periphery can be isolated to a single neural channel, but the situation is not as straightforward as in the brain. Central neurons receive input from multiple CNS regions. Frequently, these regions are interconnected, greatly increasing the complexity of the potential routes of viral spread (Fig. 9.5).

In the late 1990s, it was demonstrated that Bartha PRV could be used to define central circuits (Jasmin *et al.*, 1997; Kaufman *et al.*, 1996; O'Donnell *et al.*, 1997), although earlier studies using HSV1 in monkeys had established the feasibility of this approach (Lynch *et al.*, 1994; Middleton and Strick, 1994). Subsequent evaluation of PRV tracing within the brain addressed significant concerns about this methodology, such as injection site analysis, nonspecific spread through the cerebrospinal fluid, and viral uptake by fibers of passage (Chen *et al.*, 1999).

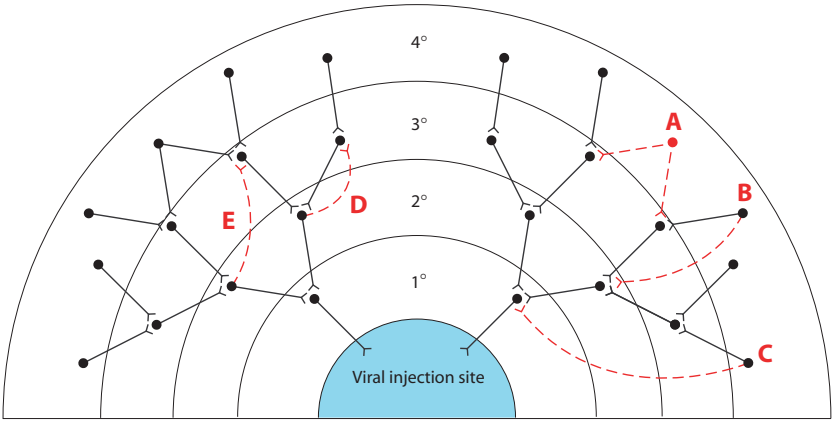

Figure 9.5. The brain's complex circuitry frequently offers multiple alternative hypotheses for the route by which a virus may have labeled a particular group of neurons. This complexity can complicate the interpretation of tracing data. In this diagram, each neuron symbolizes a neuroanatomical region with known axonal projections to a lower order site. Common types of neural connections that can complicate tracing data are shown as dashed lines. A simple time-course analysis of viral progression may differentiate between the potential routes of labeling in (C), in which two transneuronal steps separate the alternatives. However, this approach may not provide a clear answer for (B) and cannot resolve (A). The resolution of labeling in these situations may require additional neuroanatomical experiments. The reciprocally connected pair of neuronal groups depicted by (D) may lead to uncertainty as to whether a specific subset of neurons within the second-order group was labeled (1) directly from neurons in the first-order group or (2) from their target neurons in the infected third-order group, which had received virus from a different subset of second-order neurons in the same region. In (E), a similar hypothetical situation is depicted.

1. PRV Injection Site

Chen *et al.* (1999) directly addressed a number of potential pitfalls associated with Bartha PRV tracing within the brain. One of the critical issues is defining an injection site. This was problematic since after PRV was injected into brain parenchyma it rapidly entered local axons in which virions were transported away from the injection site. Several days later, when most PRV tracing experiments were terminated, immunohistochemical staining for PRV did not reveal the injection site. To avoid this problem, these investigators verified that a cocktail of PRV in a 0.05% CTb solution was useful for approximating the injection site (Chen *et al.*, 1999).

2. Bartha PRV in the Ventricular System

Another complicating issue associated with central PRV tracing studies is the possibility that the virus could enter the cerebrospinal fluid and

cause nonspecific infections throughout the brain. Chen *et al.* (1999) injected Bartha PRV into the lateral ventricle of rats and found that, after 1 or 2 days, infections were confined to specific sites and not randomly distributed throughout the brain. Highly reproducible PRV labeling occurred, within a day after ventricular injection, in a specific subset of dorsal raphe neurons immediately beneath the cerebral aqueduct. These neurons are responsible for the serotonergic axonal plexus in the ependymal lining of the ventricular system (Chan-Palay, 1976), as demonstrated by rapid labeling of the same group of neurons, as well as their ependymal axonal plexus, by CTb (mixed with PRV for injection site localization) in the same animals. The presence of PRV and/or CTb within this specific ependymal-projecting subset of dorsal raphe neurons was proposed as practical marker for screening viral tracing cases after injection near a ventricle (Aston-Jones and Card, 2000; Chen *et al.*, 1999). After 2 or more days postinjection, PRV-labeled neurons were also found scattered in other regions, such as lateral septum and hippocampus. This labeling was hypothesized to have resulted from the uptake of PRV from infected ependymal cells that had lysed. Regardless of the cause for this labeling, these experiments showed that Bartha PRV injections into the brain ventricular system did not cause widespread infections.

3. Bartha PRV in the Vasculature

Vascular leakage of Bartha PRV is another problem, since central injections invariably cause disruption of some blood vessels. To date, six studies have shown that when Bartha PRV is injected into the venous system of rat in the doses used in central tracing studies it does not produce central infections (Westerhaus and Loewy, 1999). Inoculation of similar amounts of Bartha PRV directly into the arterial supply of the brain, however, has not been tested.

4. Uptake by Fibers of Passage

Another important issue is whether Bartha PRV is taken up by fibers of passage within the injection site. Peripheral nerves take up this virus when high doses are used (Dobbins and Feldman, 1994). Only one study has examined this issue for CNS injection sites. Bartha PRV was injected into the ventrolateral medulla of rats, in the region where crossed axons from the inferior olivary nucleus travel toward the inferior cerebellar peduncle. Viral injection here resulted in robust retrograde neuronal labeling in the contralateral inferior olivary nucleus (Chen *et al.*, 1999). Whether this uptake was due to entry into injured axons or whether uptake by intact fibers of passage is not certain, but these findings highlight a potential confound when PRV is used as a central tracer. Stereotaxic injections will

cause a certain degree of damage to fibers passing near the target site—this complication should be considered when PRV is used as a central tracer.

5. Controlled Viral Tracing in the CNS

Some means of guarding against misinterpretation of nonspecific patterns of transneuronal infection include corroboration with previously characterized neuroanatomical connections, negative control lesion experiments, and positive control experiments excluding alternative pathways. Clearly, viral labeling should be viewed as specific only when underlying single neuronal connections can be verified by conventional neural tracing. If infected neurons are found in sites incompatible with a specific retrograde transsynaptic spread from the viral injection site, based on well-established neuroanatomical data, labeling should be considered nonspecific. If a hypothetical pathway cannot be convincingly constructed from the viral injection site to a particular infected group of neurons by piecing together well-characterized traditional tracing data, the specificity of labeling should be questioned.

When multiple different transneuronal routes could explain viral labeling data, combining single-neuron tracing with PRV injection can help distinguish the most likely pathway (Aston-Jones *et al.*, 2001; Farkas *et al.*, 1998). Also, lesion of a relay point in a proposed circuit should significantly decrease viral infection within a transneuronally labeled group of neurons. For example, in a study of the multineuronal circuit from the SCN to the medial prefrontal cortex, the paraventricular thalamic nucleus was hypothesized as the key relay point. Lesioning this cell group effectively blocked transneuronal labeling of the SCN after PRV injection within medial prefrontal cortex (Sylvester *et al.*, 2002). As shown in this study, lesion effects should be quantified and replicated in a sufficient number of cases to demonstrate a statistically significant reduction of viral labeling in lesioned animals versus nonlesioned animals after an identical postinjection survival time. Injection site analysis is necessary for every experiment to screen for the possibility that differences in labeling could be produced between lesioned and nonlesioned groups simply because of slight differences in the placements of viral injections. In addition, viral labeling in a positive control site should be quantified, in both lesioned and nonlesioned groups, to verify that the infection proceeded normally in alternative pathways that are not dependent upon the lesioned site as a relay.

A positive control lesion experiment, demonstrating that lesion of a relay within a different potential route does not reduce transneuronal labeling, can be helpful in verifying a hypothesized multineuronal pathway (Pickard *et al.*, 2002; Smeraski *et al.*, 2004). Lesions of alternative pathways can be critical for falsifying alternative hypotheses concerning the transneuronal route between injection site and labeled neurons (see "Accurate Interpretation

of Viral Labeling Patterns"). However, the existence of multiple parallel pathways can still complicate data interpretation in some circumstances (Aston-Jones *et al.*, 2001; Farkas *et al.*, 1998).

The approaches described above are not always practical for all tracing objectives. Sometimes, control experiments will not guarantee a clear answer regarding the specific circuit being studied by viral transneuronal labeling. However, cases should always be screened for indications of nonspecific CNS labeling.

B. Viral Tracing in the Peripheral Nervous System

Viral tracing projects designed to study the central circuits controlling motor outflow systems are considerably easier to analyze than projects designed to study CNS circuits because the route of viral entry can be experimentally limited to a single outflow channel. For example, the central parasympathetic circuits regulating pancreas, airways, or heart were studied in isolation from the sympathetic system by performing PRV tracing experiments on rats with T1 spinal transactions. These lesions completely eliminated the possibility of viral entry into the brain via the sympathetic nervous system (Haxhiu *et al.*, 1993; Loewy and Haxhiu, 1993; Ter Horst *et al.*, 1996). Since Bartha PRV does not enter the CNS via afferent systems, the data generated in these particular studies clearly produced information regarding the organization of central parasympathetic motor systems.

PRV injections into sympathetic structures sometimes resulted in unexpected labeling of vagal motor neurons, which has been greatly reduced after bilateral subdiaphragmatic vagotomy (Geerling *et al.*, 2003). Even after bilateral vagotomy, occasional cases were generated with a relatively low number of PRV-infected neurons in the dorsal vagal nucleus (e.g., 10–20 bilaterally throughout a 1-in-5 series of the full extent of the nucleus). This residual labeling may have been due to the failure to transect all of the vagal fibers.

Practical considerations have required that various neuroanatomical criteria be set for what constitutes a specific viral tracing infection after peripheral PRV injection (Sams *et al.*, 1995; Strack *et al.*, 1989b). For example, after retrograde tracing from the stellate ganglion, Jansen *et al.* (1995b) discovered labeled neurons in the red nucleus. Since this nucleus was mainly considered a somatic premotor nucleus, the labeling was interpreted as nonspecific and used to screen individual cases for nonspecific viral spread. Although it is possible that labeling in the red nucleus resulted from specific transneuronal labeling of a previously unknown sympathetic outflow pathway, the admittedly subjective criterion served as a useful index for potential nonspecific labeling in this particular study.

Finally, the neurosecretory magnocellular neurons in the paraventricular and supraoptic nuclei should be carefully examined after any type of PRV

injection into peripheral structures. Neurons in these two nuclei project exclusively to the capillary beds in the posterior pituitary. If any cell body labeling is found at these two sites, it indicates that false-positive labeling likely occurred due to uptake of PRV from the vascular system.

C. Accurate Interpretation of Viral Labeling Patterns

The data obtained from a PRV transneuronal tracing experiment can be very complex, even after only two or three rounds of retrograde transport and viral replication (Fig. 9.5). Exponential replication of the virus occurs, within a geometrically increasing population of infected afferent neurons, at every retrograde transmission. Large numbers of neurons in many sites can be labeled over several days.

Substantial increases in the number of infected neurons can lead to incorrect assumptions about the routes by which viral tracer has infected a particular group of neurons. The neuroanatomical material obtained from a PRV tracing experiment provides a complicated snapshot of all the neurons infected with PRV up to the time of death. The labeling pattern itself does not indicate the order in which particular neurons are infected. Determination of the multineuronal pathways indicated by such data is particularly problematic when an infected neuronal group is connected to numerous other infected sites. Frequently, more than one reasonable hypothesis can be generated to explain such complicated tracing data.

1. Determining the Route(s) of Transneuronal Labeling—Experimental Approaches

Multiple experimental approaches can test each hypothesis. First, a temporal estimate of viral progression can sometimes aid the generation of preliminary hypotheses about the hierarchical connections of an afferent pathway (Larsen *et al.*, 1998; Pickard *et al.*, 2002; Smeraski *et al.*, 2004). In this approach, a provisional timeline is created from the comparison of infected sites in animals killed at progressively longer postinjection time points.

Three problems, however, often prevent a simple temporal approach from discriminating between various alternative synaptic pathways. First, the rate of viral progression may be variable among experimental animals. Second, practical experience has revealed that retrograde infection does not occur in idealized waves with one stage of replication, and retrograde transfer occurring at one afferent level before infected neurons appear at a higher level. Rather, infections tend to progress in a continuous manner, with the number of neurons infected at one hierarchical level growing even after infected neurons have appeared in groups afferent to that site. Also, afferent neurons located farthest from an infected neuron require longer axonal transport times, resulting in a delay in labeling. This gradual spread

becomes even more of a blur as the infection spreads through higher order afferents. When added to the variability in rate of viral progression between different animals, this problem can obstruct the presumed logical interpretation of a simple temporal approach. Third, synaptic connections in the brain frequently do not exist in an idealized hierarchical arrangement. Multiple retrograde avenues to an afferent site and complicated reciprocal connections between higher order afferents typify brain architecture (Fig. 9.5). When two or more potential routes of viral spread are possible, temporal analysis may be useful in distinguishing between possibilities, but corroboration by other approaches is often necessary.

Besides a time-course analysis of viral spread, combination with conventional neural tracing and lesion experiments (described above) can aid in differentiating between alternative pathways. When more than one hypothetical transneuronal route can explain the spread of virus to a labeled group of neurons, selective lesion studies of the different routes can help define the circuit (see above).

2. An Example: PRV Transneuronal Labeling in Retinorecipient Sites

One example plainly demonstrates the importance of this critical analytical approach to viral transneuronal tracing. When comparing the wild-type and attenuated Bartha strain of PRV, Card *et al.* (1991) observed two qualitatively different patterns of infection after injecting two different viruses into the vitreous body of the eye. Wild-type PRV produced a rapid infection in all the sites targeted by the retinal output, such as the lateral geniculate nucleus, superior colliculus, and SCN, consistent with the idea that this virus is transported in the anterograde direction. Bartha PRV, however, produced a greatly delayed infection in the SCN and did not produce an infection in the two main retinorecipient sites~~su~~perior colliculus and lateral geniculate. Importantly, Bartha PRV infections within the SCN occurred only after long postinjection survival times (3–4 days). Given the high viral dose used~~10~~ ^{6} plaque forming units (pfu), two orders of magnitude higher than the dose at which Bartha PRV was originally used for retrograde transneuronal tracing after 4 days of survival (Strack *et al.*, 1989a, b; Strack and Loewy, 1990)~~th~~is time frame was consistent with the time required for retrograde transneuronal spread to the hypothalamus through a chain of multiple neurons.

Despite the extended time required for viral spread to the SCN, these observations were interpreted as evidence for anterograde transport of Bartha PRV. To reconcile this interpretation with the complete lack of anterograde transmission to the main retinal target sites, it was further assumed that only a subpopulation of retinal ganglion cells, which project to nonvisual sites such as the SCN, is vulnerable to a productive infection by Bartha PRV (Card, 2000; Card *et al.*, 1991). Despite a lack of evidence for anterograde transneuronal spread in other studies with Bartha PRV, this interpretation remained unchallenged.

The assumption of a very slow form of anterograde transneuronal spread, resulting in a selective infection of the retinorecipient neurons of the SCN, served as the basis for a number of subsequent viral tracing studies of this circuitry (Card, 2000; Hannibal *et al.*, 2001; Moore *et al.*, 1995; Smith *et al.*, 2000) and for investigations into the genetic properties conferring this unusual property upon Bartha PRV (Brideau *et al.*, 2000; Card *et al.*, 1992; Enquist *et al.*, 1994; Husak *et al.*, 2000; Tomishima and Enquist, 2001; Whealy *et al.*, 1993). The implication that Bartha PRV might be capable of anterograde transport casts doubt on several other investigations that provided evidence that this virus moves exclusively in a retrograde manner (Rotto-Percelay *et al.*, 1992; Strack *et al.*, 1989b).

It was not until over a decade later that an alternative explanation was tested. In the intervening years, Enquist *et al.* (1994), using a series of deletion mutants, evaluated the importance of individual genes to the anterograde spread of PRV. These important studies built a strong case for the necessity of three particular genes, deleted in Bartha PRV, for anterograde spread of PRV (see "Specific Retrograde Transport of Bartha PRV"). Further analysis of the retinal infections produced by viruses lacking two of these genes indicated that mutants deficient in anterograde transport can still infect all types of retinal ganglion cells (Enquist *et al.*, 1994; Husak *et al.*, 2000). This finding did not fit well with the proposal that Bartha PRV produces anterograde labeling in only a subset of retinorecipient nuclei by selectively infecting a small subpopulation of retinal ganglion cells (Card *et al.*, 1991).

Following these reports, Pickard *et al.* (2000) tested the possibility that the spread of Bartha PRV from the eye to the SCN and other sites might not be the result of slow anterograde spread from a specific subset of retinal ganglion cells, but, instead, due to retrograde transneuronal spread via the autonomic nerves innervating the eye. In the original tests of Bartha PRV transneuronal specificity, Strack and Loewy (1990) demonstrated that injection into the nearby anterior chamber of the eye produced robust retrograde transneuronal labeling of the sympathetic outflow to the eye. In addition, a prominent multisynaptic outflow from the SCN to the diverse sympathetic and parasympathetic targets had been demonstrated (Ueyama *et al.*, 1999). Accordingly, in both the hamster (Pickard *et al.*, 2002) and the rat (Smeraski *et al.*, 2004), it was shown that (1) enucleation of the eye 24 h after Bartha PRV injection (preventing anterograde spread of virus due to degeneration of the optic axons from the destroyed retinal ganglion cells) did not prevent later infection within the SCN and other retinorecipient sites, (2) PRV infection in autonomic preganglionic sites~~the~~ parasympathetic Edinger–Westphal nucleus and sympathetic ganglionic and preganglionic neurons—preceded the SCN infection, and (3) lesions of these autonomic sites prior to PRV injection virtually eliminated infection in the SCN. In the hamster, neurons in the retinorecipient portion of the SCN were not even the first to be infected. Rather, their target neurons in the subparaventricular zone, dorsal to the SCN, were infected before labeling occurred in the SCN (see

also Card *et al.*, 1991). In addition, the first appearance of PRV within the rat SCN did not overlap the retinohypothalamic projection (identified by concurrent anterograde axonal labeling with CTb; Smeraski *et al.*, 2004). These findings clearly disproved claims that Bartha PRV could be used as an anterograde transneuronal tracer.

3. Thorough Neuroanatomical Hypothesis-Testing

A provocative pattern of transneuronal labeling can tempt assumptions about the nature of the underlying pathway from injection site to infected neurons. As the preceding example demonstrates, however, such assumptions should not prevent the rigorous testing of alternative hypotheses. Carefully analyzing viral tracing data before asserting confidence in a particular explanation can be both complicated and time-consuming. However, combining basic viral tracing with thorough and prudent neuroanatomical analysis can significantly advance our knowledge of complicated circuits within the CNS (Aston-Jones *et al.*, 2001; Krout *et al.*, 2003; Pickard *et al.*, 2002; Smeraski *et al.*, 2004; Sylvester *et al.*, 2002).

D. False-Negative Data After Viral Transneuronal Tracing

As with any neural tracing technique, the degree of uptake and subsequent labeling of afferents to an injection site is dependent, in part, upon the amount of tracer used. Hence, with small tracer injections, a lack of labeling can be observed in sites known to provide lighter innervation to an injection site. For PRV tracing, this was first noted by O'Donnell *et al.* (1997), when injections of Bartha PRV into the mediodorsal thalamic nucleus did not produce the retrograde labeling that was expected, based on prior retrograde tracing studies, within the basolateral amygdala, a light source of innervation. Despite substantial retrograde transneuronal infections via the dense pallidal afferents, the absence of basolateral amygdala labeling suggested that virions either selectively avoided particular afferent system or stochastically entered only a proportion of afferent terminals in a given site, based on the relative amount of virus and the density of axon terminals.

This latter possibility was tested by Card *et al.* (1999), who injected a range of different Bartha PRV concentrations (10^4–10^5 pfu) into the striatum. At 2 days postinjection, a clear dose dependency was observed for extent of viral transneuronal labeling in various sites afferent to the striatum.

Viral concentration and postinjection survival time are two critical variables that affect optimal transneuronal labeling. Since only a few papers have dealt with this subject, it is not possible to make generalizations at this time regarding the optimal conditions to label any given CNS circuit. Rather, these important experimental parameters need to be empirically determined, but a reasonable starting point for most experiments would

involve injections of ~3000 virions of Bartha PRV and a survival range of 2–4 days.

VII. OTHER VIRUSES USED FOR TRANSNEURONAL TRACING STUDIES

Bartha PRV remains the only virus subjected to direct tests of its specificity as a retrograde transneuronal tracer (Card *et al.*, 1993; Chen *et al.*, 1999; Pickard *et al.*, 2002; Rinaman *et al.*, 1993; Rotto-Percelay *et al.*, 1992; Smeraski *et al.*, 2004; Strack *et al.*, 1989b; Strack and Loewy, 1990). However, various other viruses are also used for transneuronal tracing studies. Experiments with HSV1 and rabies have been used to produce transneuronal labeling with varying indications of specificity. Additional direct verifications of their directional and transsynaptic specificity could be highly useful, particularly since, unlike PRV strains, these can be used for tracing experiments in primates. In addition, restrictions on Bartha PRV usage in countries where PRV has been eradicated from most pig and cattle populations may leave these viruses as the only practical options for certain laboratories.

A. HSV1 as a Transneuronal Tracer

HSV1 has been used for transneuronal tracing in various species. Different HSV1 strains have been used for transneuronal studies in primates by Strick and colleagues (Clower *et al.*, 2001; Hoover and Strick, 1993, 1999; Lynch *et al.*, 1994; Middleton and Strick, 1994, 1996, 2001, 2002).

The transneuronal pattern of labeling produced by this virus is highly dependent on the specific strain used for tracing (Norgren and Lehman, 1998). The SC16 strain of HSV1, used in early studies by Ugolini, produced both retrograde and anterograde transneuronal labeling in the brainstem and cerebellum (Ugolini *et al.*, 1987). Another HSV1 strain, FMC, was used for retrograde transneuronal labeling of central neurons afferent to various autonomic targets in a series of studies by Blessing and colleagues (Blessing *et al.*, 1991; Ding *et al.*, 1993; Li *et al.*, 1992a, b, 1993; Wesselingh *et al.*, 1989).

The patterns of infection produced by the injection of different strains of HSV1 into monkey cortex indicated that the McIntyre-B strain preferentially caused a retrograde transneuronal pattern of labeling while the H129 strain produced an anterograde labeling pattern (Zemanick *et al.*, 1991). Subsequent analysis, however, revealed that neither virus is transported exclusively in one direction, despite a significant difference in directional preference. McIntyre HSV1 can produce transneuronal labeling in the anterograde direction (Norgren *et al.*, 1992). Also, H129 clearly produces a retrograde infection within first-order afferent neurons (Rinaman and Schwartz, 2004). This strain, unlike Bartha PRV, was not observed to spread transneuronally from retrogradely infected first-order afferent vagal

motor neurons in rats with lesioned vagal afferent fibers, following injection into the stomach wall (Rinaman and Schwartz, 2004). However, the pattern of transneuronal infection produced by H129 in this study, after a presumed anterograde transneuronal infection within the nucleus of the solitary tract (NTS), may be more consistent with retrograde transneuronal labeling of neurons afferent to this site, rather than simply anterograde spread in NTS neurons to their efferent targets [e.g., the strong infection depicted in Fig. 2 of Rinaman and Schwartz, 2004, within a dorsal part of the bed nuclei, an NTS afferent site~~ver~~sus the dense NTS innervation in a more ventral region of the bed nuclei (Ricardo and Koh, 1978)]. Further testing of the directional specificity of transneuronal labeling produced by HSV1 strain H129 should reveal whether or not this virus will be useful as an anterograde transneuronal tracer.

One drawback of viral tracing with various strains of HSV1 is the lack of neuroanatomical experiments directly addressing the specificity of transneuronal labeling. In Ugolini's original HSV1 tracing study, a significant degree of nonspecific local spread of virus was reported (Ugolini *et al.*, 1987). This nonspecific spread resulted in false-positive anterograde transneuronal labeling. Further tracing work with this strain was then conducted without direct tests of the specificity of transneuronal labeling (Ugolini *et al.*, 1989). The potential for nonspecific labeling by various strains of HSV1~~via~~ both local and transneuronal routes~~lim~~its the utility of this virus for many neural tracing objectives (Fig. 9.4).

B. Rabies as a Retrograde Transneuronal Tracer

Although the name "pseudorabies" may seem to imply a functional relationship between PRV and rabies, these two viruses are very different. Like HSV1, PRV is a member of the Alphaherpesvirinae family of neurotropic herpesviruses. It contains a double-stranded DNA genome, which is transcribed and replicated in the cell nucleus, and can cause lytic cell death shortly after infection or establish latency in vivo in neuronal tissue. The reason for the name "pseudorabies" was the CNS infection it produced in farm species at a time when few viruses (rabies being one of them) were known to invade the brain (Aujesky, 1902).

Rabies, in contrast, is a rhabdovirus~~a~~ single-stranded, negative-sense RNA virus that replicates in the cytoplasm. Unlike herpesviruses, rabies infections of the CNS, while lethal, do not appear to cause widespread cell death. Hence, this virus has been used for retrograde transneuronal labeling in various paradigms, in both rodent and primate. The earliest tracing study with rabies demonstrated anterograde transneuronal infection within the brain after injection of a challenge virus strain (CVS) of rabies into mouse olfactory epithelium (Astic *et al.*, 1993). When CVS rabies was injected into the hypoglossal nerve, retrograde transneuronal labeling was produced in rats without obvious nonspecific spread (Ugolini, 1995).

In particular, no infected glial cells were observed and infection did not appear to spread locally, even several days after the onset of infection within primary infected neurons in the hypoglossal nucleus. This result stood in striking contrast to the nonspecific viral labeling originally observed with HSV1 (Ugolini *et al.*, 1987). Studies using rabies to produce transneuronal labeling have demonstrated a potential for its use in rodent and primate neural tracing experiments (Astic *et al.*, 1993; Graf *et al.*, 2002; Grantyn *et al.*, 2002; Kelly and Strick, 2003; Moschovakis *et al.*, 2004; Tang *et al.*, 1999; Ugolini, 1995). Rabies central transneuronal tracing methodology has been thoroughly reviewed by Kelly and Strick (2000).

C. Perspectives—HSV1 and Rabies

There are two major drawbacks associated with the use of HSV1 and rabies viruses as transneuronal tracers. First and foremost, these viruses infect humans, representing a potential hazard to laboratory personnel and requiring additional precautions, especially for rabies, which requires repeated vaccinations and strict precautions (Kelly and Strick, 2000). For transneuronal studies in nonprimate species, the use of Bartha PRV does not present this problem, since it does not infect humans (Gustafson, 1975). Second, information regarding the neuroanatomical specificity of labeling produced by these viruses is incomplete. Rabies and HSV1 have not yet been subjected to many of the experimental tests used to characterize Bartha PRV as a neuroanatomical tracer (Card *et al.*, 1993; Chen *et al.*, 1999; Pickard *et al.*, 2002; Rinaman *et al.*, 1993; Rotto-Percelay *et al.*, 1992; Smeraski *et al.*, 2004; Strack *et al.*, 1989b; Strack and Loewy, 1990).

Varying degrees of transneuronal and directional specificity have been inferred from the patterns of infection observed in various tracing paradigms (Ugolini, 1995; Ugolini *et al.*, 1987; Zemanick *et al.*, 1991). For HSV1, a problematic degree of nonspecific local spread, resulting in subsequent nonspecific transneuronal labeling, has been described (Ugolini *et al.*, 1987). In contrast, some strains of rabies may spread only in the retrograde direction in some paradigms (Kelly and Strick, 2000; Ugolini, 1995), although certain strains can clearly produce anterograde transneuronal labeling (Astic *et al.*, 1993). The transneuronal specificity of infection with rabies appears promising, especially in comparison with HSV1 (Ugolini, 1995), but has yet to be directly tested.

A high degree of transneuronal specificity may not be a prerequisite for transneuronal tracing in primate circuits involving massively parallel circuitry, such as primate cortical, basal ganglia, cerebellar, and thalamic pathways. So long as the bulk of viral transneuronal transfer occurs, stochastically, in a transsynaptic manner, it is possible that nonspecific viral spread is largely constrained to immediately adjacent portions of parallel pathways within the same circuits. In any case, rabies and HSV1 are currently the only viable options for transneuronal labeling experiments in primates.

VIII. CONCLUSION

Neurotropic viruses are extremely useful neural tracers for a variety of neuroanatomical objectives. Strains with well-characterized properties, such as Bartha PRV, can be used to gain valuable new data about mammalian neural circuits. When tracing studies are executed and interpreted within known technical limitations, they can provide information currently unattainable by other methods.

Combined advances in virology and molecular biology may allow the design of viruses that will provide selective information about particular neural networks, revealing CNS circuitry in unprecedented detail.

APPENDIX

A. Safety and Practical Issues

Bartha PRV has been successfully used to eradicate PRV from most pig and cattle populations in many countries, and consequently, various rules and restrictions have been developed concerning its use. In the United States, a BSL-2 laboratory is required for use of this agent.

B. Sources of Bartha PRV and Recombinant Strains

Bartha PRV and related recombinant strains can be obtained directly from individual researchers who work with this virus. Investigators can contact Drs. Arthur Loewy (USA) and Thomas Mettenleiter (Germany).

In addition, the Center for Neuroanatomy with Neurotropic Viruses was established at the University of Pittsburgh in the summer of 2004. Under the direction of Drs. J. Patrick Card (card@ms.pitt.edu) and Peter Strick (strickp@pitt.edu), the Center will serve as a resource for investigators interested in obtaining various viral strains for tracing experiments.

C. Viral Growth, Aliquots, and Storage

The broad host range of PRV in vivo is reflected in a broad spectrum of cells that can be productively infected in vitro. One of the advantages of working with PRV is its ability to replicate to rather high titers in easily cultivable permanent cell lines. Primarily, kidney cell lines from rodents (rabbit RK-13 cells), ruminants (Madin-Darby bovine kidney, MDBK), or porcines (PSEK, PK-15) are used. However, other cell lines, e.g., monkey kidney cells (Vero), are also permissive for PRV infection. The highest titers of progeny virus are usually obtained on porcine cells, whereas RK-13 cells are preferentially used for transfection and for establishment of transgenic cells expressing

viral genes for transcomplementation of respective viral deletion mutants. MDBK cells are ideal for plaque titration since they produce the clearest and most easily visible plaques. However, other cell lines can also be used.

All the three mentioned cell lines can be cultivated in Eagle's minimum essential medium (MEM) supplemented with either 5% (for MDBK, PSEK, and PK-15) or 10% fetal calf serum (for RK-13). They routinely grow well on disposable plastic tissue culture flasks and can be propagated by trypsinization [0.8-g NaCl, 0.4-g NaCl, 1-g dextrose, 0.58-g $NaHCO_3$, 0.5-g trypsin, 0.2-g EDTA, and 1l H_2O (pH 7.1–7.2), sterile filtered] from the culture flasks, dilution at a ratio between 1:3 and 1:10 with fresh medium supplemented with fetal calf serum, and reseeding.

To grow high-titered virus stocks, cells are infected with PRV at a multiplicity of infection between 0.01 and 0.1 pfu per cell. After 1 h of adsorption, the inoculum is removed and the cells are overlaid with fresh medium. After 2–3 days, a complete cytopathic effect develops with cells first exhibiting a rounded appearance, which, as viral infection progresses, leads to lysis of the cells. After lysis of the cell monolayer, the whole culture flask is frozen and thawed, supernatant and cell debris are collected in a plastic tube (e.g., Falcon 50-ml tube) and cellular debris is sedimented by low-speed centrifugation (e.g., Heraeus Minifuge, 10 min, 6000 rpm). All virus isolation steps should be performed on ice or in a refrigerated centrifuge (+4°C). The supernatant is removed and immediately aliquoted, routinely in between 0.5- and 1-ml aliquots, and frozen at −70°C or in liquid nitrogen. Storage at −20°C leads to rapid loss of infectivity.

For determination of the infectious titer, one aliquot is thawed on ice and serial 10-fold dilutions in medium are prepared. These are then plated onto monolayer cells preferably in 6- or 24-well tissue culture dishes. As mentioned above, MDBK cells are most suited for this purpose, although other cell types can also be used. After 1-h incubation at 37°C, the supernatant is removed and substituted by medium supplemented with 5% methylcellulose: 10-g methylcellulose, 3.76-g autoclavable MEM powder suspended in 390-ml H_2O are autoclaved (use magnetic stir bar that remains in a bottle). After cooling to room temperature, 200 mM L-glutamine and 880-mg $NaHCO_3$ (dissolved in 6-ml H_2O and sterile filtered) are then added. The stock solution is stored at 4°C and is diluted 1:4 in MEM/5% fetal calf serum for use.

Cells are then incubated in a 5% CO_2 atmosphere for 2–3 days. When plaques are clearly visible, the medium is removed, the monolayer is washed 3× with PBS, and cells are then fixed with 5% formaldehyde for 20 min, washed with PBS, and stained with 1% crystal violet in 50% ethanol for 5 min. Staining solution is removed and monolayers are washed extensively. Plaques are white on a blue background.

Routinely, titers up to, and sometimes in excess of, 10^7 pfu/ml can be obtained. If higher virus titers are required, the virus suspension can be concentrated by ultracentrifugation (e.g., for 1 h at 22,000 rpm in a Beckman TST-28 rotor). It is imperative that all steps are performed either on ice or at

+4°C. The virus pellet is gently resuspended in the desired volume of either TBSal [200 mM NaCl, 2.6 mM KCl, 10 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1.8 mM $CaCl_2$] or MEM. Then, individual aliquots of 5 μl (or other desired amount) are made in plastic microcentrifuge tubes and stored at −70°C.

D. Dilution of PRV

A tube containing a single aliquot of PRV is allowed to thaw on crushed ice. For CNS injections, 2 μl of 0.1% cholera toxin β-subunit (CTb, product #103B, List Biologicals Inc., Campbell, CA) is added. The CTb is used as a marker for the injection site and can also provide limited single-neuron tracing data, in addition to viral transneuronal labeling, in the same animal. These proportions (adding 2 μl of 0.1% CTb solution to a 5-μl viral suspension) result in a final CTb concentration of about 0.03%slightly less than the 0.05% solution recommended by Chen *et al.* (1999). In situations where injection site determination is not required, a similar dilution can be made with sterile Dulbecco's Modified Eagle Medium.

It is important to note that addition of a 2-μl CTb solution dilutes the concentration of the viral suspension, a critical variable in interpreting viral tracing experiments (see discussions above). For example, adding 2 μl to a 5-μl suspension of 10^8 pfu/ml Bartha PRV will reduce the viral concentration to 7×10^7 pfu/ml. This change is relevant to the calculation of the amount of virus injected into experimental animals.

E. Injection of PRV

Injections are made with a glass micropipette that has been filled with the aid of an operating microscope. The pipette is secured to a micromanipulator attached to a stereotaxic frame, and then advanced into a specific brain target in a surgically prepared animal. The micropipette is attached by polyethylene plastic tubing to an air pressure regulator so that the virus can be ejected by applying pressure from a handheld 50-ml syringe. Commercial equipment, such as the Picospritizer (General Valve Corp., Fairfield, NJ), can be used as well. Alternatively, a glass micropipette can be glued onto a 1-μl Hamilton microsyringe (Fisher Scientific, Pittsburgh, PA) and used in a similar capacity. The advantage of using a glass micropipette is that a carefully measured volume of virus can be delivered under microscopic control. Generally, 40-nl injections of the solution described in section "Dilution of PRV" have produced good results. A 40-nl injection of PRV without added CTb (10^8 pfu/ml) contains about 4000 pfu. If diluted by the addition of CTb as described above, this same volume contains about 3000 pfu. Rats receiving PRV injections should be surveyed daily for signs and symptoms of viral infection, such as nasal inflammation, itching, and sneezing.

F. Preparation of Brain Sections for Immunohistochemical Staining

Survival periods usually range from 3 to 4 days, for CNS injections, up to as long as 7–8 days for peripheral injections, depending upon variables such as the distance of the injected target from the brain and the desired extent of retrograde transneuronal labeling. At the end of the survival period, brain sections are processed for immunohistochemical staining in the same manner as other neuroanatomical tracing methods.

Briefly, anesthetized animals are killed by perfusion through the heart with saline, followed by 4% paraformaldehyde made in 0.1 M sodium phosphate buffer (pH 7.4). The brain is removed, stored in 4% paraformaldehyde fixative for 2 days or more, and sectioned at 50 μm using a freezing microtome or cryostat. Histological sections are collected and stored in plastic tissue culture trays containing 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% sodium azide, which acts as an antibacterial agent. The quality of the histological staining tends to be reduced after longer storage periods.

G. Immunohistochemical Staining

Immunohistochemical staining follows standard protocols. For visualizing injection sites, CTb can be localized with a goat antibody supplied by List Biologicals (Campbell, CA), used at 1:40,000, and the avidin–biotin complex method (ABC, Vectastain kit, Vector Laboratories, Burlingame, CA). For visualizing PRV, monoclonal antibodies can be purchased from Chemicon (Temecula, CA). Detailed protocols for combined PRV and neuropeptide immunohistochemistry appear in several reports (Geerling *et al.*, 2003; Oldfield *et al.*, 2002; Sylvester *et al.*, 2002).

H. Disposal of PRV-Infected Material

Preparatory steps are taken before the animals are perfused~~pl~~astic bags containing absorbent material (newspaper) are used to collect all fluids. After CNS removal, animal carcasses and these fluids are disposed in biohazard waste containers. Cages, water bottles, and bedding are sterilized and cleaned in a central veterinary facility. After completion of experiments, the tissue trays containing brain sections from PRV-infected animals are submerged in bleach until the tissue dissolves.

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