Journal of NeuroVirology (1995) 1, 349–358

Influence of infectious dose upon productive replication and transynaptic passage of pseudorabies virus in rat central nervous system

J Patrick Card[1], Joan R Dubin[2], Mary E Whealy[3], Lynn W Enquist[4]

[1]Department of Neuroscience, 446 Crawford Hall, University of Pittsburgh, Pittsburgh, PA 15260; [2]CNS Research, Du Pont Merck Pharmaceutical Co., Wilmington, DE 19880-0328; [3]Zeneca, LW220, 1800 Concord Pike, P.O. Box 15437, Wilmington, DE 19850-5437; [4]Department of Molecular Biology, Princeton University, Princeton, NJ, USA

Pseudorabies virus (PRV) is a neurotropic swine alpha herpesvirus that characteristically invades the nervous system and replicates within synaptically-linked populations of neurons. The invasive characteristics and ability of this family of viruses to replicate in neurons of the central nervous system (CNS) have been exploited to map functionally related populations of neurons in a variety of systems. In this report, we examined the effects of strain and concentration on the ability of PRV to infect retinal ganglion cells and pass transneuronally through central visual circuits. We find that the ability of virulent (PRV-Becker) and attenuated (PRV-Bartha) strains of PRV to produce a productive infection of visual circuitry is directly dependent upon the infectious dose of the injected virus. Injections of at least 10^5 total plaque forming units produce 100% infectivity, whereas lower infectious doses substantially reduce the percentage of animals exhibiting productive infection via this route of inoculation. Furthermore, the virulent strain of PRV consistently infects a higher percentage of animals across a broader range of titers than attenuated virus. These data demonstrate that viral titer and strain are important variables that should be considered in the design of studies and interpretation of data derived from investigations employing this pathogen for circuit analysis.

Keywords: alpha herpesvirus; pseudorabies virus; titer; transynaptic infection; visual circuits; rat

Introduction

Pseudorabies virus (PRV) is a neurotropic alpha herpesvirus used extensively for neuronal tract tracing (Enquist and Card, 1995; Enquist, 1994). The virus invades neurons following central or peripheral inoculation, replicates in afflicted cells, and then passes transneuronally through sites of afferent synaptic contact to replicate in afferent neurons (Card and Enquist, 1994). Although PRV and the related human pathogen herpes simplex virus (HSV) have been used successfully to characterize a variety of circuits, it has become increasingly apparent that there is substantial variability between studies in the percentage of animals exhibiting productive viral replication in the CNS, even when the same strain of virus has been used. For example, Strack and colleagues (1989a, 1989b, 1990) reported that only 20% of animals receiving injections of an attenuated strain of PRV (PRV-Bartha; Bartha, 1961) into the anterior chamber of the eye, pinna of the ear, or adrenal gland exhibited productive replication of virus in the CNS. In contrast, we achieved a 100% rate of infection following injection of PRV-Bartha or a more virulent strain of PRV (PRV-Becker; Becker, 1967) into the vitreous body of the eye or peripheral organs (Card *et al*, 1990, 1991, 1992, 1993; Nadelhaft *et al*, 1992; Rinaman *et al*, 1993; Whealy *et al*, 1993; Enquist *et al*, 1994; Standish *et al*, 1994, 1995). Although obvious differences existed in the route of inoculation, neural circuitry and the volume of virus administered in these studies, there were also substantial differences in the titer of the injected virus. The latter observation seems especially relevant since previous studies using herpes simplex virus (HSV) demonstrated that hypoglossal neurons do not

Correspondence: JP Card
Received 30 June 1995; revised 31 August 1995; accepted 1 September 1995

exhibit productive replication of virus after injection of titers lower than 10^7 plaque forming units into the hypoglossal nerve (Ugolini *et al*, 1987). The present study was conducted to determine how concentration and strain of virus influence the ability of PRV to replicate in the CNS and to determine the optimal virus concentration required for analysis of synaptically linked neurons.

Results

The total concentration of injected virus clearly affected the mean time to death of animals inoculated with either PRV-Becker or PRV-Bartha. The shortest post-inoculation survival intervals were experienced by the animals that received the highest titer injections, while the lowest titer virus (10^1 pfu) had no overt effect upon any of the animals within the 300 h post-inoculation test interval. Comparison between PRV-Becker and PRV-Bartha infections showed that the percentage of rats productively infected across a range of titers was higher for PRV-Becker than for PRV-Bartha.

PRV-Becker

Increasing doses of virus produced a productive infection in progressively larger percentages of animals until a 100% rate of infection was achieved

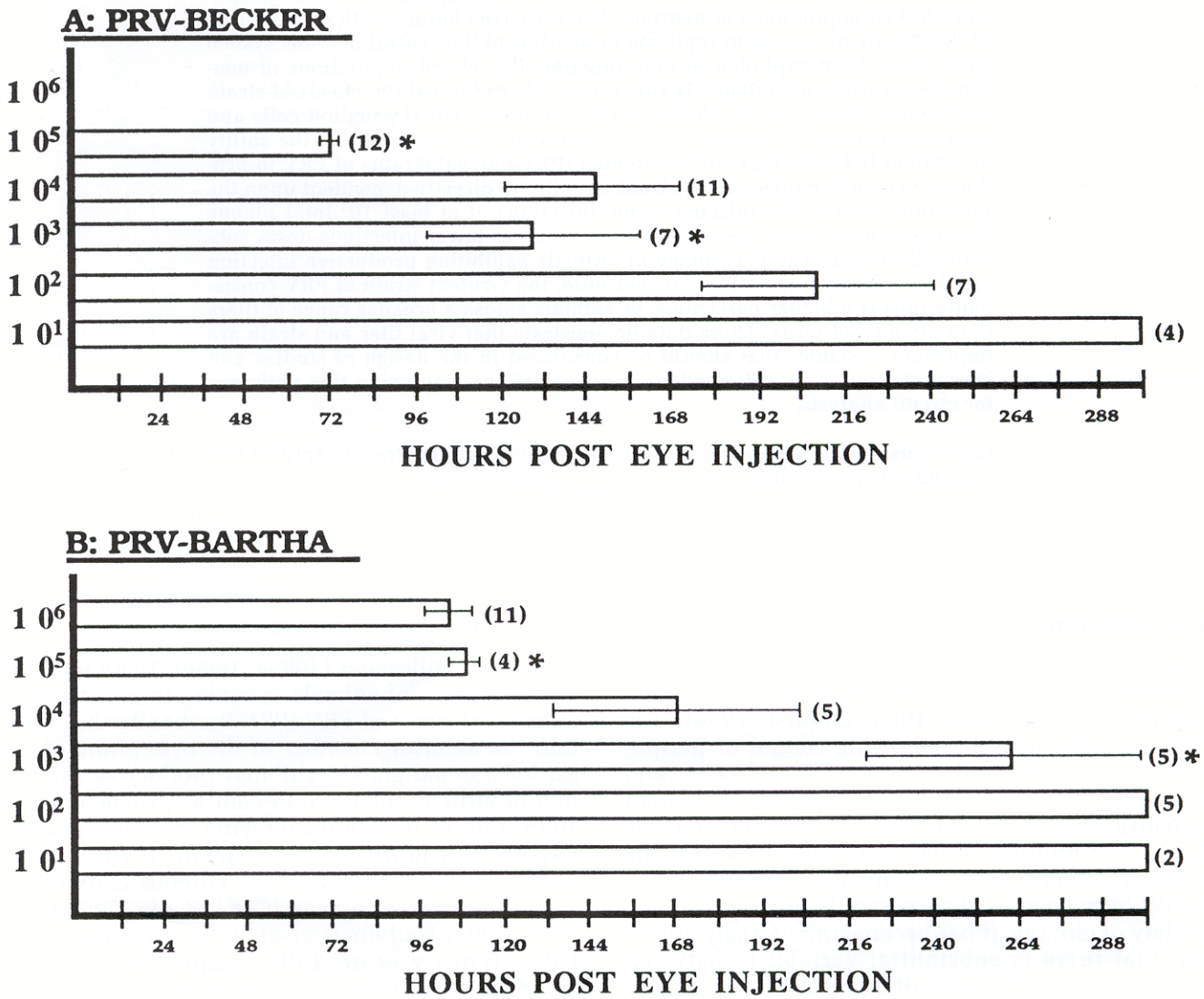

Figure 1 The mean time to death for rats receiving intraocular injection of different titers of pseudorabies virus are illustrated. Rats injected with 2 µl of either virulent virus (A: PRV-Becker) or an equal volume of an attenuated strain (B: PRV-Bartha) and their ability to survive 300 h post-inoculation was evaluated. The titer of injected virus is indicated on the vertical axes and the time in hours post inoculation is indicated on the horizontal axes. The mean time to death plus or minus the standard error of the mean for animals receiving each dosage are represented by the clear bars and the number of animals in each experimental group are shown in parentheses. An asterisk following the number of animals indicates that the mean time to death in that group of animals was significantly different from animals injected with an equivalent dose of the other strain of PRV.

with the highest dose of virus (10^5 pfu). Animals injected with the 10^5 dosage exhibited pronounced signs of infection within 60 h of inoculation and the mean time to death in this experimental group was significantly lower and less variable than that resulting from injection of reduced concentrations of PRV-Becker (Figure 1). For example, animals injected with 10^5 pfu succumbed to infection within 73 ± 2.06 h, whereas animals injected with 10^4 and 10^3 pfu survived approximately twice as long and exhibited greater variability in the post-inoculation interval (147 ± 26 h and 129 ± 29 h, respectively). Animals injected with 10^2 pfu displayed a mean time to death of 209 ± 32 h. The only experimental animals that did not show signs of infection or death following injection with PRV-Becker were those that received 10^1 pfu of virus. All of these animals survived to 300 h and showed no behavioral or physiological signs of viral infection. Furthermore, immunohistochemical analysis of the brains of these animals revealed no detectable viral antigen in any region of the neuraxis.

The presence of a productive infection in PRV-Becker injected rats was always associated with characteristic overt signs of infection not immediately attributable to an infection of the CNS. In particular, fluid excretion from both the nares and mouth was a prominent symptom of infection in chronically infected animals. These symptoms occurred irrespective of the concentration of injected virus and were associated with other behavioral changes such as hunched posture and lethargy. Although these symptoms are obviously inconsistent with a central infection, we believe they repre-

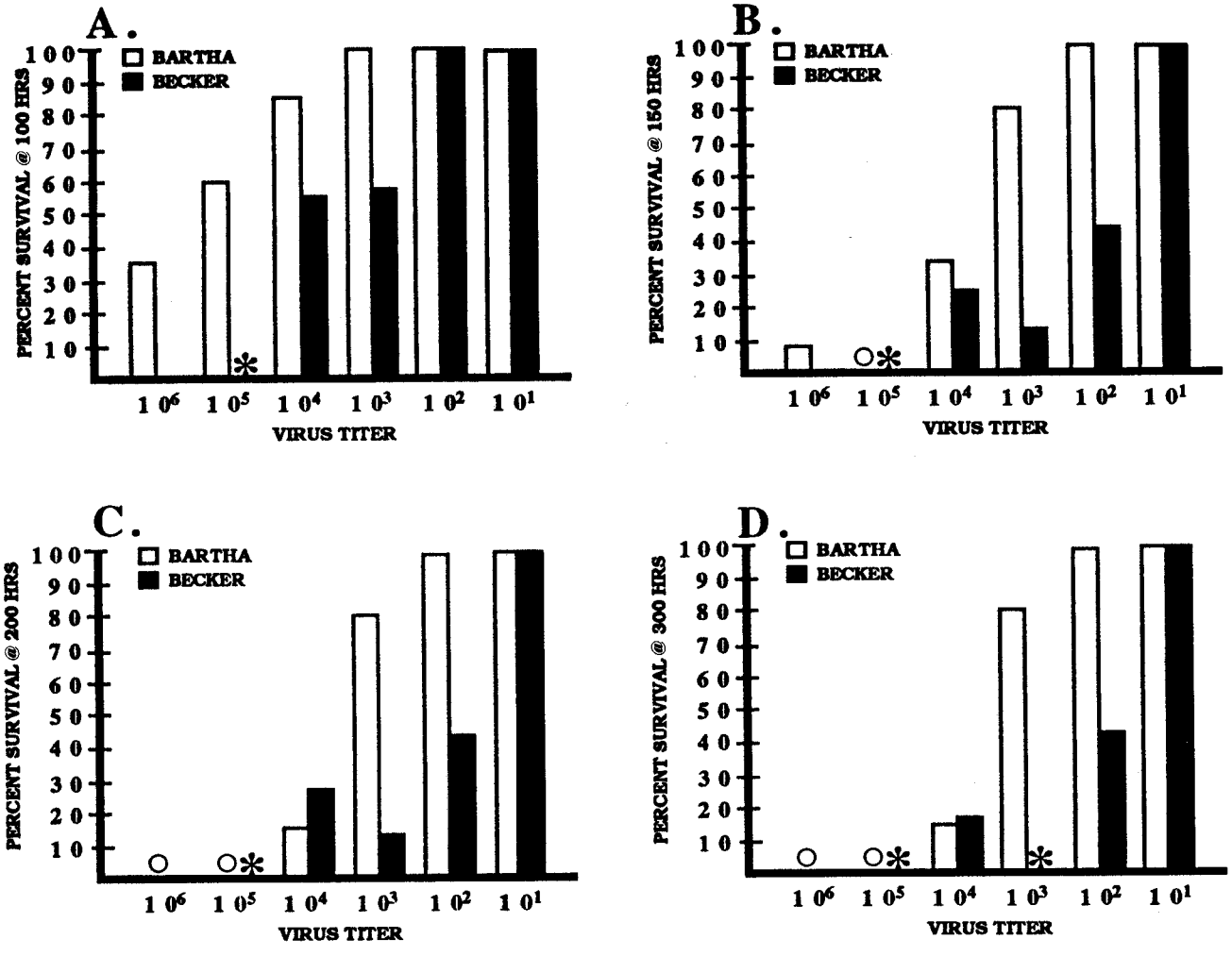

Figure 2 The percentage of animals that survived to 100 (A), 150 (B), 200 (C) and 300 (D) hours following injections with different concentrations of virulent (PRV-Becker) or attenuated (PRV-Bartha) strains of PRV are illustrated. Percentages are indicated on the vertical axes and the titer of the injected virus measured in plaque forming units is indicated on the horizontal axes. The clear bars represent percent survival of rats injected with PRV-Bartha, the filled bars represent percent survival of animals injected with PRV-Becker. * = 100% mortality following injection of indicated dose of PRV-Becker; o = 100% mortality following injection of indicated dose of PRV-Bartha.

sent a more global non-specific host response to the primary infection rather than a systemic viral infection. The survival data demonstrated that such symptoms are strictly correlated with impending mortality and with the degree of productive viral replication in visual circuitry. Furthermore, these signs of infection were largely apparent within 150 h of inoculation (Figure 2). None of the animals receiving 10^5 pfu survived to 100 h post-inoculation, but approximately 60% of animals receiving 10^4 or 10^3 pfu, and 100% of animals injected with 10^2 pfu or lower survived to this post-inoculation interval. At 150 h, less than 30% of animals injected with 10^4 or 10^3 pfu of PRV-Becker had survived, 43% of animals injected with 10^2 pfu were alive, and 100% of the animals injected with 10^1 pfu remained unaffected. In every instance of productive infection, fluid excretion from the nares and mouth and the characteristic behavioral signs of infection preceded death of the animal.

Approximately 70 h after injection of 10^4 or 10^5 pfu of PRV-Becker, viral antigen was detected in all retinorecipient regions of the CNS (Figure 3). Both the temporal and spatial aspects of CNS infection in these animals were identical to our previous reports of PRV-Becker replication in this circuitry. Large numbers of infected neurons were apparent in both the lateral geniculate nuclei (Figures 3B and E) and the tectum (Figures 3C and F) in a pattern coextensive with the known termination of retinal afferents in these regions. The most chronically infected neurons in these areas exhibited pathological changes in morphology and were associated with reactive astrocytes that also contained viral antigens. In contrast, only a moderate number of immunoreactive neurons were present in the suprachiasmatic nuclei (SCN) and the intergeniculate leaflets (IGL) of the lateral geniculate complex (Figures 3A, B, D, E) and there were few signs of neuropathology or glial infection. These findings correlate with the demonstrated temporal dissociation in the invasion and replication or PRV-Becker in functionally distinct components of visual circuitry (Card *et al*, 1991, 1992; Whealy *et al*, 1993; Enquist *et al*, 1994).

PRV-Bartha

Infection by PRV-Bartha and PRV-Becker have at least three common features. First, all rats injected with 10^1 pfu of PRV-Bartha survived the full 300 h post-inoculation interval and never showed any overt signs of infection. Second, 10^5 pfu or greater was required to infect 100% of the animals. Finally,

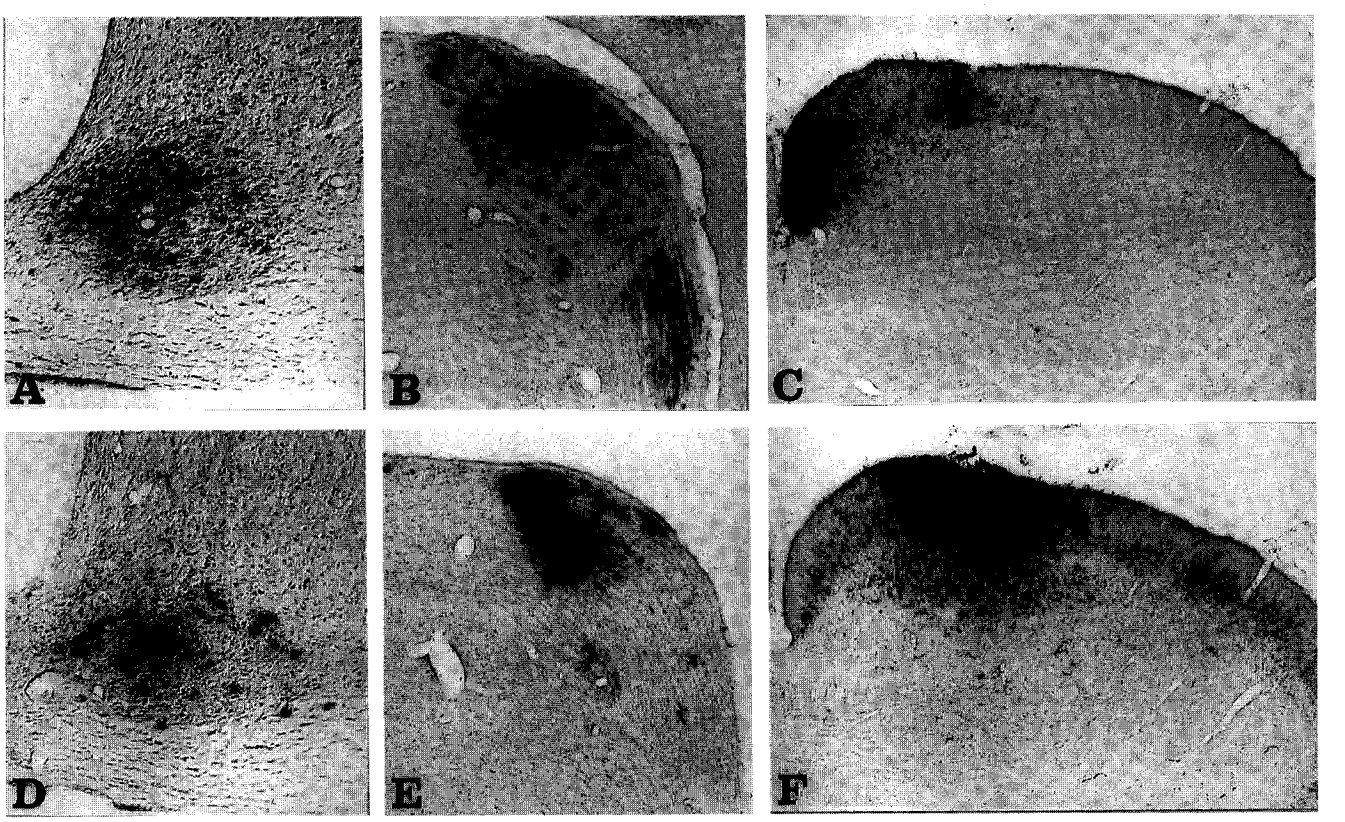

Figure 3 Immunohistochemical localization of virus in the suprachiasmatic nuclei (Figures A and D), the lateral geniculate complex (Figures B and E) and tectum (Figures C and F) after injection of different titers of PRV-Becker into the vitreous body of the eye are illustrated. Productively infected neurons were identified with a rabbit polyvalent antiserum generated against acetone inactivated PRV-Becker. Figures A, B and C are from an animal injected with 10^4 pfu of PRV-Becker and sacrificed 70 h post inoculation. Tissue illustrated in figures D, E and F illustrate productively infected retinorecipient neurons 69 h after injection of 10^5 pfu.

an analysis of percent survival at 150 h provided an accurate indication of the extent of virus-induced mortality that subsequently developed. However, PRV-Bartha infection was distinctly different from PRV-Becker infection in other important aspects. First, symptoms produced by infection with PRV-Bartha were always reduced compared to those produced by the virulent strain. Second, the percentage of animals succumbing to productive infection by any concentration of PRV-Bartha was generally less than the percentage of rats that became infected with an equivalent concentration of the virulent strain (Figure 2). Finally, at any concentration of virus, PRV-Bartha only infected a subset of the retinorecipient neurons that were infected by PRV-Becker.

As noted above, titers of PRV-Bartha that were equal to or greater than 10^5 pfu produced productive viral infection and death in 100% of injected rats within the 30 h post-inoculation survival interval (Figure 2). Mortality in this group was approximately 40% at 100 h, and reached 100% by 150 h post-injection. Greater than 80% of the animals injected with 10^4 pfu of PRV-Bartha ultimately developed fatal infections, but it took approximately 50 h longer than higher titer viruses for this extent of mortality to develop and the percentage of fatal infections resulting from injection of this concentration of PRV-Bartha never reached 100%. Rats injected with 10^3 pfu showed a substantial decrease in the percentage of animals that developed fatal infections. Eighty percent of these animals survived 300 h with no overt signs of infection, and immunohistochemical analysis of the CNS in these animals revealed no sign of viral antigen. Similarly, none of the animals that received 10^2 pfu or less of PRV-Bartha developed fatal infections or exhibited any sign of viral immunoreactivity in subsequent immunohistochemical analysis of the CNS.

Productive viral replication in the CNS of animals infected with higher titers of PRV-Bartha was confirmed in a limited number of animals that were sacrificed and subjected to immunohistochemical analysis of the CNS 92-100 h following injection of either 10^4 or 10^5 pfu of virus into the vitreous body. Both concentrations of virus produced the restricted phenotype of central infection previously shown to be characteristic of PRV-Bartha in this experimental paradigm. Substantial numbers of neurons exhibiting viral antigen were observed in the SCN and IGL (Figures 4A, B, D and E), but there were no cells displaying viral immunoreactivity in either the dorsal geniculate nucleus (Figures 4B and E) or the tectum (Figures 4C and F). Furthermore, there was little evidence of pathological changes in productively infected neurons and essentially no detectable viral antigens in reactive glia, even though the post inoculation intervals greatly exceeded the longest survival interval analyzed in the experiments employing PRV-Becker.

Overt symptoms of productive viral infection produced by intravitreal injection of PRV-Bartha were also substantially different from those produced by identical injection of PRV-Becker. Strikingly, PRV-Bartha infected animals exhibited only modest signs of viral infection despite immunohistological evidence demonstrating robust viral replication and dramatically increased levels of transynaptic passage compared to even the most severely infected PRV-Becker animals. In fact, these animals often showed essentially no other overt sign of viral infection except slight fluid excretion from the nares and/or mouth at the longest post-inoculation intervals. This reduced symptomology was consistent for animals infected with PRV-Bartha at 10^6 pfu (concentrations ten times higher than any used in the PRV-Becker analysis) and in spite of the fact that the post-inoculation interval was as much as 40 h longer than PRV-Becker infected rats.

Discussion

The propensity of alpha herpesviruses to infect neurons is well documented (Roizman, 1990), and the ability of these neurotropic viruses to replicate and pass transneuronally has made them a popular tool for defining circuits of synaptically linked neurons (Kuypers and Ugolini, 1990; Strick and Card, 1992; Enquist, 1994). Use of these viruses for circuit analysis is dependent upon the establishment of a productive, or lytic, infection with the resultant generation of infectious progeny. However, alpha herpesviruses are also capable of establishing latent infections where the viral genome persists in the host in a non-replicating state (cf. Rock, 1993 for a recent review). The choice of a latent or productive mode of replication is dictated by many factors that are not well understood. However, the existence of a portion of the viral life cycle that does not involve the production of infectious progeny has important implications for use of these pathogens for circuit analysis, a fact that is emphasized by a number of studies in which virus inoculation does not always lead to immunohistochemically detectable viral replication and transneuronal infection (Strack *et al*, 1989a, b; Strack and Loewy, 1990). The present study was conducted to determine the effect of infectious dose and viral strain upon the ability of pseudorabies virus to establish a productive infection in the CNS. We did not examine the extent of latent infection in this analysis. Our data demonstrate that both virus concentration and strain influence the ability of PRV to establish a productive infection and should be considered in designing studies that use this virus for definition of multisynaptic circuitry. Specifically, the data demonstrate that injection of at least 10^5 plaque forming units of two strains of PRV is necessary to achieve predictable and reproducible patterns of viral repli-

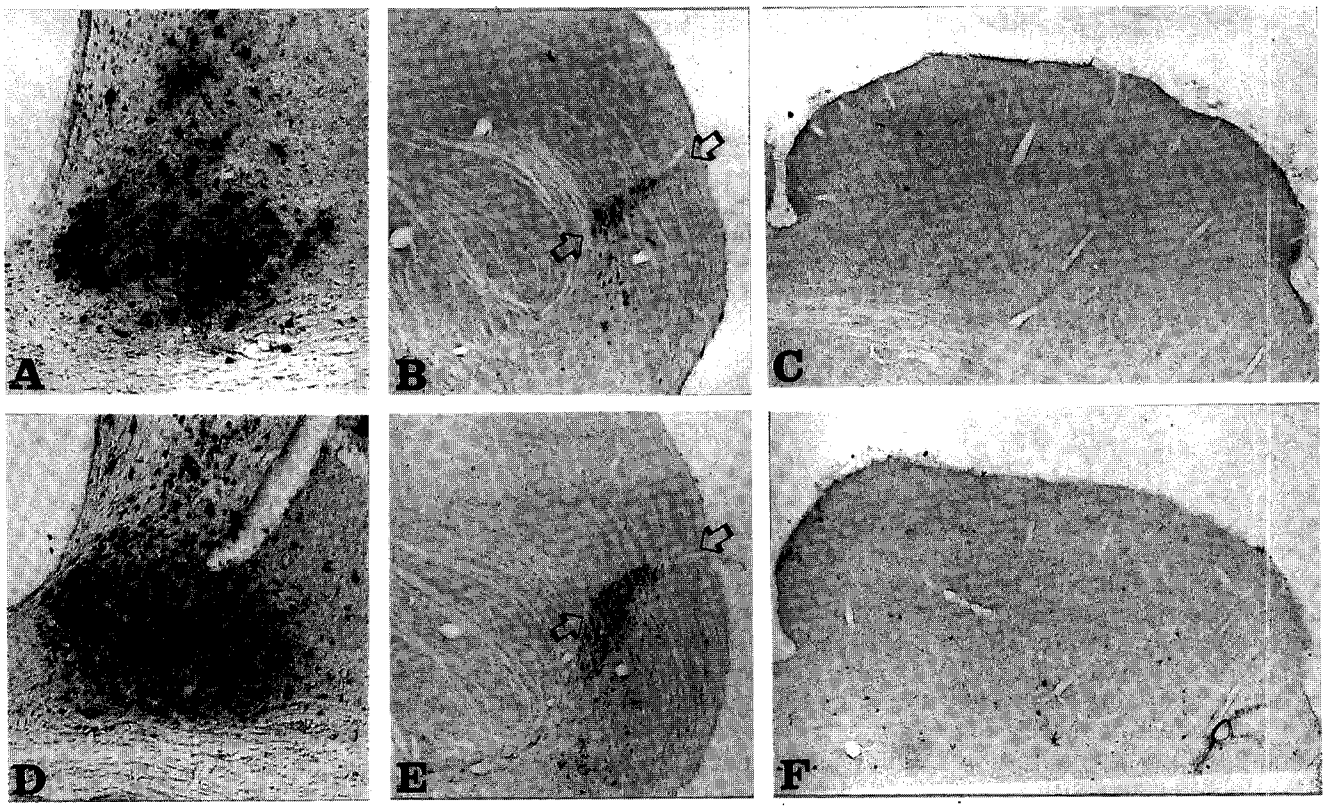

Figure 4 These photomicrographs illustrate the immunohistochemical localization of PRV-Bartha replication in neurons of the suprachiasmatic nuclei (SCN; Figures A and D), the lateral geniculate complex (LGN: Figures B and E) and tectum (Figures C and F) following intravitreal injection of different titers of PRV-Bartha. Figures A, B and C are from an animal injected with 10^4 pfu of PRV-Bartha and sacrificed 98 h later; tissue shown in Figures D, E and F are from an animal inoculated with 10^5 pfu and allowed to survive 92 h. Note the restricted pattern of infection produced by PRV-Bartha compared to that resulting from PRV-Becker injection (Figure 3). Large numbers of productively infected neurons are evident in the SCN and the functionally related subdivision of the LGN known as the intergeniculate leaflet (arrows). In contrast, no immunoreactive neurons are present in the dorsal geniculate subdivision of IGL or in the tectum.

cation in the CNS.

PRV has provided important insights into the functional organization of a variety of circuits. Although it has been used most extensively for the analysis of autonomic (Strack *et al*, 1989a, b; Strack and Loewy, 1990; Spencer *et al*, 1990; Jansen *et al*, 1992; Schramm *et al*, 1993; Standish *et al*, 1994, 1995; Vizzard *et al*, 1995) and visual circuits (Card *et al*, 1991; Levine *et al*, 1994; Moore *et al*, 1995), it has also proven useful for the definition of synaptic organization of other systems (Martin and Dolivo, 1983; Rouiller *et al*, 1986, 1989; Rotto-Percelay *et al*, 1992; Dobbins and Feldman, 1994). Careful analysis of PRV replication in these circuits has revealed unique insights into the mechanisms underlying neuronal infectivity and has demonstrated that not all neuronal systems are equally susceptible to infection. For example, analysis of the invasiveness of different strains of PRV following intravitreal injection has revealed differential replication of virus in functionally distinct components of the visual system (Card *et al*, 1991) and in cardiac circuits (Standish *et al*, 1994, 1995). The selective tropism of PRV-Bartha for certain retinorecipient areas of the CNS has been mapped to a deletion in the unique short region of the viral genome that eliminates genes encoding the gE and gI glycoproteins (Card *et al*, 1992; Whealy *et al*, 1993). Deletion of either of these genes produces the restricted phenotype, and in early investigations we entertained the hypothesis that the absence of envelope glycoproteins encoded by these genes in null mutants prevented mutant strains of virus from invading functionally distinct classes of retinal ganglion cells in the retina. However, subsequent analysis demonstrated that co-injection of gE^- and gI^- null mutants restored the capacity to produce transneuronal infection of all retinorecipient neurons (Enquist *et al*, 1994). This complementation analysis and the clear demonstration that the mutant strains of virus only replicate in a functionally distinct class of retinal ganglion cells (Card *et al*, 1992; Levine *et al*, 1994; Moore *et al*, 1995) demonstrates that the defect occurs at a stage fol-

lowing viral invasion and raises the possibility that the host neurons contribute to the selective phenotype. This possibility is further supported by the demonstration that sympathetic neurons are more susceptible to infection by the Bartha strain of PRV than motor neurons innervating somatic musculature (Rotto-Percelay *et al*, 1992).

The findings of our present analysis of PRV replication in visual circuitry, considered with the previous reports of Loewy and colleagues (Strack *et al*, 1989a, b; Strack and Loewy, 1990), demonstrate that infectious dose is a significant variable in the establishment of a productive infection in the rodent nervous system. In analyzing the transport of the PRV-Bartha strain through the sympathetic component of the autonomic nervous system, Loewy's group reported that only 20% of animals injected with 10^4 pfu demonstrated an immunohistochemically detectable productive infection 4–6 days following inoculation of peripheral targets. Our analysis demonstrated a similar rate of infection 300 h following injection of 10^4 pfu of PRV-Bartha or PRV-Becker into the vitreous body of the eye, and a further reduction in the frequency of productive infection when animals were infected with lower titers of virus. In contrast, a one log unit increase in the amount of injected virus (10^5 pfu) produced 100% infectivity and death within 150 h of intraocular injection. Similar observations have been made for infection of central and peripheral neurons with HSV type 1. Ugolini and colleagues (1987) reported that titers of HSV-1 (SC16 strain) equal to or greater than 10^7 pfu always infected hypoglossal motor neurons following nerve injection, whereas identical injection of lower titer virus produced little or no neuronal infection. In addition, Carter and collaborators (1992) found that they were unable to recover virus from trigeminal ganglia in a plaque assay following inoculation of scarified cornea of Balb/c mice with 10^2 pfu or less of the F strain of HSV-1, leading them to suggest that an inoculating dose of 10^3 pfu may represent a threshold for establishment of latent or productive infections. While there are obvious differences in the strains of virus, routes of inoculation and neuronal systems used in the above studies, there is remarkable consistency in the concentration of virus necessary to elicit productive neuronal infection in rodents.

Although injection of higher concentrations of virus ensures a higher rate of infection, it also increases the extent of virus-induced mortality. The severity of the infection is dependent upon the strain of virus and must reflect, at least in part, the efficiency of the host's immune response to a given virus. Consistent with this conclusion is our demonstration that the host often effectively contains and clears low concentrations of virus, but is apparently incapable of preventing the productive neuronal replication of higher doses of virus. Our studies of PRV infection of central visual pathways indicate that injection of concentrations of PRV-Bartha equal to or greater than 10^5 pfu produces a fatal infection within 150 h of inoculation, while injection of 10^5 pfu of the more virulent Becker strain kills animals in less than 100 h and produces more pronounced pathology (Card *et al*, 1991, 1992). It is not clear how reduced virulence and the altered neurotropism displayed by the attenuated Bartha strain are related, but data from Zuckerman and colleagues (1990) demonstrating that gC is a target antigen for cytotoxic T lymphocytes suggest that the signal sequence mutation of gC in PRV-Bartha (Robbins *et al*, 1989) along with other glycoprotein gene deletions in this strain (Lomniczi *et al*, 1987) may alter the immune response to Bartha infections. Further analysis is necessary to determine the validity of this hypothesis.

Transneuronal spread of PRV-Becker from the retina to the brainstem occurs rapidly and has pronounced pathological consequences. Although the response of glia and the recruitment of hematopoietic cells from the vasculature effectively constrains the non-specific spread of PRV-Becker in the CNS (Rinaman *et al*, 1993; Card *et al*, 1993), the rapid spread results in severe peripheral consequences that limit the usefulness of more virulent strains for circuit analysis. This has been clearly demonstrated in comparative analyses of PRV-Becker and PRV-Bartha replication in brainstem autonomic circuitry following inoculation of the stomach wall (Card *et al*, 1990). Both strains infect preganglionic parasympathetic neurons in the dorsal motor nucleus of the vagus and pass transynaptically to infect local brainstem neurons known to synapse upon these neurons. However, animals inoculated with PRV-Becker die relatively soon after the first-order transneuronal infection compared to PRV-Bartha infection and the extent of transynaptic passage and replication of the two strains differ dramatically. In particular, rats infected with PRV-Bartha characteristically exhibit extensive infection of forebrain cell groups known to give rise to descending projections to the caudal brainstem (Miselis *et al*, unpublished data) while productively infected neurons are rarely observed in these regions following identical injection of PRV-Becker (Card *et al*, 1990). These data, considered with the results from the present analysis and other studies employing PRV-Bartha indicate that the decreased pathogenicity of attenuated strains of PRV compensates for the slower spread of these strains with the ultimate result that there is more extensive transneuronal replication of virus. This feature makes the attenuated strains preferable for circuit analysis. Our results also indicate that concentration exerts an important limiting influence upon the ability of both virulent and attenuated strains of PRV to produce a productive infection.

In conclusion, although the lower virulence of the attenuated strains of PRV makes them preferable for the definition of neural circuits, one should con-

sider the restricted neurotropism exhibited by many of these strains in both the experimental design and interpretation of data derived from their use. No matter which strain is chosen, virus stocks should be titrated in each tracing paradigm to find the concentration that ensures reproducible infection, low cytopathogenicity, and maximum survival.

Materials and methods

Two strains of PRV (PRV-Becker and PRV-Bartha) were used in this analysis. PRV-Becker (Becker, 1967) is a virulent strain while PRV-Bartha is a vaccine strain harboring several mutations that reduce virulence. We have demonstrated that both strains establish a productive infection in retinal ganglion cells with subsequent anterograde transport of newly replicated virions through retinal axions, and transynaptic infection of retinorecipient neurons in the CNS (Card *et al*, 1991, 1992; Whealy *et al*, 1993; Enquist *et al*, 1994; Moore *et al*, 1995). These studies have also demonstrated that this route of inoculation produces a predictable and reproducible pattern of viral replication in 100% of animals inoculated with a minimum titer of 10^6 plaque forming units per ml (pfu ml) of either PRV-Bartha or PRV-Becker. Furthermore, productive infection of central visual pathways with either strain of virus ultimately produces death, although the post-inoculation survival interval is substantially longer in animals inoculated with attenuated virus (Card *et al*, 1992). For example, rats inoculated with approximately 10^5 pfu of PRV-Becker rarely survive longer than 72 h in this experimental paradigm whereas animals inoculated with the same concentration of PRV-Bartha routinely survive 120 h or longer (Card *et al*, 1992, 1993). Thus, this model permits a rigorous test of the effects of viral concentration and virus strain upon productive viral replication in the CNS.

Virus stocks

Stocks of both PRV strains were grown in the PK15 cell line (pig kidney fibroblasts) using previously published procedures (Card *et al*, 1991). Approximately 100 ml of each stock was clarified by centrifugation to remove cellular debris, aliquoted in freezer vials and stored frozen at –80°C until injection to reduce variability that might result from the use of more than one stock of the same virus. The titer of the PRV-Becker stock was 1×10^8 pfu ml, and the PRV-Bartha stock was 8×10^8 pfu ml. Lower titer aliquots of virus were made by thawing a vial of frozen stock and diluting it with tissue culture medium immediately prior to use so that the injected volume (2 µl) was constant in all animals. Diluted virus was subsequently titered on PK15 cells to confirm the final titer.

Animals

A total of 91 adult male Sprague-Dawley rats (Taconic) were used in the analysis. The animals weighed between 280 and 320 g at the time of inoculation and were maintained in a constant photoperiod (12 h light, light on at 0600) with free access to food and water. All viral injections were made midphase during the light phase of the photoperiod. The majority of animals (n = 73) were used in the analysis of the effect of titer on mean time to death (see following section for further detail on the experimental paradigm). Animals that survived to 300 h were sacrificed by transcardiac infusion of buffered aldehyde solutions and processed for immunohistochemical localization of virus (see below). Remaining animals were sacrificed prior to 300 h and processed for immunohistochemical localization of virus.

The experimental protocol was approved by the Animal Welfare Committee prior to the onset of the study, and all experiments were conducted in a laboratory approved for Biosafety Level 2 (BSL2) experiments. Animals were acclimated to the animal resource facility for a minimum of one week prior to use and were housed in BSL2 laboratory after inoculation.

Experimental paradigm

Eleven groups of animals were used. Five were inoculated with titers of PRV-Becker ranging from 10^1 to 10^5 pfu, and the other six groups received injections of PRV-Bartha with titers ranging from 10^1 to 10^6 pfu. The injection procedure characterized in our previous investigations (Card *et al*, 1991, 1992; Whealy *et al*, 1993; Enquist *et al*, 1994) was applied in the present analysis. Animals were anesthetized with an intramuscular injection of ketamine (60 mg/kg) and xylazine (7 mg/kg), and 2 µl of virus was injected into the vitreous body of the right eye using a Hamilton microliter syringe equipped with a 26 gauge needle. As noted above, each 2 µl aliquot contained the desired concentration of virus expressed in plaque forming units. The inoculum was injected at a rate of 1 µl per minute and the needle was left in the globe of the eye for 5 min following injection to reduce reflux into the orbit. Animals recovered under a warm light and were returned to their cages for the balance of the experiment. Percent survival within a 300 h post-inoculation interval was used as a measure of productive infection. The mean, variance, standard deviation and standard error of the mean were calculated for each group of animals, and the Student t-test was used to determine if statistical differences in mean survival existed between groups.

Immunohistochemical procedures

Animals processed for immunohistochemical localization of virus were sacrificed by transcardiac infusion of buffered aldehyde fixative (McLean and Nakane, 1974). The brain was then postfixed in the same fixative for 1 h and cryoprotected by immer-

sion in a 20% phosphate buffered sucrose solution, all at 4°C. Coronal sections through the rostrocaudal extent of the neuraxis (exclusive of the spinal cord) were cut with a freezing microtome and every sixth section was processed for immunohistocemical localization of viral antigens using a rabbit polyclonal antiserum raised against acetone inactivated PRV. Details of this procedure as it is applied in our laboratory have been published (Card *et al*, 1990).

Acknowledgements

We thank Dr Paul Husak for careful reading of the manuscript and for constructive criticism. This work was supported by grants awarded by the Samuel and Emma Winters Foundation (JPC), the National Institute of Mental Health (JPC), and the National Institute of Neurological Diseases and Stroke (LWE).

References

Bartha A (1961). Experimental reduction if virulence of Aujeszky's disease virus. Magy. *Allatorv Lapja* **16**: 43–45.

Becker CH (1967). Zur primaren Schadingung vegetativer Ganglien nach Infektion mit dem Herpes suis virus bei verschiedenen Tierarten. *Experentia* **23**: 209–217.

Card JP, Enquist LW (1994). Use of pseudorabies virus for definition of synaptically linked populations of neurons. *Methods Mol Genet* **4**: 363–382.

Card JP, Enquist LW (1995). Neurovirulence of pseudorabies virus. *Critical Reviews in Neurobiology* (in press).

Card JP, Rinaman LM, Schwaber JS, Miselis RR, Whealy ME, Robbins AK, Enquist LW (1990). Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. *J Neurosci* **10**: 1974–1994.

Card JP, Whealy ME, Robbins AK, Moore RY, Enquist LW (1991). Two α-herpesvirus strains are transported differentially in the rodent visual system. *Neuron* **6**: 957–969.

Card JP, Whealy ME, Robbins AK, Enquist LW (1992). Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. *J Virol* **66**: 3032–3041.

Card JP, Rinaman LM, Lynn RB, Lee B-H, Meade RP, Miselis RR, Enquist LW (1993). Pseudorabies virus infection of the rat central nervous system: ultrastructural characterization of viral replication, transport and pathogenesis. *J Neurosci* **13**: 2515–2539.

Carter SR, Pereira L, Pax P, La Vail JH (1992). A quantitative assay of retrograde transported HSV in the trigeminal ganglion. *Invest Ophthalmol Vis Sci* **33**: 1934–1939.

Dobbins EG, Feldman JL (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol* **347**: 64–86.

Dolivo M, Beretta E, Bonifas V, Foroglou C (1978). Ultrastructure and function in sympathetic ganglia isolated from rats infectd with pseudorabies virus. *Brain Res* **140**: 111–123.

Enquist LW (1994). Infection of the mammalian nervous system by pseudorabies virus (PRV). *Semin Virol* **5**: 221–231.

Enquist LW, Card JP (1995). Pseudorabies virus: A tool for tracing neuronal connections and the responses of the CNS to neuronal infection. In: Lowenstein PR, Enquist LW (eds). *Gene Transfer into Postmitotic Neurones: Towards Gene Therapy of Human Neurological Disorders* (in press).

Enquist LW, Dubin J, Whealy ME, Card JP (1994). Complementation analysis of pseudorabies virus gE and gI mutants in retinal ganglion cell neurotropism. *J Virol* **68**: 5275–5279.

Jansen ASP, Ter Horst GJ, Mettenleiter TC, Loewy AD (1992). CNS cell groups projecting to the submandibular parasympathetic preganglionic neurons in the rat: a retrograde transneuronal viral cell body labeling study. *Brain Res* **572**: 253–260.

Kuypers HGJM, Ugolini G (1990). Viruses as transneuronal tracers. *TINS* **13**: 71–75.

Levine JD, Zhao X-S, Miselis RR (1994). Direct and indirect retinohypothalamic projections to the supraoptic nucleus in the female albino rat. *J Comp Neurol* **341**: 214–224.

Lomniczi B, Watanabe S, Ben-Porat T, Kaplan AS (1987). Genome location and identification of functions defective in the Bartha vaccine strain of pseudorabies virus. *J Virol* **61**:l 796–801.

McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J Histochem Cytochem* **22**: 1077–1083.

Martin X, Dolivo M (1983). Neuronal and transneuronal tracing in the trigeminal system of the rat using herpes virus suis. *Brain Res* **273**: 253–276.

Moore RY, Speh JC, Card JP (1995). The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. *J Comp Neurol* **352**: 351–366.

Nadelhaft I, Vera PL, Card JP, Miselis RR (1992). Central nervous system neurons labeled following the injection of pseudorabies virus into the rat urinary bladder. *Neurosci Lett* **143**: 271–274.

Rinaman L, Card JP, Enquist LW (1993). Spatiotemporal responses of astrocytes, ramified microglia, and brain macrophages to central neuronal infection with pseudorabies virus. *J Neurosci* **13**: 685–702.

Robbins AK, Ryan JP, Whealy ME, Enquist LW (1989). The gene encoding the gIII envelope glycoprotein of pseudorabies virus vaccine strain Bartha contains a mutation affecting protein localization. *J Virol* **63**: 250–258.

Rock DL (1993). The molecular basis of latent infections by alpha herpesviruses. *Semin Virol* **4**: 157–165.

Roizman B (1990). Herpesviridae: a brief introduction. In: Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B (eds). *Virology*. Raven Press: New York, pp 1787–1793.

Rotto-Percelay DM, Wheeler JG, Osorio FA, Platt KB, Loewy AD (1992). Transneuronal labeling of spinal interneurons and sympathetic preganglionic neurons after pseudorabies virus injections in the rat medial gastrocnemius muscle. *Brain Res* **574**: 291–306.

Rouiller EM, Capt M, Dolivo M, De Ribaupierre F (1986). Tensor tympani reflex pathways studies horseradish peroxidase and transneuronal viral tracing techniques. *Neurosci Lett* **72**: 247–252.

Rouiller EM, Capt M, Dolivo M, De Ribaupierre F (1989). Neuronal organization of the stapedius reflex pathways in the rat: a retrograde HRP and viral transneuronal tracing study. *Brain Res* **476**: 21–28.

Schramm LP, Strack LM, Platt KB, Loewy AD (1993). Peripheral and central pathways regulating the kidney: a study using pseudorabies virus. *Brain Res* **616**: 251–262.

Spencer SE, Sawyer WB, Wada H, Platt KB, Loewy AD (1990). CNS projections to the pterygopalatine parasympathetic preganglionic neurons in the rat: a retrograde transneuronal viral cell body labeling study. *Brain Res* **534**: 149–169.

Standish A, Enquist LW, Schwaber JS (1994). Innervation of the heart and its central medullary origin defined by viral tracing. *Science* **263**: 232–234.

Standish A, Enquist LW, Escardo JA, Schwaber JS (1995). Central neuronal circuit innervating the rat heart defined by transneuronal transport of pseudorabies virus. *J Neurosci* **15**: 1998–2012.

Strack AM, Loewy AD (1990). Pseudorabies virus: a highly specific transneuronal cell body marker in the sympathetic nervous system. *J Neurosci* **10**: 2139–2147.

Strack AM, Sawyer WB, Platt KB, Loewy AD (1989a). CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. *Brain Res* **491**: 274–296

Strack AM, Sawyer WB, Hughes JH, Platt KB, Loewy AD (1989b). A general pattern of CNS innervation of the sympathetic outflow demonstrated by transneuronal pseudorabies viral infections. *Brain Res* **491**: 156–162.

Strick PL, Card JP (1992). Transneuronal mapping of neural circuits with alpha herpesviruses. In: Bolam JP (ed). *Experimental Neuroanatomy. A Practical Approach*. IRL Press: Oxford, pp 81–101.

Ugolini G, Kuypers HGJM, Simmons A (1987). Retrograde transneuronal transfer of herpes simplex virus type 1 (HSV 1) from motoneurones. *Brain Res* **422**: 242–256.

Vizzard MA. Erickson VL, Card JP, Roppolo JR, de Groat WC (1995). Transneuronal labeling of neurons in the adult rat brainstem and spinal cord after injection of pseudorabies virus into the urethra. *J Comp Neurol* **355**: 629–640.

Whealy ME, Card JP, Robbins AK, Dubin JR, Rziha H-J, Enquist LW (1993). Specific pseudrorabies virus infection of the rat visual system requires both gI and gp63 glycoproteins. *J Virol* **67**: 3786–3797.

Zuckerman FA, Zsak L, Mettenleiter TC, Ben-Porat T (1990). Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. *J Virol* **64**: 802–812.