IN VIVO IMAGING OF ALPHAHERPESVIRUS INFECTION
IN THE MOUSE PERIPHERAL NERVOUS SYSTEM

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ABSTRACT

Recurrent alphaherpesvirus infections in humans, such as cold sores or shingles, are associated with debilitating peripheral neuropathic pain. The mechanisms that cause such clinical manifestations are still poorly understood. Pseudorabies virus (PRV) is a related alphaherpesvirus for which swine are the natural reservoir, yet this virus is pantropic and infections of non-natural hosts are characterized by a feverish impulse to scratch to the point of self-mutilation, known as the “mad itch” syndrome. Besides its medical burden and disease, PRV is capable of invading the central nervous system by spreading through circuits of synaptically connected neurons. The neuroinvasive attributes of attenuated strains are exploited to understand fundamental aspects of the mammalian nervous system and to trace neural circuits.

The focus of this thesis was to develop an experimental model system for the direct visualization of PRV infection in vivo, and then monitor various aspects of disease progression with either wild type or attenuated PRV strains. We optimized and characterized the salivary circuit, where infection is initiated at the salivary glands, and the peripheral parasympathetic submandibular ganglia (SMG) are imaged. With single-cell resolution, we observed that ganglionic neurons infected with a virulent PRV expressing the calcium sensor GCaMP3 spontaneously generated calcium flashes in synchrony in vivo. Infections with attenuated PRV strains expressing either GCaMP2 or GCaMP3 revealed that the duration and complexity of calcium transients increased over the course of infection but remained asynchronous. Ex vivo imaging of infected salivary tissue showed that the majority of newly made particles during wild type PRV infection of SMG are invested in anterograde transport back to the initial site of infection at the glands, and many intensely bright foci are detected in the infected axon bundles. Finally, we present a model to correlate these results with the known symptomology in vivo.
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LIST OF VIRUS STRAINS

PRV Becker
Virulent wild type strain

PRV Bartha
Attenuated vaccine strain

PRV 180
VP26-RFP in Becker background

PRV 765
VP26-RFP in Bartha background

PRV 369
Diffusible GCaMP2 under CMV promoter expressed from gG locus in Bartha background

PRV 468
Diffusible GCaMP3 under CMV promoter expressed from gG locus in Becker background, VP26-RFP

PRV 469
Diffusible GCaMP3 under CMV promoter expressed from gG locus in Bartha background, VP26-RFP

PRV 151
Diffusible GFP under CMV promoter expressed from gG locus in Becker background

PRV 152
Diffusible GFP under CMV promoter expressed from gG locus in Bartha background

PRV 614
Diffusible RFP under CMV promoter expressed from gG locus in Bartha background

PRV 616
Diffusible RFP under CMV promoter expressed from gG locus in Becker background

PRV 233
gB-null, diffusible GFP under CMV promoter expressed from gG locus in Becker background

PRV 235
gB-null, Us9-null, VP26-GFP in Becker background
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CHAPTER 1

Introduction

Alphaherpesviruses and Pseudorabies Virus

Pseudorabies is an important agricultural pathogen that primarily kills livestock, and infected animals exhibit a characteristic neuropathy known as the “mad itch.” Only recently has the pseudorabies virus (PRV) been eradicated in the United States (Mayr and Claes 2008), and it continues to be an economic burden in countries worldwide. The disease was initially described by Aujezsky, and is therefore sometimes still referred to as the Aujezsky Disease virus. This Hungarian born veterinarian was the first to distinguish it from the rabies-like symptoms, from where the virus got its name “pseudo-rabies,” and classified it as a distinct infectious material. PRV was later reconciled as the causative agent of the “mad itch” based on the similar symptomology of severe pruritus and self-mutilation in cattle, rabbits, guinea pigs, rats, and mice (Shope 1931). The virus was found to be contagious only in swine, which were eventually found to be the natural host and reservoir (Shope 1934). In neonatal pigs, PRV will quickly spread systemically and cause fatal encephalitis; in adult pigs, the virus will usually go latent in peripheral sensory ganglia (Zuckermann 2000), a hallmark of herpesviruses.

The taxonomic name for PRV is suid herpesvirus 1, and it is classified within the family Herpesviridae (Pomeranz, Reynolds et al. 2005). Herpesviruses are endemic to most animal species, with at least 200 herpes viruses identified to date, and each species is the host to at least one, and sometimes several, herpesviruses. Members of this family share a common structure, encode a similar array of enzymes, utilize a common strategy of replication in the
nucleus and envelopment in the cytoplasm, and have the unique capability of residing in a latent state for the lifetime of the host after the initial infection (Pellett and Roizman 2007). As identified from electron microscopy studies (Mettenleiter, Keil et al. 2008), the distinguishing morphology of herpesviruses is an outer host-derived envelope studded with glycoproteins, an inner layer of proteins known as the tegument, and an icosahedral capsid that encloses the double-stranded DNA (Figure 1.1A).

Significant differences also exist among herpesviruses, such as a wide versus narrow host cell range, and therefore, to further classify their unique characteristics, this large family of viruses is subdivided into alpha-, beta-, and gamma- subfamilies based on different biological properties. PRV belongs to the alpha-herpesviruses, which are identified by their variable host range, short reproductive cycle, efficient spread, and establishment of latency in sensory ganglia (Pellett and Roizman 2007). They are present ubiquitously in the human population and distributed worldwide (Cohen, Straus et al. 2007). Within this subfamily, the genera Simplexvirus (for example: Herpes Simplex, HSV-1) and Varicellovirus (for example: Varicella Zoster, VZV, and PRV) encompass those with mammalian hosts. In a prototypical infection of a natural host, an alphaherpesvirus will infect an epithelial layer, such as the skin or mucosa, sort into the innervating axon terminals, and travel by retrograde transport using microtubule motors to establish latency in the peripheral sensory ganglia; upon reactivation, the alphaherpesviruses usually return to the initial site of infection on the epithelial surface by anterograde transport, but on rare occasions will spread retrogradely to the central nervous system, usually resulting in fatal encephalitis (Figure 1.1B).

PRV is one of the most commonly studied animal alphaherpesviruses, due in part to its broad host range, which includes nearly all mammals with the notable exception of higher primates (Mettenleiter 2000). It has been of interest to virologists and neurobiologists alike,
Figure 1.1. The morphology of alphaherpesviruses and a prototypical peripheral infection.

(A) The virion structure is composed of an outer host-derived envelope studded with glycoproteins, an inner layer of proteins known as the tegument, and an icosahedral capsid that encloses the double-stranded DNA. (B) Alphaherpesvirus infect an epithelial layer, sort into the innervating axon terminals, travel by retrograde transport using microtubule motors to establish latency in the peripheral sensory ganglia; upon reactivation, the alphaherpesviruses usually return to the initial site of infection on the epithelial surface by anterograde transport, but on rare occasions will spread retrogradely to the central nervous system. In this example of a pseudounipolar sensory ganglion, the virus will first be transported anterogradely in the direction of the CNS. Red circles represent PRV. (Figure inspired from an illustration created by Silvia Piccinotti)
Figure 1.1

A

- Envelope
- Tegument
- DNA
- Capsid

B

- Anterograde transport to periphery
- Retrograde transport to PNS
- Retrograde spread to CNS

Epithelial Cells  Peripheral Nervous System (PNS)  Central Nervous System (CNS)
since it can trace neuronal circuitry, and it continues to be a useful model for elucidating the biology of herpesviruses in general (Pomeranz, Reynolds et al. 2005).

**Symptomology of Alphaherpesvirus Infections and Animal Models**

Though all alphaherpesviruses have a similar life cycle of infection, each strain exhibits some unique symptoms in their natural host, and these symptoms can vary further in the non-natural host. Animal models have been developed to study the effect of host and viral factors on pathogenesis. PRV is often compared to its human counterparts VZV and HSV. It has been assigned to the same genus as VZV due to closely related homologous proteins (Mettenleiter 2000), but PRV and HSV are more easily studied side by side in related experimental models and exhibit some similar symptoms in non-native hosts. VZV causes chicken pox during the acute initial infection; upon reactivation, patients suffer from herpes zoster, and are at risk for developing post-herpetic neuralgia, a debilitating persistent pain (Arvin 2001). VZV has very restricted infectivity in non-human species, and therefore pathogenesis studies utilize the SCIDhu mouse model, in which skin, dorsal root ganglia, or T cell xenografts are infected *in vivo* (Arvin, Schaap et al. 2006). HSV is sub-divided into two different strains, both of which cause cold sores but establish latency in different peripheral ganglia. HSV-1 invades the oral region, causing facial cold sores, and resides primarily in the trigeminal ganglia. If the immune system is depressed, HSV-1 can reemerge and usually cause a painful but manageable breakout on epithelial surfaces. On rare occasions, HSV-1 will spread to the brain by a process that is not completely understood, and lead to fatal encephalitis, especially in neonates (Whitley 2001). HSV-1 is pantropic and can establish latency in peripheral ganglia of rodents, though reactivation is extremely low (Roizman and Sears 1993). PRV is also pantropic, and causes the characteristic “mad itch” clinical manifestation. The murine model for PRV latency requires
mice to be pre-treated with high titers of neutralizing antibodies (Osorio and Rock 1992); however, in the absence of pre-treatment, non-native hosts are highly susceptible for PRV infection. Several animal models of PRV pathogenesis exist that have elucidated how the virus spreads, how the host immune system responds, and which PRV genes are virulence factors.

The mouse flank scarification model has been useful in simulating a naturally occurring alphaherpesvirus infection, since infection is initiated at a peripheral epithelial site, the virus is sorted into sensory and motor axon terminals of PNS ganglia, and finally virions spread to the CNS. There is a robust immune system response that allows the study of host innate and adaptive immune recruitment in infection. It was originally developed for the study of HSV and reactivation (Simmons and Nash 1984), and then adapted for the study of PRV (Brittle, Reynolds et al. 2004). No cranial neurons directly innervate the skin, and therefore analysis of pathogenesis and spread in the PNS is possible before CNS involvement. From studies in this system in which infection of a wild-type virulent PRV strain was directly compared with a highly attenuated PRV strain, we have come to understand that the wildtype and attenuated strains have two different mechanisms of neuroinvasion and lethality in mice: Specifically, the virulent strain induces the classic symptomology of pruritis with rapid onset of death, but no viral antigen is found in the brain and there are no signs of CNS pathology. In contrast, mice infected with the attenuated strain survive much longer, do not exhibit any peripheral neuropathy, but do present CNS abnormalities near death and have abundant infectious virus in the brain (Brittle, Reynolds et al. 2004). The cellular basis to explain these differences continues to be investigated, and recent in vitro electrophysiology data suggested electrical coupling could account for the neuronal response to infection (McCarthy, Tank et al. 2009). In Chapter 2, I will describe the salivary circuit model that I optimized for PRV infection and demonstrate the applicability of ex vivo and in vivo techniques for observing disease progression.
Chapter 1

The PRV Strains Becker and Bartha and the Host Immune Response

The linear double-stranded DNA genome of PRV is characterized by two unique regions (unique long, UL, and unique short, US) and the US region is flanked by internal and terminal repeat sequences (Figure 1.2A). The sequence and gene arrangements of both wild-type and attenuated strains have recently been fully sequenced and characterized (Szpara, Tafuri et al. 2011). Several wild-type PRV strains are widely studied, including PRV Kaplan, NIA-3, and Becker, and in this thesis, all studies conducted with a virulent PRV strains have been derived from PRV Becker. The most common attenuated strain used in the laboratory is named PRV Bartha, which has been used as a live attenuated vaccine in the swine industry. PRV Bartha was not genetically engineered but was isolated after multiple passages of a virulent field isolate in cultured chicken cells and embryos, and selected to grow well in culture and produce immunity without disease (Enquist 2002; Pomeranz, Reynolds et al. 2005). By identifying and studying the attenuating mutations in PRV Bartha, much has been learned about what genes are responsible for virulence using both animal models and in vitro characterizations. In particular, three independent mutations contribute to its reduced virulence (Pomeranz, Reynolds et al. 2005): point mutations within UL21, a signal sequence mutation in the glycoprotein C (gC) gene, and a large deletion within the US region that encompasses the genes encoding gE and US9, as well as a large portion of the gI and US2 coding regions (Figure 1.2A).

Several of the PRV Bartha mutations have important characterized phenotypes. The UL21 tegument gene was shown to confer defects that affect infectious particle production and thereby delayed kinetics in retrograde spread, but efficiency of transneuronal spread could be restored when this locus was repaired with a wild-type sequence (Curanovic, Lyman et al. 2009). Furthermore, the large deletion of gE, gI, and US9 has received a lot of attention because these gene products are particularly important for anterograde directional spread within axons;
Figure 1.2. PRV Bartha harbors several mutations and is defective for anterograde spread.

(A) This simplified schematic of the PRV genome highlights a subset of mutations found in the PRV Bartha strain that have been characterized to contribute to its reduced virulence: point mutations within UL21, a signal sequence mutation in the glycoprotein C (gC) gene, and a large deletion within the US5 region that encompasses the genes encoding gE and US9, as well as a large portion of the gI and US2 coding regions. (B) The virulent PRV Becker strain can spread in both the retrograde and anterograde directions. The attenuated PRV Bartha strain can only spread in the retrograde direction and is defective for anterograde transport and spread, a phenotype that seems to be caused primarily by the large deletion in its US5 region.
Figure 1.2

A

Subset of Bartha mutations: gC U_{L21} \Delta gE/gI/US9/US_{2}

\[ \text{UL region} \quad \text{Internal Repeats} \quad \text{US region} \quad \text{Terminal Repeats} \]

B

Becker infection:

Anterograde transport and spread

Retrograde spread and transport

Bartha infection:

No anterograde transport or spread

Retrograde spread and transport
without them virions are not sorted into axons, and therefore are defective for anterograde spread (reviewed in (Ekstrand, Enquist et al. 2008)). The retrograde-only spread is a hallmark of PRV Bartha infection (Figure 1.2B), and this attribute has made PRV Bartha particularly useful for the neuroscience circuit tracing community, which will be discussed in the next section. Studies with HSV-1 have shown the gE and gi proteins form a heterodimer that acts as an Fc receptor to bind human immunoglobulin G (IgG) and evade the adaptive immune response (Lubinski, Nagashunmugam et al. 1998; Chapman, You et al. 1999), but HSV-1 gE/gI does not bind murine IgG in vivo (Nagashunmugam, Lubinski et al. 1998). Homologous PRV gE/gI proteins also display Fc receptor activity toward porcine IgG (Favoreel, Nauwynck et al. 1997); however, in wild-type PRV infections of mice, animals succumb to virulent PRV infection before the antibody response is elicited (Brittle, Reynolds et al. 2004). The gC protein along with cellular factors appear more critical to protecting PRV from complement attack in swine cells (Maeda, Hayashi et al. 2002), and since PRV Bartha harbors a gC mutation, this may also contribute to its avirulence.

There appears to be a staged non-neuronal cellular response to PRV infection of the CNS. There is focal infiltration of CD45 positive leukocytes to specific regions of CNS infection, and the number of immune cells correlates to the number of infected neurons; monocytes appear first followed by lymphocytes recruited from the vasculature (Rassnick, Enquist et al. 1998). PRV is also able to evade the IFN-mediated immune response by reducing expression of interferon stimulated genes, thus disarming the induction of an IFN-β antiviral state (Brukman and Enquist 2006). When the total set of upregulated and downregulated genes were analyzed in rats infected with either PRV Becker or Bartha, the majority of genes fell within the category of genes related to the immune response; the acute initial response to infection was similar between the two strains, but extensive CNS invasion of the attenuated strain mediated a delayed adaptive immune reaction (Rinaman, Card et al. 1993; Paulus, Sollars et al. 2006).
Vivo infections with a highly attenuated PRV Bartha derivative, recruited microglia were shown to disintegrate the membranes of infected neurons, followed by immune cell assembly around compromised neurons which resulted in the complete clearance of infected neurons from early-infected brain regions while the virus continued to spread (Denes, Boldogkoi et al. 2006).

Circuit Tracing of the Nervous System Using PRV

Apart from their medical burden and disease, alphaherpesviruses fit into a broader class of neurotropic viruses that are exploited by the neuroscience community to trace neuronal circuits. Elucidating neural connections has become a fundamental component of advancing our understanding of the functional organization of the CNS (Enquist and Card 2003). Understanding the connectivity within complex neural networks in the CNS is of interest to neuroscientists who seek to relate behavioral data to the underlying anatomical circuitry (Briggman, Abarbanel et al. 2006). Before neurotropic viruses were as widespread in their use, traditional methods for tracing circuits relied on enzymes or dyes, such as horseradish peroxidase or wheat germ agglutinin; however, these tracers presented limitations because either they were retained within a neuron and could not diffuse to connected neurons, or they quickly diluted when they spread, impeding the delineation of multi-synaptic circuits (Ekstrand, Enquist et al. 2008). Thus, neurotropic viruses superseded these techniques as self-amplifying tracers of neural circuitry, as they are able to travel transsynaptically in specific circuits with multiple connected neurons.

PRV is used extensively in the circuit tracing community, and the attenuated strain PRV Bartha is particularly exploited to construct derivatives that express various genes, since it is amenable to genetic manipulation and the insertion of large segments of foreign DNA. PRV Bartha spreads faithfully among functionally connected neurons by cell to cell contact, and the precise site of egress and entry is posited to be at synapses, though evidence is only
circumstantial. Nonetheless, careful studies have confirmed PRV’s utility as a reliable tracer, since it can reproduce tracing studies of non-viral tracers and infection does not spread interaxonally to non-synaptically connected, physically adjacent neurons (Ekstrand, Enquist et al. 2008). Surrounding glia can be infected, but these support cells appear to be non-permissive to PRV replication and therefore do not serve to produce infectious virus for spread (Rinaman, Card et al. 1993; Tomishima and Enquist 2002). In addition, PRV Bartha does not appear to significantly affect the electrophysiological properties of infected neurons in vivo: When PRV Bartha expressing EGFP was used to trace circuitry in vivo, and then the electrophysiology of constituent neurons were recorded in vitro using whole-cell patch-clamp techniques, there was no significant difference in physiological properties of infected and non-infected neurons (Smith, Banfield et al. 2000).

Many PRV Bartha recombinants are available that express unique fluorescent reporter proteins and different fluorescent tags to analyze circuits and define synaptic architecture (Enquist and Card 2003). Because of the increased survival of the animals when infected with PRV Bartha, there is considerably more spread within the CNS than with a wild type PRV (Enquist 2002). By the same token, PRV Bartha derivatives infect all neuronal types indiscriminately, and in order to tease apart specific circuits within an interweaved complex circuit, conditional PRV tracing strains have been developed to replicate only in a subpopulation of specified neurons. One of the most popular is PRV Ba 2001, and the strategy for this virus is based on the Cre-lox recombination system: The viral strain was constructed such that a certain essential gene, thymidine kinase, requires Cre-promoted recombination to grow, and this strain can then be injected into a transgenic mouse that expresses Cre under the control of specific neuronal promoters (DeFalco, Tomishima et al. 2001). More recently, a new approach with the Cre recombinase system has been developed using the Brainbow cassette, in which the genome
carries a default fluorescent reporter but additional fluorescent reporters can be irreversibly expressed with the addition of Cre (Livet, Weissman et al. 2007; Card, Kobiler et al. 2011): A PRV Bartha derivative (PRV 263) was constructed to express the Brainbow cassette, and this strain was injected in combination with a unique lentivirus vector that produced Cre expression in catecholamine neurons. The viral genome expressed the default red reporter in infected cells without Cre, but in the subpopulation of targeted neurons with Cre, the red reporter gene was excised from the genome and expression of yellow or cyan reporters was enabled; in addition, recombined virus continued to spread transsynaptically, thereby labeling those neurons linked to the Cre-expressing catecholamine neurons (Card, Kobiler et al. 2011). This technology promises to provide new insights into functionally defined systems.

While PRV strains which express unique or recombinatorial fluorescent reporters are useful to dissecting connections within neuronal circuits and subpopulations, these techniques do not yield information about the strength of synaptic connections or activity of infected neurons, and there is still room for continued improvements on the available circuit tracing viral strains. In Chapter 3, I will describe the construction of a PRV Bartha tracing strain that expresses a calcium indicator, and when combined with functional calcium imaging in the living animal, it is useful tool for revealing activity in synaptically connected CNS neurons in vivo.

Limitations of In Vitro Methods for Studying PRV Transneuronal Spread

In recent years, dissociated primary neurons or compartmentalized chambers have been developed as alternative methods to in vivo animal models for most of the basic research aimed at elucidating mechanisms of PRV spread and the effect of infection on neuronal physiology. These techniques have proved tremendously useful in making great advances in our understanding of the viral genetic components needed for directional spread (for example,
(Ch'ng, Spear et al. 2007)), as well as effects of PRV on the electrophysiology of neurons (McCarthy, Tank et al. 2009). Nonetheless, with all of these studies, the question always arises as to the relevance of the results to the actual behavior of the virus in the context of an infection in a living animal.

Primary neurons that are harvested from embryos are grown in a simplified and artificial environment, absent of infiltrating immune cells and without natural pre- and post-synaptic partners. The neuronal media is supplemented with glutamine, B-27, and nerve growth factor to aid the growth and maturation of the neurons (Curanovic, Ch'ng et al. 2009), yet these cells still only survive for a few weeks, and the media is obviously different in composition from the blood or other animal bodily fluids. In addition, in the context of infection in dissociated neurons, PRV is added directly to the cell bodies and axons simultaneously, which is very different from a natural infection in which PRV invades the periphery and enters the nervous system through innervations to the epithelia. The trichamber system developed in the lab allows PRV to be added strictly to axon endings (Curanovic, Ch'ng et al. 2009), which is one step closer to mimicking the in vivo situation, yet the process of viral sorting is accelerated without the epithelial layer, and additional portions of the axon are exposed to infection rather than only axon terminals. There are also several examples in which an in vivo observation is not entirely reproduced with in vitro conditions, such as the complete gE anterograde spread defect (Ch'ng and Enquist 2005) and the H129 anterograde-only phenotype (Curanovic, unpublished data).

Therefore, pairing in vitro results with animal models is essential for validating and providing conclusive evidence of a particular observed phenomenon. Animal models of infection like the flank and eye scarification model allow the course of infection to take place in the animal, then pathogenesis is assessed by brain sections or clinical symptoms. Since these studies have looked at how infection progresses in the natural context of the animal, many have
elucidated mechanisms underlying the spread of infection and cause of death, especially when different viral strains, such as virulent and attenuated, are compared side by side (Brittle, Reynolds et al. 2004). Yet the scope of these models has been restricted to observing the progression of infection indirectly, since spread and virulence are measured through symptomology, or in sacrificing the animal to assay viral titers and distribution. Even in PRV’s history of use as a neuroanatomical tracer in vivo, studies have traditionally been done by infecting living animals and subsequently studying fixed and labeled tissues. While these experimental paradigms have provided important data, they preclude real-time investigations of disease progression or infection in the context of the activated host immune cells. Some recent advances have been made in imaging the effects of HSV-1 infection in the mouse using bioluminescence (Luker, Prior et al. 2003). In Chapter 4, I will describe how I applied in vivo imaging to directly observe the calcium profile of neurons infected with PRV Becker.

Scope of This Dissertation

While in vitro and in situ methods have enormous value and continue to be exploited to answer scientific questions, the overarching goal of my thesis was to extend these various findings to the natural context of an animal model of infection, with the additional attempt to image aspects of PRV infection directly in the living, anesthetized mouse. I optimized the mouse salivary circuit as my experimental model system for infecting and imaging PRV in vivo and ex vivo in the peripheral nervous system. Using this system has allowed us to validate some previous in vitro findings, such as transport kinetics, but we also gained valuable insight into how infection is seeded at the inoculation site. Similarly we corroborated an in vitro result regarding the effect of PRV Becker infection on neuronal physiology, yet we were able to further substantiate the correlation between symptomology and disease progression in vivo.
CHAPTER 2

The Mouse Salivary Circuit: A Model System for PRV Infection *In Vivo*

The Parasympathetic Salivary Circuit

Secretion of saliva is mediated by a complex interplay between the parasympathetic and sympathetic efferent nerves (reviewed in (Garrett and Kidd 1993)). While these two autonomic components travel to the glands by separate routes, once in the glands, the non-myelinated axons from each type of nerve intermingle and travel through the interstices of the glandular acinar cells in association with Schwann cells, forming Schwann-axon bundles (Garrett and Kidd 1993). There are also sensory nerves in the salivary glands, and these afferent nerves pass mainly through the mandibular division of the trigeminal ganglia (Chibuzo and Cummings 1980).

Salivation is a complex process that involves a balance between the sympathetic stimulation that leads to a viscous secretion with high protein content, and the parasympathetic outflow that produces more copious, watery saliva during digestion (Garrett and Kidd 1993; Iversen, Iversen et al. 2000).

Since salivation is a reflex activity, central influences from higher centers act on the salivary centers (Garrett 1987). One of the salivary centers was identified using HRP retrograde labeling of projecting preganglionic fibers to the glands, and its position was located to the reticular formation in the pons, on the ipsilateral side of each salivary gland (Hiura 1977). Part of the parasympathetic pathway, this salivary center in the brainstem is referred to as the superior salivatory nucleus (SSN). The SSN fibers follow intricate passages to their destinations, which include the pterygopalatine ganglia (PtG) that innervate the lacrimal glands, as well as the
Figure 2.1. The parasympathetic salivary circuit: from the superior salivatory nucleus (SSN) to the submandibular ganglion (SMG) to the salivary gland. The SSN axons (represented in light blue) leave the brainstem with the intermediate nerve of the facial nerve (cranial nerve VII), then branch off with the chorda tympani, join the lingual nerve, and finally diverge to contact the SMG (the brainstem diagram was adapted from (Fitzgerald)). The SMG, boxed in red, in turn innervate the salivary glands. ICP, inferior cerebellar peduncles; CST, corticospinal tract; PCML, posterior column-medial lemniscal pathways.
Figure 2.1

- salivary ducts
- chorda tympani
- lingual nerve
- salivary glands
- SMG
submandibular ganglia (SMG) that innervate the salivary glands (see Figure 2.1). The SSN connection to the lacrimal glands was discovered using transneuronal viral tracing with the PRV Bartha strain, and revealed a ventral lacrimal and a dorsal submandibular subgroup of presynaptic neurons in this brainstem nucleus (Toth, Boldogkoi et al. 1999). The central nervous system (CNS) nuclei that project to the SSN were also described using a retrograde labeling study with a PRV Bartha strain expressing β-galactosidase (Jansen, Ter Horst et al. 1992); the authors found labeling across the CNS, including several hypothalamic regions and part of the amygdala, suggesting that there is a broad interplay of various CNS areas in the control of salivation.

**Innervation and Structure of the Submandibular Ganglia**

The SMG consist of a network of ganglia located along the salivary ducts and further distributed within the glands. The precise location and size of each ganglion is not stereotyped like other autonomic ganglia, such as the superior cervical ganglia, but rather there exists a general pattern of SMG arranged along the ducts, with each ganglion comprising anywhere from 50 to 500 neurons (Yamakado and Yohro 1977). The establishment of presynaptic innervations to the SMG from the SSN has been found to follow a similar process of reorganization as that observed in the neuromuscular junction (NMJ), namely there are excess inputs during prenatal development that are subsequently pruned after birth during maturation. The formation of the NMJ has been extensively studied and the mechanisms of assembly, maturation, elimination, and maintenance are well-understood (reviewed in (Sanes and Lichtman 1999)). The SSN to SMG connection was studied because it provides a model of neuron to neuron contact. Using intracellular recordings, the SMG of neonatal rats were found to be multiply innervated, but by adulthood (5 weeks old), 75% of the ganglion cells were innervated by a single preganglionic
fiber (Lichtman 1977). While the number of axons innervating each cell decreases, the total number of synaptic boutons increases as new terminals are formed by each preganglionic axon on its individual target neurons; thus, there is a remodeling of synaptic connections rather than simple elimination (Lichtman 1977; Purves and Lichtman 1978). Follow-up experiments demonstrated that a preganglionic axon typically innervates several neurons within a ganglion that are not necessarily adjacent but rather intermingled with other cells that are innervated by different axons (Lichtman 1980). Thus, individual axons from the SSN in the brainstem branch upon arrival at the SMG to innervate several ganglionic neurons, and the presynaptic axon then further ramifies to make multiple synaptic contacts on one neuron, but each neuron receives only one principal presynaptic input (Figure 2.2).

Each neuron in the SMG is ensheathed by two or more satellite cells, and these glial cells appear to be active players in the rearrangement of synaptic contacts, not simply passive or supportive participants. Specifically, the number of satellite cells associated with each neuron increases during maturation, concomitant with synaptic reorganization, and the satellite cell number per neuron is highly correlated with neuronal volume (Pomeroy, Zurakowski et al. 1996). In addition, when the position and of the satellite cells was examined over weeks to months, progressive changes in the glial positions were noticed; in particular, the glial nuclei were found to aggregate in regions of the neuronal surface where presynaptic terminals were concentrated, suggesting that these satellite cells are in involved in the synaptic remodeling process (Pomeroy and Purves 1988).

The structure and dendritic arborization of autonomic ganglia differ depending on the type and species. Some neurons lack dendrites while other nerve cells harbor hundreds of dendritic branches; sympathetic neurons of smaller mammals have fewer dendrites than the homologous neurons in larger animals, and the dendritic complexity correlates with the
Figure 2.2. Innervation of the SMG. Individual axons from the SSN in the brainstem branch upon arrival at the SMG to innervate several ganglionic neurons, and the presynaptic axon then further ramifies to make multiple synaptic contacts on one neuron, but each neuron receives only one principal presynaptic input.
Figure 2.2

Axon 1  Axon 2  Axon 3

Presynaptic input from brainstem

SMG neurons

22
geometry and extent of innervation (Purves and Lichtman 1985). When the parasympathetic SMG neurons were examined across several species, those of mice and rats were found to lack dendrites altogether (Snider 1987). Likewise, electron microscopy and staining of presynaptic boutons revealed that the synapses were made on short protuberances emanating from the neuronal cell bodies (Lichtman 1977). There are two types of intraganglionic neurons, classified as large and small, with large neurons exhibiting spine-like processes and small neurons lacking dendritic specializations completely, and there are no interneurons, facilitating the distinction of presynaptic terminals and SMG neurons (Yamakado and Yohro 1977).

**The SMG as a Model System for PRV infection**

Due to the simple anatomy and superficial location of the SMG beneath the skin of the neck in rodents, these ganglia are easily accessible for optical imaging and have therefore been used extensively for repeated imaging experiments to locate and study the same nerve cells in a living mouse at different time points (Purves and Lichtman 1987; Purves, Voyvodic et al. 1987). We chose to capitalize on these features and develop the mouse SMG as a model system for the study of PRV spread, using both *ex vivo* and *in vivo* techniques. Since the SMG supply the parasympathetic innervation to the salivary glands and receive input from the SSN, they are at the interface between the peripheral nervous system (PNS) and the CNS. The PNS to CNS junction is particularly interesting because, during an infection in the natural host, alphaherpesviruses usually enter sensory nerves innervating the cells of mucosal membranes and establish latency in the sensory PNS ganglia, such as the trigeminal ganglia in case of HSV-1 infection for example (Roizman and Sears 1993). Upon reactivation, the virus is carried by anterograde axon transport back to the epithelial surface of initial infection and rarely accesses the CNS. Since invasion of the CNS causes fatal encephalitis, the host immune system is critical
in the establishment of latency; thus, special modes of immune regulation are in place at this junction (Lafon 2009). HSV-1 latency in studied extensively in the mouse model; however, in the non-natural host, PRV always proceeds lytically. PRV infections in swine usually occur by skin epithelial or mucosal transfer, and therefore infections of the salivary circuit are unlikely in a natural context; nonetheless, the circuit comprises all of the same components of a peripheral circuit, and has the key advantage of being both optically accessible for two-photon imaging in the live animal, as well as physically accessible for quick removal of infected tissues for \textit{ex vivo} imaging. The \textit{in vivo} and \textit{ex vivo} imaging techniques will be explained in sections to follow.

\textbf{Surgical Procedure for PRV Infection of the Salivary Glands.}

When PRV is used for tracing studies, the virus is preferentially injected at a peripheral site or organ that is retrograde to the desired ganglia or nuclei to be studied, since PRV has an affinity for axon terminals rather than direct uptake at neuron cell bodies in vivo (Card, Enquist et al. 1999). Since I was interested in imaging PRV infection in SMG neurons, I chose to inject PRV into the salivary glands. I optimized a surgical procedure for injecting PRV to the salivary glands that had been initially developed by a previous postdoc in the lab (Feierbach, Piccinotti et al. 2006). The surgical procedure will be described in detail (see also the published protocol paper (Granstedt, Kuhn et al. 2010)).

All experimental protocols related to animal use were approved by the Institutional Animal Care and Use Committee of the Princeton University Research Board under protocol number 1850 and were in accordance with the regulations of the American Association for Accreditation of Laboratory Animal Care and those in the Animal Welfare Act. Adult mice underwent surgery in the Schultz building biocontainment facility using aseptic technique. Male mice between 7-10 weeks old were anesthetized with a freshly prepared solution of ketamine.
Chapter 2

(100 mg/kg)/xylazine (10 mg/kg), administered by the intraperitoneal (IP) route. I chose to work with males only because of the published finding that the estrous cycle of female mice affects PRV infection of the CNS (Weiss, Dobbs et al. 2001). The mice needed to be at a surgical plane of anesthesia prior to incising the neck region; therefore, the animals were closely monitored for response to a painful stimulus created by forceful pinching of the toe. If an animal was not at a sufficient level of anesthesia to begin the experiment, as indicated by movement of the animal or increased respiratory rate upon pinching the toe, then an additional dose of ketamine/xylazine was administered. Ophthalmic ointment was placed in both eyes.

Once the mouse reached a plane of anesthesia where no pain response to a forceful toe pinch was elicited, the mouse was transferred to a custom-made surgical platform, which consisted of a square piece of sturdy cardboard for stability that was overlayed with duct tape. This base was reused for every surgery and only replaced if there was tearing or damage to the tape. Then a fresh piece of folded thick paper towel was taped on top, and a rubber band was taped to one side. The mouse was put on its back with its front limbs taped down and the rubber band drawn over its upper jaw and front teeth so that the head was titled back and the neck region was taught (Figure 2.3A). The neck of the mouse, from the base of the chin to just above the ribcage, was shaved using a platinum razor blade. The shaved area was then prepared for surgery with aseptic technique. Disinfectant scrub (e.g. Betadine) was applied, being careful not to allow scrub to drip down onto the animal's eyes. The disinfectant scrub was alternated with 70% isopropyl alcohol 2-3 times over the surgical area. A disinfectant solution was applied last. An approximately 1.5 cm incision was made in the neck to visualize the salivary glands. The incision was made with a sterile scalpel blade on a sterilized scalpel handle, with the skin grasped using sterilized forceps in order to ensure a shallow incision (Figure 2.3B). The glands were separated from the skin using tissue separators.
Figure 2.3. PRV infection of the salivary glands. Each panel represents the main steps involved in the surgical procedure of PRV infection of the salivary glands. (A) Animal is anesthetized and placed on its back with all front limbs taped down. (B) Since the glands are located just below the surface of the skin, only a minor incision in the neck region is required. (C) A Hamilton syringe is used to inject and deliver viral inoculum.
Four separate 1-2 µl injections of PRV inoculum were made into each salivary gland for a total of 5-6 µl per side (Figure 2.3C). The incision was closed with nylon or silk sutures by a simple interrupted suture pattern. The mouse was then administered buprenorphine (100 µg/kg) via the subcutaneous route for prophylaxis against post-surgical pain (Roughan and Flecknell 2002). We chose buprenorphine as the analgesic because other types of analgesic, such as COX-2 inhibitors derived from non-steroidal anti-inflammatory drugs, inhibit PRV infection (Ray, Bisher et al. 2004). Buprenorphine was administered once the surgery was completed but prior to full recovery from anesthesia, to lessen the pain caused by the minor surgery. No additional doses were administered in subsequent days to avoid any interference with the progression of viral infection, and we did not expect the animals to experience much pain from the surgery since the incision was minor. Each infected mouse was housed in a separate cage clearly marked with a biohazard sticker, and placed in the BSL-2 holding room. Infected mice were then minimally monitored, so as to not affect the viral pathogenesis.

Since the symptoms caused by PRV in rodents have been studied for a number of years, we knew what signs of pathogenesis to expect as the infection progressed (Brittle, Reynolds et al. 2004). Since the timing of the onset of symptoms can vary depending on where the infection is initiated, I made my own informal observations of how the diseases progressed after PRV infection of the salivary glands. Mice infected with a virulent strain of PRV (Becker-derivative) showed few symptoms in the first 24 hours after infection. After 24 hours, especially closer to 48 hours, the animals showed increasing pain or distress, as evidenced by outbursts of scratching in the neck region where the virus was injected, as well as a hunched posture and lack of eating and drinking. The animals were always dead before 72 hours, and therefore we did not allow the virulent infections to last beyond 48 hours. In the event that an animal scratched and opened their stitching, the animal was euthanized by CO₂ asphyxiation and
cervical dislocation. However, animals infected with an attenuated strain of PRV (Bartha-derived) did not show any of the symptoms exhibited by the virulent virus. Instead, the animals appeared no different from uninfected animals for the first 3-4 days. However, these animals still died from the infection by 5 or 6 days. During the final days of infection, the animals experienced neurological symptoms, like seizures, but they did not exhibit signs of pain or distress. Therefore, we did not allow the attenuated infections to last beyond 4 days. We minimized the amount of observations done to the infected animals so as to let the infection proceed in a natural context without interference. We only observed them to catch the appropriate endpoint and to ensure that the wound was not opened.

Two-photon Imaging of Infected SMG *In Vivo*

At the desired time post-inoculation of the submandibular glands (24-96 hours post infection, depending on strain of PRV), an infected mouse was removed from the Schultz biocontainment facility and transported in a secondary containment device (an orange biohazard bag, in accordance with Princeton University BSL-2 pathogen biosafety protocols) to a two-photon imaging facility for *in vivo* imaging analysis with a two-photon microscope (Denk, Strickler et al. 1990; Denk and Svoboda 1997). Each microscopy imaging session lasted approximately 2-3 hours. One mouse at a time was anesthetized for approximately 10 minutes in an isoflurane induction chamber using 2.5% isoflurane. The mouse was then transferred to a custom-built imaging stage (Figure 2.4A). Isoflurane was delivered to the mouse using a small animal anesthesia system equipped with a scavenger system and attached to a pressurized oxygen cylinder; this system contained a precision isoflurane specific vaporizer equipped with a Bain non-breathing circuit attached to a small rodent-sized nose cone covered by a latex diaphragm with a slit sufficient in size to fit snugly around the muzzle of the mouse. An
Figure 2.4. Setup for in vivo imaging of PRV infection. (A) Custom-built stage is equipped with heating blanket, magnetic retractors, isoflurane nose cone, and a micromanipulator that hold the imaging platform. (B) Neck region of an anesthetized mouse is exposed. SMG (light blue) are located along the salivary duct (d) and send projections to the salivary glands (g) where the virus was injected. The platform (p) elevates the ganglia for imaging under two-photon microscopy.
induction of 1.5% isoflurane was maintained during the entire course of imaging to keep the animals under anesthesia. The mouse was placed in dorsal recumbency on the imaging stage, with all limbs tapped down. The body temperature was maintained at 37°C with a homeothermic blanket system.

The sutures were removed with stitch scissors and the incision re-opened in order to access the salivary tissue and the SMG. We used tissue-separator scissors to separate the glands from the skin. The imaging site on the neck was further exposed utilizing magnetic retractors attached at the edge of the wound margin. Using fine forceps to cut connective tissue, we located the SMG anterior to the salivary glands and lifted the ganglia onto a small metal platform that is controlled by a micromanipulator (Figure 2.4B). The tissues were kept moist by regular perfusion of the imaging site with warm, sterile lactated Ringers saline or artificial cerebral spinal fluid. Following imaging, mice were euthanized by cervical dislocation. The in vivo imaging technique was applied to look at the effects of PRV infection on neuronal physiology using calcium imaging, and the results will be described in Chapters 4 and 5.

**Ex Vivo Imaging of PRV Infection in the SMG**

In addition to optimizing the protocol for imaging infected SMG in vivo, I sought to establish another method for visualizing infected tissue in a system that closely approximated an in vivo infection but was simplified for imaging in an environment outside the living animal. Therefore, I developed a procedure to image tissue explanted from an infected animal, which I have termed an acute explant or an ex vivo preparation, using the salivary circuit that has been previously described. The animal was deeply anesthetized, and material encompassing the glands, ducts, and SMG was removed; to explant this sample, layers of connective tissues were pulled away with fine forceps to separate the glands, ducts, and SMG from surrounding
Figure 2.5. Setup for ex vivo imaging of PRV infection. (A) Simplified schematic of explanted tissues that include the glands, ducts, and SMG. Layers of connective tissues are pulled away in order to expose the tissues of interest, and the cut site is downstream near the digastric muscle. The injection site of PRV inoculum is in the salivary glands, and the SMG are imaged using a standard inverted epifluorescent microscope. (B) Schematic of glass-bottom dish that is coated with sylgard. A hold is cut out over the glass coverslip and the sample is flattened and pinned down firmly using fine needles that are held in place by piercing the sylgard.
structures, and a cut was made near the digastric muscle (Figure 2.5A). The animal was immediately sacrificed by thoracotomy and the tissue was further dissected to expose the SMG. A glass-bottom dish was prepared by coating with sylgard and cutting a hole over the glass coverslip in order to visualize the infection on a standard inverted epifluorescent microscope. The sample was pinned down firmly using fine needles that could be held in place by piercing the sylgard (Figure 2.5B). The needles also served to flatten the tissue to increase the optical range of the objective through the thick sample. The area was filled with warmed Neurobasal media, supplemented with 1% (vol/vol) penicillin/streptomycin-glutamine (Invitrogen), and B27 Supplement (Invitrogen). Depending on the type of analysis performed, the objective was heated and a stage top incubator system was put in place to maintain a stable temperature and humid environment. In that case, after the dissection and preparation of the sample for imaging, the dish was left to equilibrate for 15 minutes on the stage before the imaging was started, and therefore the time between when the tissue was explanted to when imaging began was approximately 30 to 40 minutes.

**Mechanism of Viral Uptake after PRV Infection of the Salivary Glands**

When PRV is injected into the CNS, viral uptake is thought to occur primarily at axon terminals with limited replication at the site of injection, since PRV is highly neurotropic and has a particular affinity for axon terminals (Card, Enquist et al. 1999). To determine if this also held true in the salivary circuit when PRV is injected into the salivary glands, I performed experiments both *in vivo* and *ex vivo* to address this question. Specifically, I want to distinguish whether viral particles were primarily taken up at axon terminals innervating the glands during the initial infection, or if particles first replicated in the glandular acinar cells before sorting into axon terminals. I made use of an available PRV strain that does not express the fusion protein
Figure 2.6. *In vivo and ex vivo imaging of SMG infected with gB null strains.* (A) Infection with PRV 235 and *in vivo* imaging of SMG at 48 and 72 hours post infection (h.p.i.). Several cells are infected, as seen by bright puncta located in and surrounding the nuclei of infected cells. The diffuse and non-specific coloring is autofluorescence. (B) Infection with PRV 233 and *ex vivo* imaging of SMG at 48 h.p.i. The GFP and phase contrast panels of the same region of interest are shown. Several infected cells are detected, though fewer than expected at this time point.
Figure 2.6

A  PRV 235 in vivo

48 h.p.i.  72 h.p.i.

B  PRV 233 ex vivo at 48 h.p.i.

GFP  Phase
glycoprotein B (gB). PRV strains that lack gB but are grown on complementing cells are able to infect once and replicate in those primary infected cells, but cannot spread to secondary neurons or neighboring cells, since virion-incorporated gB is required for egress and entry, and therefore transneuronal spread (Curanovic and Enquist 2009). In the salivary system, we would expect that if virus primarily gets taken up at axon terminals, then most cells in the SMG would be infected even with a gB null strain. However, if there is first replication in the glands, and subsequent axonal take up, then no cells in the SMG would be infected, since the gB null strain would replicate in the glands but not spread to axon terminals.

Starting first in vivo, I infected 3 male and 3 female mice with PRV 235, a Becker-derived strain that lacks gB as well as Us9, restricting its ability to send new particles into axons, and expresses GFP fused to VP26, the minor capsid protein. Images were then taken at 24, 48, and 72 hours post infection. There was no significant difference between time points in the number of infected cells, and overall fewer infected cells were observed in the SMG at 48 and 72 hours than in infection conditions with a wild type strain in which all cells within an SMG are infected by 48 hours (Figure 2.6A). Using the ex vivo imaging setup, I also infected mice with PRV 235 and detected a handful of infected cells in the SMG at 48 hours. Then I infected two mice with PRV 233, another Becker-derived strain that lacks gB alone and expresses diffusible GFP in the gG locus. The results were similar, with several infected cells detected at 48 hours, but many fewer than expected (Figure 2.6B). Taken together, we can conclude from the data that PRV does get taken up directly at axon terminals without the need to first replicate in the glands; however, the fact that we detect fewer numbers of infected cells at 48 hours compared to a wild type infection suggests that PRV also replicates in the glands before sorting into axon terminals. This is consistent with the observation that increasing numbers of cells are infected at increasing times post infection with a virulent or attenuated strain that has gB.
Time Course of Infection in the Salivary Circuit

To understand the rate at which PRV travels through the salivary circuit using my injection protocol, I performed a time course of infection. At the time of this experiment, I was maintaining a colony of transgenic YFP mice (Feng, Mellor et al. 2000), and I used females aged 3 to 5 months old. I inoculated mice with either PRV 151 (virulent Becker derivative expressing diffusable GFP in the gG locus) or PRV 152 (attenuated Bartha derivative expressing diffusable GFP in the gG locus), at a titer of $1 \times 10^8$ (not concentrated), with three animals per time point per virus. I harvested the salivary glands, SMG, and brainstem, at 16, 24, 32, 40 and 48 hours post infection, and included an additional time point of 72 hours post infection for PRV 152, since the animals survive longer. I first perfused the animals with PBS in order to remove the blood, then pooled similar tissues from the three mice at each condition and followed the published protocol for titering on PK15 cells (Brittle, Reynolds et al. 2004). Briefly, pooled tissues were stored in Eppendorf tubes with 500 µl of viral DMEM and flash frozen in liquid nitrogen. Then the samples were thawed and the tissue minced with scissors then pulverized with sterile disposable pestle. The homogenates were treated with two additional cycles of freezing in liquid nitrogen and thawing in the 37°C water bath. After centrifuging at 13,000 RPM for 2 minutes, the clarified supernatant was transferred to a fresh Eppendorf tubes, sonicated, and titered by plaque assay on PK 15 cells. The results are shown in Figure 2.6.

The data show that PRV 152 has detectable titers in the brainstem at 32 hours post infection, before PRV 151. Both PRV strains present similar titers at 40 hours, and by 48 hours, PRV 151 appears to have higher titers in the glands, SMG, and brainstem compared to PRV 152 (Figure 2.6). Since the time I performed this time course, I slightly altered my protocol to use only younger male mice and concentrated viral stocks, as previously described. Nonetheless, the results of this time course still hold significance. The virulent PRV 151 strain seems to establish a
Figure 2.7. Time course of infection in the salivary circuit. Bar graph of titers recovered from infected tissues at various time hours post infection (h.p.i.). Mice were infected with either the virulent strain PRV 151 (red) or the attenuated strain PRV 152 (blue). Then the glands (G), SMG (S), and brainstem (B) were harvested and tissues from 3 mice per time point per virus were pooled and processed for analysis of viral titers.
Figure 2.7

Time course of infection in the salivary circuit

- G : Glands
- S : SMG
- B : Brainstem

Graph showing log_{10} PFU/ml over time (16 h.p.i., 24 h.p.i., 32 h.p.i., 40 h.p.i., 48 h.p.i., 72 h.p.i.) for PRV 151 (red) and PRV 152 (blue) in glands (G), salivary glands (S), and brainstem (B) compartments.
robust anterograde infection back in the initial site of infection at the glands, whereas the attenuated PRV 152 spreads efficiently to higher order neurons in the CNS. In addition, it appears that the titers in the SMG increase over time during PRV 151 infection, but only initially to some degree during a PRV 152 infection and then the titers even appear to decrease slightly. The possible significance of these results will be further discussed in Chapter 6 in conjunction with other data from upcoming chapters.

Summary

Here I have described the development and characterization of a model system for PRV infection in the salivary circuit that can be used for in vivo and ex vivo analysis of infected cells and tissues. The infection protocol was based on a previously reported protocol (Feierbach, Piccinotti et al. 2006). I further optimized certain aspects in order to minimize variability between animals, such as concentrating the viral inoculum and using only male mice aged 7-9 week old. In addition, I performed a time course of infection in order to examine the rate at which a virulent and attenuated PRV strain travel through the circuit. By using a PRV strain in which the fusion protein gB is deleted, I was able to distinguish the method of viral uptake during infection of the salivary glands. The data suggests that PRV both replicates at the initial site of infection in the glands, and is also taken up directly at axon terminals. Both routes of infection contribute to the increasing numbers of infected neurons observed in the SMG at increasing times post infection. These characterizations and optimizations laid the groundwork for further analysis in vivo and ex vivo, which will be described in Chapters 3, 4, and 5.
Chapter 3

Ex Vivo Tracking of PRV Particles

Background and Objective

Alphaherpesviruses transit over long distances in the nervous system. Particles are transported bi-directionally within axonal compartments using molecular motors. Defining the processive rates of viral transport and spread in axons can contribute to increasing our understanding of viral pathogenesis. The dynamics of individual virion transport during PRV infection have been assessed in vitro in dissociated chick dorsal root ganglia (Smith, Gross et al. 2001), but not in tissue. We utilized a model system that more closely approximated an in vivo infection, and then compared the observed movement to what has been published in vitro. Therefore, we developed and optimized a protocol to image explanted tissue from mice infected with PRV, which we have termed ex vivo preparation, using the salivary circuit that has been previously described. We then manually tracked the particles across many frames and measured their processivity and transport dynamics.

Procedure for Imaging of Viral Particles in Explanted Tissue

Adult male mice were infected by injection into the salivary glands with PRV 180, a virulent PRV strain isogenic with PRV Becker in which the capsid was fluorescently tagged with monomeric red fluorescent protein (mRFP). The animals recovered and the infection was allowed to proceed for 12, 24, or 48 hours. At the desired time point, a piece of tissue was explanted that included the glands, ducts, and submandibular ganglia (SMG). The SMG were
dissected, exposed, and pinned down into a Sylgard-coated, glass-bottom dish for epifluorescence imaging (Figure 3.1A). The dish was filled with warmed, supplemented Neurobasal media.

While imaging, the orientation of the tissue was kept constant across all experiments so that directionality could be determined. The imaging window was kept at a fixed height of 100 pixels and width of 300 pixels with the axon bundles aligned horizontally, but the precise position within the tissue varied since every animal has a different number of SMG that are uniquely positioned and the infection proceeds asynchronously; nonetheless, we tried to position the imaging window in an area between the SMG and the salivary glands (Figure 3.1A, magnified panel). Movies were acquired on a standard inverted epifluorescent microscope with a 60× Plan Fluor Ph3 objective (Nikon) at 37°C in a 5% (vol/vol) CO2 enriched atmosphere using a stage top incubator system (Quorum Scientific). The fluorescence exposure was set to 500 milliseconds, resulting in an acquisition rate of approximately 2 frames per second, and the resolution was 3.75 pixels per micron. Three animals were imaged per time point at 12, 24, and 48 hours post infection (h.p.i.).

Particles were then manually tracked in infected axons, and 100 particles were tracked per animal using the ImageJ plug-in MTrackJ (Meijering, Dzyubachyk et al. 2012). The data was pooled across the three animals collected for each time point. The viruses were visualized by tagging the viral capsid with mRFP, which was determined to be the optimal fluorophore for imaging in tissue. The emitted fluorescence was below the diffraction limit on the microscope, and therefore each particle resembled a punctum of several graded bright pixels above background noise. The exact location was hard to resolve but the ImageJ software allowed sub-pixel resolution by computing the centroid, or intensity-weighted mean position of the bright pixels that constitute the virion. All particles transiting to the left were regarded as anterograde-
Figure 3.1. Ex vivo imaging setup and tracking of viral particles. (A) A glass-bottom dish was prepared by coating with sylgard and cutting a hole over the glass coverslip in order to visualize the infection on a standard inverted epifluorescent scope. The sample was pinned down firmly and flattened using fine needles that could be held in place by piercing the sylgard. Magnified panel shows the approximate imaging window and the directions defined as anterograde and retrograde. (B) Panels represent a cropped area taken from a sample movie, every 10 frames. The exact time of acquisition within the movie is indicated. In this view, three particles are tracked: particle 1 (orange) is moving anterograde, particle 2 (yellow) is stalled, and particle 3 (green) is moving retrograde. Scale bar is consistent in all frames and equates 5 µm.
Figure 3.1

A

Glass-bottom dish with sylgard

B

Anterograde

Retrograde

Salivary Glands

SMG

Imaging window

T = 4.12 s

T = 6.55 s

T = 9.22 s

T = 11.89 s

T = 14.64 s
moving particles and those transiting to the right were deemed retrograde-moving particles. Particles that did not move for the duration of the movie were labeled as stalled. Figure 3.1B is a cropped area taken from a sample movie and displays panels every 10 frames. In this particular view, particle 1 (orange) is moving anterograde, particle 2 (yellow) is stalled, and particle 3 (green) is moving retrograde (Figure 3.1B). Once the coordinates of each viral particle position across all frames in a movie were determined, the data was further analyzed using custom code written in Matlab to measure velocity and processivity, and to extract other features.

**Net Transport and Directionality of Tracked Particles**

We first looked at the net transport and directionality (Figure 3.2). The trajectory of each particle was subdivided into anterograde, retrograde, or stalled parts. Stalls were empirically defined as coordinates in frames where the mean displacement over the two neighboring time points was less than 0.8 pixels (0.21 µm). The transport distance was calculated to be the distance in microns that the particle traveled before stalling, in each direction. The distances were binned and plotted in a histogram.

Several interesting features emerged (Figure 3.2): First, we observed that at 12 hours, more particles were moving anterograde than at 24 or 48 hours, and conversely fewer were moving retrograde. At 12 hours, 84% of particles were moving anterograde, while only 16% were traveling retrograde. At 24 and 48 hours, 67% and 64% of particles were traveling anterograde, respectively, and 31% and 32% of particles were traveling retrograde. There is also a high peak at 12 hours in the bin of the furthermost anterograde distance, indicating that the great majority of particles traveled without stalling through the entire imaging window. In contrast, at 24 and 48 hours, there is a broader distribution of distances, and the peak in the furthermost anterograde bin at 48 hours includes fewer particles than at 12 hours. In addition,
Figure 3.2. Quantitative analysis of particle transport and directionality ex vivo. Histogram of net transport, or the distance in microns that each particle traveled before stalling at 12, 24, and 48 hours post infection (h.p.i.). Anterograde and retrograde directions were analyzed separately, and percentage of particles transiting in each direction is indicated.
Figure 3.2

Net transport and directionality

12 h.p.i.

100
50
0
20
40
60
80
100

Distance of transport (μm)

Number of particles

Anterograde 84%

Retrograde 16%

24 h.p.i.

Anterograde 67%

Stalled 2%

Retrograde 31%

48 h.p.i.

Anterograde 64%

Stalled 4%

Retrograde 32%
for both 24 and 48 hours there is a peak at 0, representing a population of particles were present that were completely stalled, and this peak is absent from the 12 hour time point. At 24 hours, 2% of particles were completely stalled, and this increased to 4% of particles at 48 hours (Figure 3.2).

**Measurement of Duty Cycle**

We then looked at the duty cycle, or the fraction of time that the particles spent transiting versus stalling (Figure 3.3). Movements in both anterograde and retrograde directions at each time point were combined to define the motion or active state as a fraction of the total time. The trajectory of each particle was normalized by setting a trajectory in which there were no stalls to 1 and a trajectory in which the particle was completely stalled to 0. The values were then binned and plotted on a histogram. Again, a difference emerged at 12 hours, as the mean value for the duty cycle was 0.74 ± 0.013 (mean ± standard error of the mean (s.e.m.) across particles), whereas at 24 and 48 hours, the mean value was 0.59 ± 0.017 and 0.53 ± 0.016, respectively. Therefore, more particles are in motion at 12 hours and those that are being actively transported transit for longer periods of time (Figure 3.3).

**Velocity of Moving Virions**

To measure the velocity, we split up the anterograde and retrograde motions, and calculated the microns traveled per second in each direction during the frames that the particles were in motion (Figure 3.4). We did not find an appreciable difference between the various time points in the anterograde direction. The mean anterograde speed was 1.94 ± 0.032 (mean ± s.e.m. across particles), 1.85 ± 0.041, and 1.90 ± 0.041 microns per second at 12, 24, and 48 hours respectively. This is close to what has been published *in vitro* (Smith, Gross et al. 2001). In
Figure 3.3. Quantitative analysis of the duty cycle ex vivo. Histogram of the duty cycle, or the fraction of time that the particles spent transiting versus stalling at 12, 24, and 48 h.p.i. Movements in both the anterograde and retrograde directions were combined to define motion. The trajectory of each particle was normalized by setting a trajectory in which there were no stalls to 1 and a trajectory in which the particle was completely stalled to 0. The mean values at each time point are indicated (mean ± standard error of the mean).
Figure 3.3

Duty cycle

Mean: 0.74 ± 0.013

Mean: 0.59 ± 0.017

Mean: 0.53 ± 0.016
the retrograde direction though, the 12 hour time point had a lower mean, suggesting that particles moved slower. The mean retrograde speed was $0.82 \pm 0.028$, $1.06 \pm 0.028$, and $1.04 \pm 0.025$ microns per second at 12, 24, and 48 hours respectively. However, we noticed a bimodal distribution, especially at 12 and 24 hours (Figure 3.4), suggesting that a subgroup of particles may have a slower velocity and another subgroup may have a faster velocity, and the peak of the second group was consistent across time points. Therefore particles are still able to transit as fast in the retrograde direction at every time point, but a pool of particles may be transported slower.

**Analysis of Stalling Behavior**

We did some further analysis on the stalls that occurred during runs. As previously mentioned, no particles were completely stalled at 12 hours, but as the time after infection increased, more particles appeared to stall and never regain momentum. In looking at those particles that stalled but then did restart in either direction, we found that the stall frequency for 12, 24, and 48 hours was $0.014 \pm 0.00048$ (mean ± s.e.m. across particles), $0.014 \pm 0.00043$, and $0.014 \pm 0.00043$, respectively. In other words, the numbers of stalls per second was consistent across time points, and particles were just as likely to stall. However, when we looked at the duration of the stalls, we found a difference. We generated a histogram of the stall durations and found the mean values to be (in seconds): 12 hours, 1.40 (with median = 0.5); 24 hours, 2.13 (median = 0.75), and 48 hours, 2.03 (median 1.0). Thus, even though the particles may fall off their molecular motors just as frequently at all times post infection and therefore stall, they spend more time stalled at 24 and 48 hours than earlier on in infection. As another way of looking at these measurements, we calculated the duration of runs, and separated anterograde and retrograde directions. As expected, the 12 hour time point had longer
Figure 3.4. Quantitative analysis of particle velocity *ex vivo*. Histogram of particle velocity in anterograde and retrograde directions at 12, 24, and 48 h.p.i. The microns traveled per second were calculated in each direction during the frames that the particles were in motion. The mean speed values in each direction at each time point is indicated.
Figure 3.4

Velocity of runs

<table>
<thead>
<tr>
<th>Time (h.p.i.)</th>
<th>Mean Speed of Runs (μm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Anterograde: 1.94 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>Retrograde: 0.82 ± 0.028</td>
</tr>
<tr>
<td>24</td>
<td>Anterograde: 1.85 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>Retrograde: 1.06 ± 0.028</td>
</tr>
<tr>
<td>48</td>
<td>Anterograde: 1.90 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>Retrograde: 1.04 ± 0.025</td>
</tr>
</tbody>
</table>
durations of runs in the anterograde direction, and the mean values (in seconds) at 12, 24, and 48 hours were 2.81 ± 3.80 (standard deviation), 2.39 ± 3.26, and 2.37 ± 3.05, respectively. The standard deviation was high because the runs were short, on average. In contrast, there was little difference in the retrograde direction: the mean values (in seconds) were 0.76 ± 0.81 (standard deviation), 0.79 ± 0.72, and 0.82 ± 0.82, for 12, 24, and 48 hours, respectively. Also, it appeared that the run durations were much shorter in the retrograde direction than in the anterograde direction. The fact that the processivity is more interrupted in the retrograde direction may contribute to the slower mean velocity described earlier.

**Ex vivo particle tracking of a PRV Bartha derivative**

A separate group of adult male mice were infected by injection into the salivary glands with PRV 765, an attenuated PRV strain isogenic with PRV Bartha in which the capsid was fluorescently tagged with mRFP. Due to a deletion in its genome, this strain can only spread in the retrograde direction, and therefore newly replicated particles will not be sorted into axons and transported anterogradely. The animals recovered and the infection was allowed to proceed for 24 or 48 hours. As before, the same piece of tissue was explanted that included the glands, ducts, and SMG, and the subsequently dissected and exposed SMG were pinned down into a Sylgard-coated, glass-bottom dish for epifluorescence imaging (Figure 3.1A). In contrast to the PRV 180 infection, we could not identify any directed particle movement in axons of tissue infected with PRV 765 at 24 or 48 hours post infection. The only particle motion that we observed resembled random undirected Brownian motion.
Discussion

Here we present qualitative and quantitative data of particle transport and processivity in explanted tissue. We infected with a fluorescently-tagged, virulent PRV strain, and detected directed particle movement in axons of infected tissue. Since the tissue orientation was held constant and the imaging window was set to be between the SMG and glands (Figure 3.1A), we inferred that viral particles traveling in the anterograde direction were those that had been newly replicated in the SMG neurons and were therefore heading back to the glands to reseed the infection in the glandular cells. Conversely, the viral particles traveling in the retrograde direction were thought to be those that had replicated in the glandular cells and were headed to the SMG. Our first time point for imaging was 12 hours post infection, and at that time many particles were already traveling anterogradely. The particles that we observed traveling retrogradely at 12 hours were probably not original particles injected from the inoculum, but presumably those particles from the inoculum that first replicated in the glands before being taken up at axon terminals. We were surprised to discover that a large quantity of viral particles were traveling anterogradely at 12 hours, but this suggests that in this system particles are efficiently sorted into innervating axon terminals in the glands, and the first amplification step most likely occurs in the SMG. The second amplification step then occurs in the glands from the particles that first replicated there before spreading to the axons, and the third amplification may take place in the glands again from the particles that first replicated in the SMG and then reseeded the glands. This is consistent with the observation that increasing numbers of SMG neurons are infected at increasing times post infection.

When we infected with an attenuated PRV Bartha derivative, we did not observe any directed particle movement at 24 or 48 hours. At those time points, the virulent strain is primarily moving anterogradely, however the attenuated strain is restricted for anterograde
spread due to a deletion in its genome. Therefore, the fact that anterograde particle transport is undetectable during an infection with the attenuated strain is expected. In addition, there may not be any detectable retrograde movement because this strain cannot reseed the glands, and the amplification that occurs in the SMG leads to particles being transported retrogradely to the brainstem. Perhaps if we looked at an earlier time after infection, such as 6 or 12 hours, we may observe retrograde particle movement from virions that first replicated in the glands during the initial infection before being sorted into axon terminals. We still observe increasing numbers of infected cells at various times after infection, and this may be due to PRV Bartha’s delay in replication and spread (Curanovic, Lyman et al. 2009).

In preparations with both the virulent and attenuated strains, we detected bright foci in which fluorescent puncta were swirling around, but we could not identify whether these foci were varicosities, infiltrating immune cells, or infected glial cells, or some other structure. Varicosities were demonstrated to occur during a virulent PRV infection model in vitro; the formation was dependent on gD and these were thought to function as egress sites (De Regge, Nauwynck et al. 2006). No such characterizations have been made with PRV Bartha, and this attenuated strain is known to have mutations in gD (Szpara, Tafuri et al. 2011). In addition, functional varicosities for egress are unlikely to form during PRV Bartha infection because this strain lacks Us9 and therefore cannot travel anterograde and also cannot send down axons the glycoprotein gB (Tomishima and Enquist 2001), which is part of the fusion machinery and required for egress. Perhaps these foci could be infected Schwann cells, which are located in the bundles of these unmyelinated axons. In tissue infected with PRV Becker, there were so many of these intensely bright foci closely apposed to axons that the field of view was saturated with them. There were far fewer in the PRV Bartha infections. Further work is needed to define the nature of these structures and understand their potential importance during PRV infection.
Since PRV is used extensively as a neural circuit tracer, the spread of PRV is often conceptualized as proceeding transsynaptically and unidirectionally, since specific circuits are labeled and the infection will progress until the animals dies. Yet it is important to remember that the tracing strains are derivatives of PRV Bartha, a highly attenuated vaccine strain that lacks certain genes for anterograde spread, and therefore this strain can only travel in the retrograde direction. Wild type strains like PRV Becker, on the other hand, can transit in both directions. In peripheral circuits where the infection begins at a distal site, such as an epithelial surface or organ, the organization naturally leads to retrograde spread initially, since sensory or autonomic ganglia extend innervating axon terminals to their targets but do not reside there. As a result, one may still envision these strains traveling from the starting point of injection to higher order neurons by constantly spreading, without stopping. However, it is known that PRV Bartha and PRV Becker have different modes of lethality (Brittle, Reynolds et al. 2004), and PRV Becker does not actually spread far into the CNS. Consistent with the fact that PRV Becker acts primarily on the peripheral nervous system before death, I have presented data for that suggests this strain spends more time traveling to and from the initial site of infection than spreading to the CNS.

The ex vivo preparation described above was developed as a model system that more closely approximates an in vivo infection for the measurement of transport dynamics of viral particles. The salivary circuit has proved to be useful because of its accessibility for infection and harvesting of tissues. The infection resembles a more natural scenario since it proceeds in the animal, and therefore the virus is subject to host factors. In addition, the viral particles are required to be sorted into axon terminals and spread occurs among natural synaptic partners. These features and context are absent in the simplified in vitro systems, and thus much remains to be elucidated from in vivo infection conditions with further ex vivo analysis.
CHAPTER 4

*In Vivo* Calcium Imaging with G-CaMP2 in the Submandibular Ganglia

**Background and Objective**

Recording the activity of multiple neurons in intact circuits remains challenging for *in vivo* study because current tools cannot simultaneously report activity and connectivity. Electrophysiological recordings and fluorescent calcium indicators can inform about activity but not large-scale circuit connectivity. Genetic tools offer complementary ways of distinguishing and targeting cell types (Luo, Callaway et al. 2008), but a combination of functional imaging and circuit tracing is still missing. Conversely, commonly used chemical tracers (Wouterlood, Vinkenoog et al. 2002) and neurotropic viruses (Loewy 1998) can identify neuroanatomical circuits but do not document activity. An ideal tool should simultaneously report activity and connectivity with sensitivity and reliability for long periods of time *in vivo*.

Transient increases in calcium [Ca$^{2+}$] levels are a useful correlate of neuronal activity. Calcium transients can be measured by injected dyes or by genetically encoded fluorescent [Ca$^{2+}$] indicator proteins (FCIPs). G-CaMP2 is an FCIP composed of a circularly permuted green fluorescent protein (GFP) linked to calmodulin (CaM) and the CaM target sequence M13 (Nakai, Ohkura et al. 2001). In the presence of [Ca$^{2+}$], CaM binds M13, resulting in increased fluorescence (Wang, Shui et al. 2008; Akerboom, Rivera et al. 2009). The fluorescence intensity increases as a function of [Ca$^{2+}$] concentration. G-CaMP2 is a calcium sensor that is stable at physiological pH and mammalian temperature (Ohkura, Matsuzaki et al. 2005). Several publications have demonstrated the use of G-CaMP2 in mammals *in vivo* (Diez-Garcia, Akemann et al. 2007; Hoogland, Kuhn et al. 2009).
Though FCIPs can reliably monitor neural activity, they need a vehicle to cross synapses and thereby report connectivity. Neurotropic viruses have proven useful to this end (Loewy 1998). One publication reported the construction of a gE-deleted PRV-Kaplan recombinant containing the ratiometric calcium indicator TN-L15, and demonstrated its use by imaging explanted retina (Boldogkoi, Balint et al. 2009). As described in preceding chapters, another useful variant is PRV-Bartha: this attenuated, retrograde tracer travels along chains of synaptically connected neurons, and many PRV-Bartha derivatives with fluorescent labels have been widely implemented for circuit tracing (Ekstrand, Enquist et al. 2008).

In PRV’s history of use as a neuroanatomical tracer in vivo, studies have traditionally been done by infecting living animals and subsequently studying fixed and labeled tissues. Using two-photon microscopy (Denk, Strickler et al. 1990) and the optically accessible submandibular sample preparation for in vivo imaging, we sought to virally deliver a fluorescent calcium sensor to this circuit, providing the first opportunity to image PRV live in vivo to explore both connectivity and activity. Thus, by inserting the calcium sensor G-CaMP2 into the genome of the classical PRV-Bartha tracing strain and monitoring activity in vivo, we present the first use of PRV to express a fluorescent calcium indicator protein and report fluorescence-based neuronal activity in the living animal. We confirmed that this recombinant, PRV369, could reliably detect single action potentials in vitro. We then studied fluorescence-based activity in vivo in the SMG. We identified a window of 48 hours after inoculation in which PRV369 can be used to look at calcium signals in these ganglia. Infected neurons responded to external electrical and sensory stimuli. After 72 hours we detected changes in intracellular [Ca$^{2+}$] concentrations and duration of [Ca$^{2+}$] transients, indicating cell or tissue responses to infection. PRV369 can be used for in vivo investigation of physiological neuronal circuit activity and subsequent effects of infection with single cell resolution.
Figure 4.1. PRV369 stably encodes G-CaMP2 in the gG locus. (A) Map of the gG region of PRV, for the background strain PRV-Bartha, the mRFP1-containing derivative PRV614, and the G-CaMP2-containing derivative PRV369. The retrograde-limited spread phenotype of PRV Bartha stems from a deletion in this region, which results in the fused open reading frame of Gl-Us2. The lines below the gene names are marked with 1 kb spacing. Cross marks indicate regions of homologous recombination used to generate PRV369. (B) Nucleocapsid DNA from strains PRV Bartha, PRV614, and PRV369 were digested with Sal1 and probed by Southern blot with a 1.9 kb fragment of Us3 and gG (gray box below Bartha genome in (A)). Fragments observed were the expected sizes, based on Sal1 cut sites denoted by arrows in (A): PRV Bartha fragment of 2.6 kb, PRV614 fragments of 1.5 and 3.4 kb, and PRV369 fragments of 1.6 and 3.5 kb. (C) Equivalent single-step growth kinetics of PRV Bartha and PRV369 in epithelial cells in vitro. PK-15 cells were infected at an MOI of 10, with input virus inactivated by a low pH citrate wash after 1 hour. Each time point was performed in triplicate and titered in duplicate. The average titer for each virus is plotted along with the standard error of the mean (SEM) for each time point and virus.
Figure 4.1

A  Genome maps of gG region

PRV Bartha  

US3  gG  gD  gI-US2

PRV614  
(mRFP1 in gG)

US3  mRFP1  gG  gD  gI-US2

PRV369  
(G-CaMP2 in gG)

US3  G-CaMP2  gG  gD  gI-US2

SalI SalI SalI

B  SalI digest and Southern blot of gG region

PRV Bartha  PRV 614  PRV 369  kb

10  8  6  5  3  2  1.5  1  0.5

C  Single-step growth curve

Plaque forming units (PFU)

10^10  10^8  10^6  10^4  10^2

0  6  12  18  24

--- PRV Bartha
--- PRV369 (GCaMP-2)
**PRV369 infected cells stably express G-CaMP2**

PRV369 was constructed by homologous recombination and replacement of mRFP1 in the glycoprotein G (gG) locus of PRV614, a PRV-Bartha-derived tracing strain (Figure 4.1A). Viral protein gG is not required for spread *in vivo* (Smith, Banfield et al. 2000), and many of the commonly used PRV viral tracing strains employ insertions at this locus (Banfield, Kaufman et al. 2003). The G-CaMP2 open reading frame is transcribed from the cytomegalovirus immediate-early (CMV-IE) promoter (see (Banfield, Kaufman et al. 2003) for more details). Correct genomic insertion of the G-CaMP2 cassette was confirmed by Southern blot analysis (Figure 4.1B). Western blots revealed no change in expression of upstream and downstream proteins in comparison to the parental PRV614 strain. PRV369 grows comparably to PRV-Bartha in cultured epithelial cells (Figure 4.1C).

**Fluorescence traces correlate with neuronal activity *in vitro***

To test whether changes in fluorescence correlated with firing of action potentials (APs), we infected dissociated mouse superior cervical ganglion (SCG) neurons and recorded action potentials with sharp electrodes while imaging corresponding fluorescence changes with two-photon microscopy. A typical infected SCG neuron is shown in Figure 4.2A. The electrical recording shows action potentials and the optical recording displays the corresponding \([\text{Ca}^{2+}]\) transients. We found that in all recordings, every action potential correlated with a change in fluorescence (representative trace in Figure 4.2A). This virally-delivered indicator reliably reported single action potentials *in vitro*.

We analyzed the amplitude of fluorescence fluctuations in response to action potential firing. Single action potentials were common, whereas bursts of two or more action potentials were scarce and irregular. Single, double, or triplet spikes could easily be separated in the \([\text{Ca}^{2+}]\)
Figure 4.2. PRV369 sensitively and accurately detects graded neuronal activity in dissociated neurons in vitro. (A) Sample recording of an impaled neuron, with diagram indicating electrode position on an actual image of an SCG neuron (left). In response to every action potential (bottom trace), a calcium event was detected in the fluorescence trace (top trace). (B) Analysis for one neuron of the percent fluorescence change for single, double, or triple action potentials. Fluorescence increases proportionally to action potential number. Ticks indicate action potentials by electrophysiology. Bold line indicates average. (C) Quantitative comparison across multiple neurons. The amplitude of the fluorescence peak is significantly different between one, two, or three action potentials: the average ΔF/F was 7%, 19%, and 27%, respectively. (D) Comparison of fluorescence decay from peak. There is no statistical difference in decay between one, two, or three action potentials. The average t_{1/2} was 0.9, 1.1, and 1.1 seconds, respectively. AP, action potential; ΔF/F, relative fluorescence change.
A  Recording in dissociated mouse SCG neurons

B  Fluorescent fluctuations in a single cell

C  Fluorescence comparison across cells

D  Measurement of $t_{1/2}$ across cells
transients by the maximal relative fluorescence change (Figure 4.2B). In one cell we detected an average fluorescence change of 8.4±1.6% for single action potentials, 19.3±5.5% for spike doublets, and 32% for a triplet. We defined spikes as doublets and triplets if the interspike interval was less than 300 milliseconds (Figure 4.2B). Across four cells, we compared the average maximal amplitude of fluorescence change and found a statistically significant difference between fluorescence levels for one, two, and three action potentials (Figure 4.2C). We then measured the t1/2 from the peak, or the time it takes for the fluorescence to decay halfway from peak amplitude (Figure 4.2D). As expected, there was no statistically significant difference between one, two, and three action potentials (one vs. two, p=0.1; two vs. three, p=0.8), indicating that G-CaMP2 was not saturated with calcium. These in vitro characterizations ensured that this viral strain appropriately expressed and delivered G-CaMP2 in infected cells. Therefore we progressed to studying fluorescence-based activity under physiological conditions in vivo.

**Infection by PRV369 enables visualization of spontaneous activity in vivo**

Using two-photon microscopy, we imaged the spontaneous activity in a known neuronal circuit in vivo. As previously described, the submandibular ganglia (SMG) are peripheral parasympathetic ganglia that innervate the salivary glands and receive input from the superior salivatory nucleus in the brainstem; this circuit has already been defined in viral tracing experiments (Jansen, Ter Horst et al. 1992; Toth, Boldogkoi et al. 1999). We imaged the SMG in the anesthetized mouse at 24, 48, and 72 hours after PRV369 inoculation (Figure 4.3A-C). The ganglia are located along the salivary duct and are accessible for imaging in vivo. We perfused the area with warmed mammalian Ringer’s solution and heated the animal to maintain physiological temperature. We used a miniature platform to raise ganglia away from
Figure 4.3. Infection with PRV369 reports spontaneous activity \textit{in vivo}. Traces of spontaneous activity are shown at 24 (A), 48 (B), and 72 (C) hours post inoculation (h.p.i.). The number of infected cells increases with time, and there is also an increase in the number of calcium events. The average fluorescence change of these changes \textit{in vivo} is 50\%. Scale bar = 20\mu m.
Spontaneous activity at 24, 48, 72 hours post injection

Figure 4.3
surrounding tissues, and only ganglia that were within a few millimeters of the gland were able to be lifted on the platform. Fluorescence changes related to spontaneous activity were easily detectable in vivo (Figure 4.3A-C). The number of infected neurons appeared to increase with time. To count the total number of labeled neurons, we pooled data from accessible ganglia in 3 animals at each time point. At 24 hours we counted a total of 65 labeled cells; at 48 hours, 133 cells; and at 72 hours, 280 cells. [Ca$^{2+}$] signals were detectable at a high signal-to-noise ratio because of the high relative fluorescence change of G-CaMP2 upon [Ca$^{2+}$]-binding (Nakai, Ohkura et al. 2001). These results show that infection by PRV369 was successful in vivo and allowed optical analysis of an infected neuronal circuit in a living animal.

**Time course of infection reveals changes in calcium activity**

Infection by PRV-Bartha activates host defenses (Rinaman, Card et al. 1993; Rassnick, Enquist et al. 1998; Brittle, Reynolds et al. 2004), which may influence the behavior of neurons. Therefore we sought to define an optimal window for using PRV369 to image native network activity in the SMG. We observed the rate of spontaneous calcium events at 24, 48, and 72 hours post inoculation (h.p.i.) in the salivary glands (Figure 4.4A). Analysis of the number of calcium events at each time point revealed a change in event frequency as time after infection increased: At 24 and 48 h.p.i., the majority of infected neurons generated events sparsely, but by 72 h.p.i., more cells were active and at higher rates (examples in Figure 4.3). In addition, we observed a statistically significant difference in the average duration of calcium events at 72 h.p.i. compared to 24 or 48 h.p.i. (Figure 4.4A). We further calculated the fraction of time that labeled cells spent at elevated intracellular calcium levels and observed a difference between 24 and 48 h.p.i., with a larger difference occurring at 72 h.p.i. (Figure 4.4B). At 72 h.p.i. the cells displayed more complex patterns in the calcium events, indicative of unusually large bursts of
Figure 4.4. Quantitative analysis of spontaneous calcium flux. (A) The average duration of a calcium event increases with the time after inoculation, with 72 h.p.i. exhibiting the most significant increase. The average duration is 7.6±1.1, 10.5±0.7, and 15.8±0.6 seconds for 24, 48, and 72 h.p.i., respectively. (B) The average percent of time that a cell is in a state of elevated intracellular calcium concentration also increases with time after inoculation. The average percent time was 1.0±0.3, 2.5±0.5, 6.3±0.5% for 24, 48, and 72 h.p.i., respectively. Labeled cells at 72 h.p.i. spend significantly more time in an elevated calcium state. (C) Probability distribution that the occurrence of a calcium event will last a certain length of time (in seconds) for 24, 48, or 72 hours post inoculation (h.p.i.). At 72 h.p.i., the occurrence of long-lasting calcium events is more frequent than earlier time points, and only at this time point do calcium events last longer than 50 seconds.
Figure 4.4

A

Ca²⁺ event duration (s)

24 h.p.i. | 48 h.p.i. | 72 h.p.i.

B

% time of elevated Ca²⁺ concentration

24 h.p.i. | 48 h.p.i. | 72 h.p.i.

C

Probability

Ca²⁺ event duration (s)

0-5 | 10-15 | 20-25 | 30-35 | 40-45 | 50-55 | 60-65 | 70-75 | 80-85 | 90-95

24 h.p.i. | 48 h.p.i. | 72 h.p.i.
activity (compare traces in Figure 4.3A-C). We conclude that in the SMG, PRV369 is most useful for measuring circuit activity within 48 hours after inoculation. Since G-CaMP2 fluorescence of infected SMG neurons appeared by 24 hours, there is a window of about 24 hours in which to observe and analyze neuronal activity with minimal effects of infection.

**Labeled SMG neurons respond to electrical and sensory stimuli in vivo**

We next used PRV369 infection to record stimulus-evoked (non-spontaneous) activity in the SMG *in vivo*. We electrically stimulated infected ganglia to evaluate the responsiveness of the infected neurons to external stimuli. We delivered an electrical pulse train via a wire electrode placed on the presynaptic axon bundle emanating from the brainstem to innervate the SMG. This stimulus triggered synchronous calcium transients in a subset of the neurons in the field of view (Figure 4.5A). Occasionally, an axon initial segment was clearly visible and also responded with a transient signal upon stimulation (Figure 3.5A, dotted purple trace). As expected, the calcium event triggered in the axon initial segment was of shorter duration, compared to the calcium event in the cell body of the same neuron (Figure 4.5A, solid purple trace). In general, $\Delta F/F$ values for electrically stimulated calcium transients were on average smaller and shorter-lasting than spontaneous transients, especially at later time points of infection. Moving the electrode to different position on the presynaptic axon bundle activated a different subset of neurons. Antidromic stimulation yielded similar results, and neurons were responsive to electrical stimulation at 24, 48, and 72 hours after inoculation.

We tested the sensory response of the SMG at 48 h.p.i. It has been reported that hot ($T = 50^\circ$C) distilled water delivered to the oral cavity elicits a salivatory reflex (Bartsch, Habler et al. 1996). We applied $40^\circ$C distilled water to the oral cavity and observed a sensory-evoked
Figure 4.5. PRV369-infected SMG neurons respond to external electrical and sensory stimuli.

(A) An electrical stimulus (arrow below traces) elicited a sharp and transient response in a subset of neurons. The diagram indicates where the tungsten electrode (orange) was placed on the innervating presynaptic axons from the brainstem (black bundle). Occasionally, the axon initial segment was visible (dotted purple trace) and triggered a shorter calcium event compared to the cell body of the same neuron (solid purple trace). (B) When hot distilled water (T = 40°C) was delivered to the oral cavity (arrow below traces) as a natural sensory stimulus for salivation, SMG neurons produced a strong and synchronous response. Scale bar = 20µm.
Figure 4.5

A  Electrical stimulation

B  Sensory stimulation
response in PRV369-labeled neurons. A subset of neurons exhibited a strong response (Figure 4.5B) and sensory-evoked events were of similar duration to spontaneous events. Similar groups of neurons fired during repetitions of the sensory stimulus. In one animal, across three repeated sensory stimulations, 12 out of 15 fluorescent cells showed sensory responses; 2/3 of the responsive cells exhibited calcium transients 100% of the time. Sensory responses were not seen when the oral cavity was stimulated with distilled water at room temperature (23 ºC), demonstrating that the responses were temperature-specific. Taken together, these results demonstrate the utility of PRV369 infection for analysis of electrically evoked responses and physiologically relevant sensory stimulation.

Discussion

Since PRV 369 is isogenic with PRV152 (Bartha GFP) (Smith, Banfield et al. 2000) and PRV614 (Bartha RFP) (Banfield, Kaufman et al. 2003), two of the most frequently used PRV-based viral circuit tracers, the use of PRV369 is facilitated in circuits already elucidated by these tracers. PRV369 can also be used in dual-infection experiments with these isogenic parental strains (Kim, Enquist et al. 1999; Card and Enquist 2001; Banfield, Kaufman et al. 2003). The prior use of PRV in delineating central nervous system (CNS) circuits (reviewed in (Aston-Jones and Card 2000) and (Ekstrand, Enquist et al. 2008)), and recent advances in live imaging with other genetic calcium indicators in the CNS (see for example (Wallace, Meyer zum Alten Borgloh et al. 2008) and (Mank, Santos et al. 2008)), suggest the potential usefulness of PRV369 for revealing activity in synaptically connected CNS neurons in vivo.

In contrast to PRV369, the published As1-PRV08 calcium sensor virus was constructed in a mutated PRV-Kaplan strain background, which the authors found to have higher infectivity and more cytotoxicity than several PRV-Bartha-derived strains tested in their study (Boldogkoi,
In addition, As1-PRV08 expresses the TN-L15 calcium sensor (Heim and Griesbeck 2004), a FRET-based [Ca^{2+}] indicator. In matched in vivo comparisons (Hendel, Mank et al. 2008), G-CaMP2 was found to have faster kinetics than TN-L15, including a 4-fold faster off-rate for calcium. In response to a strong stimulus, Boldogkoi et al. found an average 13% increase in the citrine:CFP fluorescence ratio in ganglion cells infected by As1-PRV08 (Boldogkoi, Balint et al. 2009). In SCG neurons infected with PRV369 in vitro, the average fluorescence change was 7% for a single action potential, and 27% for triplets. In the SMG in vivo, signals of up to 100% relative fluorescence change were observed.

SMG neurons could not be impaled for intracellular recording or bolus-loaded with calcium dyes in vivo without damaging the neurons. The SMG is surrounded by extracellular connective tissue, and each neuron is ensheathed by satellite cells (Pomeroy and Purves 1988). Most studies have characterized SMG neuron activity with electrodes ex vivo (for example, Lichtman 1977) or extracellular recording in vivo (Bartsch, Habler et al. 1996). One published method for intracellular recording of SMG neurons under in vivo conditions necessitated a separate recording chamber and partial extraction of tissue (Suzuki and Kusano 1978). PRV369 is thus noteworthy for allowing characterization of neuronal activity in a circuit that is otherwise difficult to access by previous recording methods. In the future, PRV369 should prove useful for studying local circuits in which multiple connected neurons are visible within the same field of view, allowing noninvasive and simultaneous characterization of network activity in multiple cells.

The endogenous activity of PRV369-labeled neurons can be monitored for at least 24 hours with limited effects due to infection. Most SMG neurons generated signals sparsely at 24 and 48 hours post inoculation into the salivary glands, consistent with previous published data that spontaneous firing is rarely observed from these cells (Suzuki 1981). However, the number
of infected cells and firing frequency increased significantly by 72 hours. In addition, the average duration of a [Ca\textsuperscript{2+}] event lasted significantly longer at late time points in infection. As suggested by cytological changes observed in other circuits, the later time point may correlate with the initiation of host defenses stimulated by infection (Rinaman, Card et al. 1993). The increased activity we observe at 72 hours post inoculation marks the first live, in vivo observation of these long-suspected effects of viral infection. One previous report monitored herpes simplex virus infection in transgenic report mice using titer-dependent bioluminescence techniques, but not at the level of single cells (Luker, Schultz et al. 2006). We envision that further experiments with PRV369 in infected circuits in vivo will allow exploration into the nature of the host response to infection. In summary, PRV369-labelling of connected neurons allows for reliable and sensitive detection of endogenous circuit activity early in infection, and will provide a long-sought means to simultaneously reveal both connectivity and activity at single cell resolution, in intact neural circuits in vivo.

Materials and Methods

**Cells and virus construction.** PRV 369 was constructed by homologous recombination between a plasmid containing a G-CaMP2 expression cassette and the gG locus of the PRV-Bartha genome. The plasmid pN1-G-CaMP2 (pN1-RSET-mG1.6#X-1) was a gift of Dr. Junichi Nakai (Nakai, Ohkura et al. 2001), and contains the coding sequence of G-CaMP2 in place of EGFP in the pEGFP-N1 plasmid, under the control of a cytomegalovirus immediate-early (CMV-IE) promoter (Clontech; GenBank Accession # U55762). A 990bp fragment of PRV gG was isolated by PstI and Ndel digestion of the previously described pBB04 plasmid (Smith, Banfield et al. 2000). This vector was cloned into the DraIII site of pN1-G-CaMP2, downstream of the simian virus 40 (SV40) polyadenylation sequences. This plasmid was linearized and co-transfected into
swine epithelial (PK15) cells with DNA from PRV614, a PRV Bartha derivative with mRFP1
inserted into the gG locus (Banfield, Kaufman et al. 2003). Homologous recombination of the G-
CaMP2 cassette into the gG locus of PRV614 occurred between the CMV-IE promoter and the
flanking gG sequence, replacing the original 2.3 kb mRFP cassette at this locus with the 2.5 kb G-
CaMP2 cassette. Resulting recombinants were plaque-purified on PK15 cells by selection of non-
red plaques. Southern blot analysis using a 1.9 kb piece of gG (EcoRI – HindIII fragment) as a
probe confirmed that the GCaMP2 cassette had recombined correctly into the PRV genome,
producing the recombinant PRV369. Restriction fragment length polymorphism (RFLP) analysis
using BamHI, KpnI, and PstI was used to compare PRV Bartha, the parental PRV614, and PRV369,
confirming the correct integration of the G-CaMP2 cassette. Western blot for the upstream gene
Us3 and downstream gene gD revealed no changes in expression of these proteins relative to
the parental PRV614 strain. PRV614 and related tracing strains have a previously described
decrease in Us3 expression relative to PRV-Bartha (Demmin, Clase et al. 2001).

_Dissociated neuronal cultures of superior cervical ganglia (SCG)._ SCG neurons were used
for _in vitro_ characterization of PRV369 for ease of maintenance in dissociated culture. Methods
for harvesting and culturing SCGs have been described in detail elsewhere (Curanovic, Ch'ng et
al. 2009). Briefly, SCG ganglia were harvested from mouse embryos at embryonic day 15,
dissociated, and allowed to settle on tissue culture grade plates coated with 0.5 mg/ml poly-
ornithine and 10 μg/ml laminin. Neurons were cultured in Neurobasal™ media containing, 1X
Penicillin-Streptomycin-Glutamine solution, 1X B-27 supplement, and 50 ng/ml nerve growth
factor (all from Invitrogen), and allowed to mature for 10 days before using in experiments.

_Electrophysiology._ Experiments _in vitro_ were performed at room temperature. We
recorded spontaneous activity from 16 to 18 hours after infection. The effect of PRV infection on
dissociated superior cervical ganglia neurons has been recently characterized (McCarthy, Tank
et al. 2009). For intracellular recordings, quartz micropipettes were pulled (P-2000, Sutter Instrument, Novato, CA) to a resistance of 40-60 MΩ and filled with 10 μM fluorescein in 3 M KCl. Recordings were acquired at 5 kHz (Axopatch 200B, Axon Instruments).

**Infection of mouse submandibular ganglia (SMG).** All animal procedures were performed in accordance with the guidelines of the National Institutes of Health and were approved by local authorities (Princeton University Institutional Animal Care and Use Committee). The model for mouse SMG infection was previously published (Feierbach, Piccinotti et al. 2006), and more details can be found in Chapter 2 of this thesis. Briefly, mice aged roughly 7-10 weeks were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal administration. A midline incision was made along the neck, exposing the salivary glands. Using a Hamilton syringe, concentrated PRV inoculum (1x10⁹ pfu/ml) was injected into both submandibular glands (5 µl/side). The incision was sutured and the mice were given a dose of buprenorphine (100 µg/kg concentration) against post-surgical pain. The mice were allowed to recover for various times post inoculation.

**Calcium imaging in vivo.** The protocol for imaging infected SMG in living mice was adapted from Purves and Lichtman (Purves and Lichtman 1987; Purves, Voyvodic et al. 1987), and more details are given in Chapter 2 of this thesis, as well as in the published protocol (Granstedt, Kuhn et al. 2010). Briefly, mice were anesthetized in an isofluorane chamber and then transferred in supine position to a custom-built stage with heating pad, rectal temperature probe, and nose cone for isoflurane inhalation. A ventral midline incision was made along the neck and the skin was pulled back with retractor. The SMG were exposed and lifted on a small custom-made metallic platform, controlled by a micromanipulator. The area was perfused with warmed mammalian Ringer’s solution containing (in mM): 154 NaCl, 5.6 KCl, 2.4 NaHCO₃, 2 Tris...
buffer pH 8.0, and 2.2 CaCl₂, adjusted to pH 7.4 with HCl. The animal’s temperature was
maintained at 35°C.

Electrical and sensory stimulation. We used a tungsten electrode (1 MΩ resistance) to
electrically stimulate pre- and post-synaptic axons by 10 pulses of 60 V, delivered every 0.1 ms
at 100 Hz (ISO-Flex triggered by Master-8, A.M.P.I.). For the sensory stimulation, we injected
aliquots (300 µl) of hot water (40ºC at outlet) at a pressure of 8 psi to the mouth by air-pressure
from a picospritzer (Pressure System Ile, Toohey Company) while simultaneously imaging the
SMG. The tubing was heated electrically.

Two-photon microscopy. Two-photon fluorescence imaging was performed on a custom-
built microscope with a tunable Mira 900 Titanium:sapphire laser (Coherent) at 910 nm
excitation wavelength and a GaAsP photomultiplier tube (H7422P-40, Hamamatsu). The
microscope was controlled by CfNT software (M. Müller, Max Planck Institute for Medical
Research, Heidelberg, Germany). We used a 40x, 0.8 N.A. water-immersion objective (HCX APO,
Leica) to record movies with 256 x 256 or 128 x 128 pixel at 2 ms/line scan rate.

Image analysis. The two-photon raw data was first opened in ImageJ (W. S. Rasband,
ImageJ, National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/). We
defined an infected neuron as 30% brighter than background fluorescence from an area outside
of the ganglia in the same field of view. We established this threshold because uninfected cells
have punctate autofluorescence intensities that range from 10-20% above background. A region
of interest was drawn around each infected cell, and the mean intensity over time was saved
and further analyzed using Igor Pro 6 software (WaveMetrics, Inc.,
http://www.wavemetrics.com). For ∆F/F measurements in plots, the background fluorescence
was calculated by fitting a curve. Peaks were identified using a custom-written program in
Python (Python Software Foundation). The analysis program used algorithms based on a
previous publication (Yang, He et al. 2009). The program identified a baseline and recognized peaks as points that were 0.75 intensity units above the baseline and 10% above baseline noise ratio.
CHAPTER 5

Evidence for Electrical Coupling In Vivo During a Virulent PRV Infection

Background and Objective

For more than a century, wild-type PRV has been known to cause the “mad itch” syndrome in animals that are non-native hosts, characterized by a strong impulse to scratch feverishly which results in severe self-inflicted wounds and eventual fatality. Due to its economic impact on swine and cattle, PRV has been studied in detail to understand the molecular basis for this symptomology. Viral genes and protein products have been targeted as virulence factors, and these research efforts have led to a successful vaccine (see (Mettenleiter 2000) for review). Remarkably, it appears that the wild-type PRV strains hardly spread into the CNS since the animals die so quickly and therefore do not cause acute encephalitis; rather the infected peripheral circuits are the only players in determining the severe clinical manifestations. In stark contrast, the attenuated strain PRV Bartha spreads extensively throughout the CNS but does not cause any pruritus, and the animals live significantly longer, though they still succumb and present some neurological symptoms at late stages of infection (Brittle, Reynolds et al. 2004).

It has been documented in the literature for many decades that virulent strains of PRV cause spontaneous electrical discharges in infected neurons of peripheral sympathetic ganglia and these have been correlated with symptomology (Dempsher, Larrabee et al. 1955). An accumulation of numerous studies has led to the hypothesis that the increased electrical activity due to PRV infection originates in the presynaptic terminals due to spontaneous acetylcholine release that triggers synchronized firing in postsynaptic ganglionic neurons (Dempsher and Riker
However the mechanism of this phenomenon still remained obscure until a new perspective on this issue came into view when McCarthy et al. examined the electrical activity of neurons infected with various strains of PRV in vitro using patch clamp recordings of individual dissociated sympathetic neurons (McCarthy, Tank et al. 2009). The results showed that starting at 8 hours post infection, neurons infected with virulent PRV strain had elevated firing rates that could not be interrupted, but the attenuated strain did not cause such a phenotype; however, later in infection, all of the neurons fired synchronously and continuously in both conditions. By 9 hours post infection, coupling events were noticed with the virulent strain as indicated by transfer of small molecular weight dyes between adjacent neurons, whereas the attenuated strain took an additional 10 hours to exhibit signs of electrical coupling. Large molecular weight dyes could be transferred from one neuron to another in infections with either virulent or attenuated strains by 18 hours post infection. Importantly, the viral membrane fusion protein glycoprotein B (gB) was shown to be required for elevated firing rates and fusion of infected neurons (McCarthy, Tank et al. 2009). While these results contributed to understanding the mechanism by which PRV may induce neuropathy, notably through fusion of neuronal membranes, the relatively simplified in vitro system still left unanswered questions as to the correlation of these results to in vivo models.

Therefore, we sought to investigate whether electrical coupling occurred in the context of an animal infection by imaging infected neurons in the living, anesthetized animal. To look at neural activity, we made use of the florescent genetically encoded calcium sensor G-CaMP3 (Tian, Hires et al. 2009), which is a modified GFP protein that flashes green in response to calcium transients induced by neural activity. It is an improved version of G-CaMP2 (Nakai, Ohkura et al. 2001) with increased signal to background ratio. We constructed a virulent strain that expressed G-CaMP3 (PRV 468), and performed all experiments in vivo. Around 48 hours
post infection, we observed spontaneous calcium flashing events in ganglia infected with PRV 468, reminiscent of the previously described spontaneous discharge of infected ganglia in situ (Dempsher, Larrabee et al. 1955); yet by looking at the single-cell level, we could detect that the calcium transients of many infected neurons were synchronous and quasi-cyclical. We quantitatively analyzed the correlation of the calcium flux between infected neurons, and characterized the synchrony of onset of calcium peaks as well as other parameters. By cutting the axons leading to the brainstem, we determined that the impetus for flashing did not originate outside the ganglia in the projecting brainstem neurons. We constructed an attenuated PRV Bartha derivative expressing GCaMP3, and did not observe the synchronous calcium flashing even at 72 hours post infection. Therefore, we propose a mechanism of electrical coupling in the unmyelinated axons projecting from the SMG neurons to the salivary glands to explain the differences between the wild-type and attenuated strains and to account for the phenotype in vivo.

Construction of PRV Becker and PRV Bartha Strains Expressing G-CaMP3

We inserted the calcium sensor G-CaMP3 into the genome of the virulent wild-type strain PRV Becker by homologous recombination, generating the recombinant strain PRV 468. We also inserted G-CaMP3 into the genome of the classical attenuated tracing strain PRV Bartha by homologous recombination, generating the recombinant strain PRV 469. The G-CaMP3 open reading frame is transcribed from the cytomegalovirus immediate-early promoter and the sequence was inserted in the glycoprotein G (gG) locus of the viral genome. Viral protein gG is not essential for spread in vivo (Smith, Banfield et al. 2000), and many of the commonly used PRV viral tracing strains employ insertions at this locus (Banfield, Kaufman et al. 2003). We confirmed correct genomic insertion and protein expression of the G-CaMP3 cassette for both
Figure 5.1. Construction and characterization of PRV 468 and PRV 469. (A) To confirm correct genomic insertion after homologous recombination, nucleocapsid DNA from strains PRV Becker, PRV Bartha, PRV 468 and PRV 469 were digested with Sal1 and run on a gel. Fragments observed were the expected sized and the shift due to the insertion is indicated with an arrow. (B) We confirmed protein expression of the G-CaMP3 cassette with a Western blot. As a viral protein control, we probed for VP5, the major capsid protein, and as a cellular protein control, we probed for beta-actin. (C) PRV 468 and 469 have equivalent single-step growth kinetics of parental strains PRV Becker and PRV Bartha in epithelial cells in vitro. The average titer for each virus at each time point is plotted along with the standard deviation. The titers values were normalized for each strain to the input inoculum.
Figure 5.1

A

B

C

Mock PRV BE PRV BA PRV PRV PRV

GCaMP-3

VP5

beta-Actin

PRV BE

PRV 468

PRV 469

PRV 468

PRV 469

Mock

log_{10} PFU/ml

hours post infection

Becker

Bartha

468

469

kb

10 8 6 4 2 1 0.5
strains with a restriction digest with SalI (Figure 5.1A) and a Western blot (Figure 5.1B), respectively. PRV 468 and 469 grow comparably to parental strains in a single-step growth curve assayed in cultured epithelial cells (Figure 5.1C).

**Spontaneous Calcium Flashing Observed During Infection with Virulent PRV468**

We infected adult male mice with PRV 468 and monitored the spontaneous calcium activity in the peripheral salivary ganglia of living, anesthetized mice using two-photon microscopy. The preparation for *in vivo* imaging was the same as previously published (Granstedt, Szpara et al. 2009). We empirically chose 40 to 48 hours post infection as our imaging window because we wanted to look at a time point when all the neurons in the peripheral ganglia would be infected but before the disease was too advanced and animals were moribund, which usually occurs past 50 hours after infection with the virulent strain Becker, as determined from unpublished observations of symptomology onset in this particular system. We immediately noticed that many of the neurons in the ganglia were firing synchronously (Figure 5.2A).

Upon further inspection of the calcium traces from each cell, not every infected cell was necessarily involved in the synchronous activity, and the amplitude and duration of each calcium event varied (Figure 5.2A). Nonetheless, all participating cells appeared to begin each new calcium peak simultaneously. We quantified the correlation of calcium flux between neurons using a histogram of Pearson’s coefficients of correlation (Figure 5.2B). In this analysis, the pairwise coefficients between neurons in a ganglion were computed, and the coefficients were then binned in 0.1 increments to generate a distribution. All features of the calcium traces, such as amplitude, shape, and noise, were included in the analysis between each pair. The distribution clearly indicated a strong positive correlation (Figure 5.2B). Thus, when one cell
Figure 5.2. Neurons infected with PRV 468 display a synchronous flashing phenotype.

(A) Many of the infected neurons in the ganglia flash synchronously. Though the amplitude and duration of each calcium event varies, all participating cells appear to begin each new calcium peak simultaneously. (B) Quantification of the correlation of calcium flux between neurons using a histogram of Pearson’s coefficients of correlation. Pairwise coefficients between neurons in a ganglion were computed, and coefficients binned in 0.1 increments to generate a distribution. All features of the calcium traces, such as amplitude, shape, and noise, were included in the analysis between each pair. (C) Distribution of inter-peak intervals in a histogram with 1 second bins. (D-E) Trace and analysis from a movie acquired using a line scan. (D) Difference in peak timing during a line scan; values are centered on zero and mean is indicated. Inserts in (E) are magnifications of the trace to show that one cell would flash before the other, but it was not necessarily always the same cell the flashed first, and often they flashed synchronously.
Figure 5.2

A Virulent Becker-GCaMP3 (PRV468)

B

C

D

E

\( \Delta F/F \) 400%

\( \Delta F/F \) 300%

\( \Delta F/F \) 100%

Mean = 68 ms

Inter peak interval (s)

Number of measurements

Number of peak intervals

% number of pairwise correlation coefficients

Difference in peak timing (s)
experiences an increase in calcium flux, there is a high likelihood that another cell will also experience an increase in calcium flux.

The other notable feature of the synchronous calcium flashes was the continuous quasi-cyclical pattern (Figure 5.2A), which would persist for the entire duration of our imaging session without interruption. The pattern appeared to be generated at fairly regular intervals, though there was noise and occasional short delays in the frequencies. Therefore, we analyzed the distribution of inter-peak intervals in a histogram with 1 second bins (Figure 5.2C). In this example, there was a prominent peak at 10 and 11 seconds, which demonstrates the overall regularity of the peak generation.

The example shown in Figure 5.2A-C was taken at a time resolution of approximately 2 frames per second. These low time resolution experiments revealed that the calcium transients in different neurons of a ganglion infected with PRV 468 were highly correlated and synchronized. To further analyze the synchronicity of the infection-evoked calcium signals and to determine whether existing delays could be below our limit of detection, higher time resolution recordings were necessary. We first increased the time resolution 8 fold, superimposed the traces and still could not detect any delay since the synchrony matched perfectly even at this time resolution. Therefore we chose to acquire movies at the fastest possible time resolution of 1000 frames per second (ie. 1 millisecond per frame) using a line scan. At this time resolution, we were able to see some slight delays between traces (Figure 5.2E with inserts). We calculated the differences in peak timings by looking at the second derivative and taking its zero in the rising curve, and then generated a histogram with 50 millisecond bins of the differences in peak timings by subtracting the values of one cell from the other (Figure 5.2D). In this particular example, there were 29 measurements that were centered on zero, but the mean difference was 68 milliseconds.
Figure 5.3. Superimposed calcium traces from four individual cells. Peaks were overlaid by aligning the zero value of the second derivative of the curves, and these values were set to time 0. Cell 1 does not flash. Cell 2, 3, and 4 show 16 peaks superimposed each. The averaged peak is shown in red.
Figure 5.3

Cell 1

Cell 2

Cell 3

Cell 4
Each Cell has a Unique Calcium Peak Shape

As previously mentioned, we observed that each participating, synchronously flashing cell exhibited a unique calcium trace profile in which the calcium flux varied in amplitude and duration from cell to cell. We further examined the individual traces from each cell to determine whether a particular cell was internally consistent. Therefore, using the line scan data, which is the highest time resolution of acquisition, and the normalized ΔF/F traces, we superimposed peaks from one cell’s trace for four different cells (Figure 5.3). The peaks were overlaid by aligning the second derivatives of the curves, and these values were set to time 0. The averaged peak is shown in red. In this example, cell 1 displays very weak fluorescence changes that are barely above the background noise. Cell 2, 3, and 4 show a superimposition of 16 peaks each. It is noteworthy that each of these cells displays a unique shape, amplitude, and duration to its calcium peaks that are remarkably consistent. For example, cell 2 exhibits calcium flashes that average 150% above background at their peak, and these calcium levels plateau for a full second before beginning to decrease and return to background. Cell 3 also peaks around 150% above background, but the calcium levels immediately begin to decrease after peaking. Cell 4 displays much higher changes in calcium flux, averaging close to 500% above background. These unique features of individual neuron calcium traces warrant further investigation and illustrate how adjacent neurons still retain some independence in the regulation of their calcium flux.

The Flashing Phenotype is Reproducible Across Many Mice

Between 40 to 48 hours post infection, every neuron in the SMG was infected and the flashing phenotype could be detected across many mice examined. Since the flashing followed a cyclical reproducible pattern, we analyzed the inter peak intervals across several mice (Figure 5.4). We found that the calcium flashes generally occurred every 5-15 seconds (Figure 5.4A-E).
Figure 5.4. Quantitative analysis of inter peak intervals across several mice. (A-F) The flashing phenotype was reproducible across many mice and the calcium flashes generally occurred every 10-15 seconds (A-E), though occasionally it was longer (F). We were able to acquire data from two different ganglia in one mouse, and found that the ganglia were similar, but each still had a unique inter peak interval distribution (A-B).
Figure 5.4

A. Mouse 1
   SMG 1

B. Mouse 1
   SMG 2

C. Mouse 2

D. Mouse 3

E. Mouse 4

F. Mouse 5
Occasionally it was longer (Figure 5.4F). In one animal, we were able to acquire data from two different ganglia, and therefore we were curious to know whether the inter peak interval measurement would be identical or separate. We found that the ganglia were similar, but each still had a unique inter peak interval distribution, suggesting that the ganglia are independent from each other and not interconnected (Figure 5.4A-B).

**The Synchronous Calcium Signals are Generated Independently from Brainstem Projections**

The very close synchrony of calcium flux between cells suggested to us that electrical coupling may be occurring somewhere within the salivary circuit, for example in the neurons of the SMG, the neurons of the superior salivatory nucleus (SSN) in the brainstem, or in SMG axons that project to the salivary glands. Qualitatively, we did not detect syncitial formation of SMG neurons or enlarged multi-nucleated soma. In addition, we observed synchronous calcium flux among cells that were not necessary neighboring one another. Since each SMG neuron is ensheathed by one or several satellite cells, it is possible that these specialized glial cells act as barriers to fusion between cells. The fact that an individual infected neuron had a unique shape to its calcium peaks (Figure 5.3) gave us further evidence that each cell body probably remained separate. Therefore, we initially hypothesized that electrical coupling may be present upstream in the SSN.

These SMG ganglia receive input from the SSN in the lateral reticular formation of the pons. The neurons that comprise this structural nucleus of cells are in close proximity, and the preganglionic axons branch to innervate one of more neurons in the SMG but each neuron in the SMG receives input from only one cell in the SSN. Therefore, we hypothesized that the infection may be causing electrical coupling, or microfusion events, among the soma of the nucleus in the brainstem that sends projecting axons to the SMG, as this could help to explain
**Figure 5.5. Axon cuts of brainstem projections.** (A) The animal was prepared as usual and spontaneous activity imaged for several minutes. (B) Histogram of Pearson’s coefficients indicates a strong correlation before the cut. (C) Synchronous activity after the cut. (D) Schematic of where the cut was made. We used a hemostat to clamp and hold the preganglionic bundle of axons and surrounding tissue, then cut everything below. We always verified that all tissue had been cut afterwards by removing the hemostat and ensuring that nothing remained below. (E) Histogram of Pearson’s coefficients indicates a strong correlation after the cut.
Figure 5.5

A. Before cut

B. Binned Pearson's Correlation Coefficients

C. After cut

D. Salivary glands

E. Histogram of Binned Pearson's Correlation Coefficients

Axons to brainstem
why synchrony was distributed across a ganglion. We did not think electrical coupling would be occurring in the projecting axon bundle since these axons are myelinated, though they could be mechanisms acting on presynaptic terminals within the SMG. To look at the SSN involvement, we performed axon cuts during our imaging session. We prepared animals for imaging as before, and imaged spontaneous activity for several minutes (Figure 5.5A). Once we detected the synchronous phenotype, we used a hemostat to clamp and hold the preganglionic bundle of axons and surrounding tissue, then cut everything below. Figure 5.5D illustrates where the cut took place. We then immediately resumed our imaging, and found that the synchronous phenotype was unaffected (Figure 5.5C). We always verified that all tissue had been cut afterwards by removing the hemostat and ensuring that nothing remained below. We tried to relocate the same region of interest as before the cut, but it was difficult to find the exact same cells and configuration since the tissue moved and rotated during the clamping and cutting. Nonetheless, we were confident that we found the same ganglion as before the cut. As before, we quantified the correlation of calcium flux between neurons using a histogram of the Pearson’s coefficients. Both before and after the cut indicated a strong correlation (Figure 5.5B,E). We did notice that the amplitude of the normalized traces after the cut were smaller than before the cut. When we looked at the raw values, we detected an overall increase in calcium levels after the cut.

**Calcium Monitoring with the Attenuated PRV 469**

We infected animals and monitored spontaneous calcium activity using PRV469, a PRV Bartha derivative, at around 72 hours after infection. We chose a time point that was 24 hours later than the observations we made with the virulent strain because we knew that mice infected with attenuated PRV strains survive longer. From our previous published data using
Figure 5.6. Infection with the attenuated PRV 469 does not elicit synchronous flashing.

(A) The spontaneous calcium signals of PRV 469 infected neurons are qualitatively unique and autonomous to each cell. (B) Histogram of Pearson’s coefficients indicated no correlation between cells since values are centered on zero. (C) Histogram of inter-peak intervals did not reveal any significant peak above noise.
Figure 5.6

A) Attenuated Bartha-GCaMP3 (PRV469)

B) Binned Pearson’s Correlation Coefficients

C) Number of peak intervals
PRV369, an attenuated strain which expresses G-CaMP2, at 72 hours after infection we observed that most of the SMG neurons were infected and these infected neurons exhibited complex calcium events that were unique to each cell and asynchronous (Granstedt, Szpara et al. 2009). With the improved calcium sensor G-CaMP3, we observed similar complex calcium events, but the changes in fluorescence ($\Delta F/F$) were brighter and often exceeded 200% (Figure 5.6A). We still did not observe any qualitative signs of electrical coupling between neurons in the SMG, and quantitatively, the calcium traces did not reveal any synchronous activity between neurons. The histogram of Pearson’s coefficients did not indicate any correlation (Figure 5.6B). In addition, the histogram of inter peak intervals did not reveal any peak above noise, signifying that there was no cyclical pattern of flashing among the calcium traces (Figure 5.6C).

**Discussion**

Here we report a striking phenotype of peripheral neurons infected *in vivo* with a virulent strain of PRV expressing GCaMP3 in which infected neurons have synchronized calcium flashes around 48 hours after infection. By our various quantitative measurements, the calcium flux between cells was highly correlated. Only at the fastest acquisition possible did we detect 50 to 100 millisecond delays in the calcium flashes between pairs of neurons. This synchrony suggested to us the possibility of electrical coupling. Changes in intracellular calcium levels have been reported during PRV infection of neurons *in vitro*, and infected neurons exhibited synchronous fluorescent flashes that were thought to result from electrical coupling since the onset coincided with increased firing rates (Kramer and Enquist 2012). In addition to the synchrony of calcium flux between neurons, it was notable that the calcium flashes were cyclical. In fact, the pattern was regular enough that we measured a frequency range between 0.1 to 0.2 Hertz, though the average frequency varied per ganglion. Bursts of spontaneous
activity have been recorded in sympathetic superior cervical ganglia (SCG) infected with PRV, and when the electrical activity of pre- and post-ganglionic nerves are recorded simultaneously, action potentials appear on top of excitatory postsynaptic potentials, thereby precluding pacemaker potentials; the spontaneous activity is known to be of cholinergic origin since it is reversibly blocked by curare (Dempsher and Riker 1957; Dolivo, Honegger et al. 1979).

When we cut the projections from the SSN and imaged immediately afterwards, the phenotype was unaffected, suggesting that the impetus to fire was not originating upstream in the nuclei of the brainstem. Spontaneous discharges from SCG infected with a virulent PRV strain have been described for a while in an in situ preparation and the onset has been shown to correlate with the mad itch symptomology (Dempsher, Larrabee et al. 1955). Follow up studies in the same peripheral autonomic ganglia using pharmacology demonstrated that the increased infection-induced electrical activity originated in the presynaptic terminals within the ganglia (Dempsher and Riker 1957; Kiraly and Dolivo 1982), and a nicotinic acetylcholine receptor antagonist abolished the postsynaptic firing activity without abolishing the spontaneous discharges from the preganglionic nerve (Liao, Maillard et al. 1991). In addition, denervation of the preganglionic nerve prior to infection with PRV resulted in infection of the SCG with animals showing the typical signs of mad itch, yet no spontaneous discharges were recorded in the ganglia, though the ganglia were still excitable (Dempsher and Riker 1957). Since the axon cuts reported here were done acutely, imaging was resumed immediately before denervation occurred, similar to the in situ preparation for SCG. Therefore we cannot exclude the possibility that presynaptic terminals within the SMG play a role in the observed phenotype. Nonetheless, because of the clear difference we observe between virulent and attenuated strains, we propose a model to account for our observed phenotype that primarily concerns the postganglionic nerve trunk.
We did not observe any qualitative signs of multi-nucleation between neurons in the ganglia, and the infected cells that participated in the synchronous activity still maintained distinct shapes in their calcium traces. While fusion between neuronal cell bodies has been reported *in vitro* (McCarthy, Tank et al. 2009), we surmise that perhaps electrical coupling in SMG cell bodies does not happen in the *in vivo* condition because of the basic organization in which one or more specialized satellite glial cells form envelopes around individual neurons, creating distinct functional units that are largely isolated from the surrounding neurons and their attending satellite glial cells (for review, see (Hanani 2010)). The architecture may help to limit diffusion of viral particles between SMG neurons. Glial cells in the CNS have been reported to be susceptible to PRV infection but they do not allow production of new viral progeny that could then spread to adjacent cells (Tomishima and Enquist 2002). Schwann cells in the PNS that surround unmyelinated axons can also be infected with PRV, and capsid assembly in their nuclei has been visualized ultrastructurally, though whether this leads to a productive infection is still debated (Dolivo, Beretta et al. 1978).

In contrast to the virulent strain, infection with the attenuated PRV 469 strain did not exhibit the same flashing phenotype. Instead, the neurons remained autonomous in their calcium flux changes and there was no correlation between neurons based on quantitative measures. These results differ from *in vitro* observations, in which even a PRV Bartha derivative did ultimately cause fusion pores to form (McCarthy, Tank et al. 2009), but are consistent with our previous published work using an attenuated PRV strain expressing GCaMP2 (Granstedt, Szpara et al. 2009). Other *in vivo* systems which have been exploited for transsynaptic analysis using PRV Bartha derivatives have reported no dramatic alteration in the physiological properties of infected neurons (Smith, Banfield et al. 2000). Furthermore, when PRV Bartha was still in its infancy as a neuronal circuit tracer, *in vivo* studies were done to compare the spread of
the virus with wheat germ agglutinin, which cannot spread across neuronal cell bodies, and PRV remained confined to first order neurons (Strack and Loewy 1990). The attenuated strain can only spread retrogradely and therefore will not send new viral particles down the axons back to the initial site of infection. This restriction is due to a large deletion in its genome, which removes the coding sequence of several genes, of which three membrane proteins, glycoprotein E (gE), gI, and Us9, have been found to be critical for anterograde sorting of viral components and anterograde spread in vivo (Card, Whealy et al. 1992; Yang, Card et al. 1999; Brideau, Card et al. 2000). In addition, the Us9 membrane protein controls the axonal localization of other membrane proteins, such as gB (Tomishima and Enquist 2001). The absence of gB in the axons of PRV Bartha infected neurons is noteworthy because gB is an essential component of the viral fusion machinery and is required for spread (Babic, Mettenleiter et al. 1993; Curanovic and Enquist 2009). Therefore fusion would not be able to occur in the absence of gB, and in fact, electrical coupling was absent in vitro in cultured sympathetic neurons infected with a gB null strain (McCarthy, Tank et al. 2009).

Based on the results with the virulent and attenuated strains, we hypothesize that electrical coupling may be occurring in downstream axon bundles projecting to the salivary glands. We are currently designing experiments to construct a gB null strain expressing GCaMP3 and examine the calcium profiles of neurons infected with this strain in vivo. We expect that the flashing phenotype will disappear if our model is true. In vitro, PRV was shown to spread transneuronally specifically at sites of axo-axonal contact (Ch'ng, Spear et al. 2007). Furthermore, in various in vitro models, capsids have been observed to egress at discrete sites along the axon, such as varicosities (De Regge, Nauwynck et al. 2006; Curanovic and Enquist 2009). In vivo, axo-axonal coupling exists in the hippocampus, in the absence of infection, and this mechanism is used for ultrafast neuronal communication in the hippocampus (Schmitz, Schuchmann et al. 2009).
One computational analysis of this phenomenon in the CNS revealed that the axonal gap junctions induce synchronous oscillations and soma potentials are evoked antidromically from the axon (Maex and De Schutter 2007). In the PNS, ultrastructural studies have demonstrated sites of axo-axonal synapses between sympathetic and parasympathetic nerves innervating the same peripheral target (Ehinger, Falck et al. 1970), and these sites of contact have functional significance (Smith, Warn et al. 2002). Sensory and autonomic postganglionic axons are usually unmyelinated, and groups of axons are surrounded by Schwann cells in troughs termed Remak bundles (Campana 2007; Hanani 2010); therefore, it is conceivable that a mechanism for infection-induced axo-axonal coupling in the salivary circuit could exist.

We provide some of the first direct evidence in vivo that supports the proposed mechanism of electrical coupling during a virulent PRV infection of peripheral ganglia that may relate with symptomology. By observing the calcium profiles of many neurons simultaneously with single-cell resolution, we were able to characterize synchronous patterns of activity that were highly inter-correlated among infected neurons. This phenotype is reminiscent of the spontaneous bursting activity in infected sympathetic ganglia associated with behavioral pruritis (Dempsher, Larrabee et al. 1955). Since various human herpes virus infections lead to severe neuropathic pain like disseminated herpes zoster or postherpetic neuralgia, understanding the mechanism by which this class of viruses induces alterations in neuronal physiology can help the development of preventative and therapeutic treatments.

**Materials and Methods**

*Cells and virus construction.* PRV 468 and PRV 469 were constructed by homologous recombination between a plasmid containing a G-CaMP3 expression cassette and the gG locus of the PRV Becker and PRV Bartha genomes, respectively. The plasmid pN1-G-CaMP3 was a gift
of Dr. Charles Zuker, and contains the coding sequence of G-CaMP3 in place of EGFP in the pEGFP-N1 plasmid, under the control of a cytomegalovirus immediate-early (CMVIE) promoter (Clontech; GenBank Accession # U55762). The pN1-GaMP3 plasmid was linearized and co-transfected into swine epithelial (PK15) cells with DNA from PRV616, a PRV Becker derivative, or PRV 614, a PRV Bartha derivative, and both these parental strains have mRFP1 inserted into the gG locus (Banfield, Kaufman et al. 2003). Homologous recombination of the G-CaMP3 cassette into the gG locus of PRV616 or PRV 614 occurred between the CMV-IE promoter and the simian virus 40 (SV40) polyadenylation sequences, replacing the original 2.3 kb mRFP cassette at this locus with the 2.5 kb GCaMP2 cassette. Resulting recombinants were plaque-purified on PK15 cells by selection of non-red plaques. Then we co-infected at a high MOI the resulting strains with a PRV Becker or PRV Bartha derivatives that expressed an mRFP capsid fusion protein, PRV 180 and 765, respectively, and plaque purified three times so that the final strains PRV 468 and PRV 469 express diffusible G-CaMP3 as well as a red capsid tag. This enabled us to independently confirm infection of cells. Restriction fragment length polymorphism (RFLP) analysis using Sal1 was used to compare PRV Becker, PRV Bartha, PRV 468, and PRV 469, confirming the correct integration of the G-CaMP3 cassette. Nucleocapsid DNA was prepared using standard protocols (Szpara, Tafuri et al. 2011). To check for robust protein expression of the inserted cassette, we performed a western blot using the following antibodies: polyclonal rabbit anti GFP (Invitrogen A11122), mouse anti major capsid protein VP5 (Enquist lab clone 3C10), and the monoclonal mouse anti Beta-Actin (Sigma A1978). For the single-step growth curve, PK-15 cells were infected at an MOI of 10, with input virus removed by saline washes after 1 hour. Each time point was performed in triplicate and titered in duplicate.

Infection of mouse submandibular ganglia (SMG). All animal procedures were performed in accordance with the guidelines of the National Institutes of Health and were
approved by local authorities (Princeton University Institutional Animal Care and Use Committee). The model for mouse SMG infection was previously published (Granstedt, Kuhn et al. 2010), and more details can be found in Chapter 2 of this thesis. Briefly, mice aged roughly 7-10 weeks were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal administration. A midline incision was made along the neck, exposing the salivary glands. Using a Hamilton syringe, concentrated PRV inoculum (1x10^9 pfu/ml) was injected into both submandibular glands (5 μl/side). The incision was sutured and the mice were given a dose of buprenorphine (100 μg/kg concentration) against post-surgical pain. The mice were allowed to recover for various times post inoculation.

*Calcium imaging in vivo.* The protocol for imaging infected SMG in living mice was repeated from our previous publication (Granstedt, Szpara et al. 2009), and more details are given in Chapter 2 of this thesis, as well as in the published protocol (Granstedt, Kuhn et al. 2010). Briefly, mice were anesthetized in an isofluorane chamber and then transferred in supine position to a custom-built stage with heating pad, rectal temperature probe, and nose cone for isoflurane inhalation. A ventral midline incision was made along the neck and the skin was pulled back with retractors. The SMG were exposed and lifted on a small custom-made metallic platform, controlled by a micromanipulator. The area was perfused with warmed artificial cerebral spinal fluid containing (in mM): 127 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1 MgCl₂, 25 glucose. The animal’s temperature was maintained at 35°C.

*Two-photon microscopy.* Two-photon fluorescence imaging was performed on a custom-built microscope with a tunable Mira 900 Titanium:sapphire laser (Chameleon - Coherent) at 920 nm excitation wavelength and a GaAsP photomultiplier tube (Hamamatsu). The microscope was controlled by ScanImage (Pologruto, Sabatini et al. 2003), a freely available software written
in Matlab (Mathworks). We used a 40x, 0.8 N.A. water-immersion objective LUMPlanFl/IR (Olympus) to record movies at various scan rates.

Image analysis. The two-photon raw data was analyzed directly in Matlab using custom-written code. To correct for the movements due to breathing, the images were aligned and registered automatically. Then the software drew regions of interest (ROI) around each infected cell and these ROI were subsequently confirmed manually. ROI for cells bodies missed by the software were added manually. The mean pixel intensity in each ROI was then extracted for every time points to obtain a time-series fluorescence trace for each ROI (ΔF/F). Slow timescale changes in the fluorescence traces (e.g. due to photo-bleaching or small drift in the z focal position) were removed by determining the distribution of fluorescence values in a 20 second interval around each sample time point and subtracting the 8th percentile value. The value of 20 seconds was chosen because it is much longer than the duration of a calcium transient observed. The 8th percentile value was found to be appropriate to isolate the baseline. The baseline-subtracted fluorescence traces for each ROI were then further analyzed to measure the correlation between the fluorescent signals of different neurons. Correlations were from Pearson’s correlations. The pairwise correlation coefficients of a given ganglion were displayed in histograms of 0.1 width bins. Inter-peaks intervals analysis on low time resolution data only (movies taken at T=1 and 2ms per line, 256*256, 256*128 and 256*64 pixels) was performed using a threshold method. ΔF/F traces were smoothed using a high frequency filter and times at which ΔF/F reached the threshold value were measured. Subtractions of successive time values gave the inter-peak interval values. The results for each cell were displayed in histograms of 1 second bins. We performed high time resolution experiments by scanning single lines (line scans at t=1 and 2ms) crossing usually 3 or 4 cell bodies. Regular images of the whole field of view were recorded before and after the experiment to check for possible long time-scale drift in X, Y
or Z. The segments of the line associated with each cell body was then treated as a ROI and processed in Matlab, as described above. The peak timing analysis relying on arbitrary threshold values was not appropriate for measuring the calcium transient delays between cells. To address the timing of an individual cell’s calcium transients, a method independent of the actual ΔF/F values was necessary, and the onset of the signal or the inflection point was measured. We choose to quantify the timing of the calcium transient by measuring the time values when the second derivative of ΔF/F cancels (while going from positive to negative value, i.e. in the rising portion of the fluorescence signal). These time values were then used to measure inter-peak intervals and cell-to-cell delays, and to re-align all peaks from a given cell body.
CHAPTER 6

Discussion

Herpetic Neuropathic Pain

A clinical hallmark of HSV and VZV infections in humans is the associated peripheral pain described by afflicted patients. HSV-1 can cause recurrent orolabial lesions which are preceded by sensations of pain, burning, tingling, or itching, which last for several hours before the appearance of small clustered vesicles, known as cold sores, on the lip (Whitley and Roizman 2001). Reactivation of VZV after primary infection and latency causes herpes zoster, a localized, vesicular rash that is preceded by several days of localized neuropathic pain; individuals who have experienced shingles are then at risk for developing postherpetic neuralgia, a sustained debilitating pain that can persist for years despite clearance of viral shedding (Arvin 1996). Understanding the mechanistic basis for these symptoms continues to be an active area of research that is of considerable interest for the design of therapeutic interventions.

Though PRV does not infect humans, because it is closely related to both HSV and VZV and can easily be studied in culture and in animal models, it continues to be utilized for elucidating pathways directly involved in the manifestation of clinical symptoms. Laboratory mice infected with virulent PRV exhibit the characteristic “mad itch” syndrome, or pruritis, in which they will scratch themselves with increasing frequency during the course of disease progression, to the point of self-mutilation. In addition, studies with PRV are greatly aided by the availability of an attenuated vaccine strain, PRV Bartha, and mice infected with this strain do not display any signs of peripheral neuropathy. Since the mutations of PRV Bartha have now
been fully mapped (Szpara, Tafuri et al. 2011), this strain provides a powerful tool for genetic studies aimed at linking mutations to overt behaviors.

The overall aim of this thesis has been to develop an in vivo model for the direct visualization of PRV infection, and then monitor the effects of wild type or attenuated PRV strains on the physiology of neurons. The model developed and characterized here was the salivary circuit, where infection is initiated at the salivary glands, and the peripheral parasympathetic submandibular ganglia (SMG) are imaged. The glands also receive motor sympathetic efferent input from the superior cervical ganglia, as well as somatic sensory afferent innervations from the trigeminal ganglia (Chibuzo and Cummings 1980). The afferent supply is considered to be of the same origin as the trigeminal sensory innervations of the oral and nasal mucosa, and therefore this model is also relevant to disease models of HSV-1, which naturally infects the trigeminal ganglia. We observed that mice infected with PRV to the salivary glands display the characteristic pruritis, and therefore this system recapitulates several aspects of the flank scarification model, with the primary advantage of easy manipulation and direct access to infected peripheral ganglia for imaging. Whereas neurons of the dorsal root ganglia are pseudo-unipolar and lack synapses, the SMG receive synaptic input from the brainstem, and accordingly this system can also be utilized for studies aimed at understanding transsynaptic spread. The time course of infection through the circuit and the mechanism of viral uptake were characterized.

**Model for PRV Infection in the Salivary Circuit**

When we looked at peripheral ganglionic neurons infected with a virulent PRV expressing the calcium sensor GCaMP3, we observed that infected neurons exhibited spontaneous synchronous calcium flashes. This phenotype persisted even when the presynaptic
Figure 6.1. Schematic of how PRV infects the salivary circuit. The simplified diagram above shows the basic organization of the glands, SMG nerves, and pre-ganglionic axons which lead to the brainstem. Window (A) is a magnified schematic panel of the SMG nerve during PRV infection, and we hypothesize that electrical coupling is occurring between adjacent unmyelinated axons (red lines). We observe bright foci in the axon bundles (red ovals), and these could either sites of microfusion, varicosities, or infected Schwann cells. Window (B) illustrates that SMG cell bodies are ensheathed by satellite glial cells (purple), forming individual functional units, and this organization may limit spread of infection in the ganglion between neuronal soma (PRV is represented by red circles).
During virulent PRV infection:

Figure 6.1
trunk was completely severed. Infections with attenuated PRV strains expressing either GCaMP2 or GCaMP3 revealed that the duration and complexity of calcium transients were exaggerated at increasing times post infections, yet as opposed to the reported effects of wild type PRV, PRV Bartha-infected neurons were never seen to flash synchronously. We quantitatively analyzed these results to show that the calcium transients were positively correlated between neurons infected with a virulent PRV but not with an attenuated PRV strain. Ex vivo imaging of infected tissue showed that the majority of newly made particles during wild type PRV infection of peripheral salivary ganglia are invested in anterograde transport back to the initial site of infection at the glands, and many intensely bright foci are detected in the infected axon bundles.

Based on the in vivo and ex vivo data presented in this thesis, and in the context of the supporting literature, we have developed a model for how PRV may alter neuronal physiology in vivo that could account for the known symptomology. We hypothesize that the virulent PRV Becker strain may induce electrical coupling events primarily in the SMG nerve of unmyelinated axons that project to the glands, and these sites of microfusion may be occurring at varicosities (Figure 6.1A). The SMG cell bodies are enveloped by satellite glial cells that may restrict direct spread between neuronal soma (Figure 6.1B). In contrast, the attenuated PRV Bartha cannot spread anterograde and therefore will not be able to induce electrical coupling in the unmyelinated axons but will continue instead to spread retrogradely to neurons in the CNS.

There is circumstantial evidence in the literature to suggest that virulent alphaherpesviruses may spread laterally in peripheral sensory ganglia (see (Smith 2012) for review) (Figure 6.2). In the mouse flank model, herpes simplex virus and PRV inoculations at a single site result in spread throughout the innervated dermatome, suggesting disseminated spread in the sensory dorsal root ganglia (DRG) (Weeks, Ramchandran et al. 2000; Brittle, Reynolds et al. 2004). Fusion of neurons and satellite cells has been demonstrated in human
Figure 6.2. Lateral spread in DRG. Circumstantial evidence suggests the possibility of alphaherpesvirus spread between sensory and visceral afferents. This simplified schematic represents a very basic overview of how sensory (blue) and visceral (green) afferents are co-resident in DRG, and how lateral spread could occur (double-headed arrow). Motor efferents (red) on the other hand send projections through the ventral root and would be infected through different pathways, including the initial site of infection.
DRG xenografts in mice infected with varicella zoster virus (Reichelt, Zerboni et al. 2008). In addition, virulent PRV infection of the mouse flank results in substantial viral titers recoverable in visceral organs, but not in an infection with attenuated PRV Bartha (Brittle, Reynolds et al. 2004); infection of sensory neurons that project to internal organs could be accounted for by lateral spread in the DRG since these visceral afferent neurons reside concomitantly in sensory ganglia (Smith 2012) (Figure 6.2). Our model could account for these observations.

Spontaneous Electrical Discharge

Since the clinical manifestations due to PRV infection in non-native hosts are complex and multifactorial, there are several avenues of research that may be worth investigating based on qualitative observations from the data presented in this thesis. The observation at single cell resolution that many infected neurons spontaneously elicited synchronous calcium transients deserves to be followed-up. The spontaneous generation of electrical activity in neurons infected with PRV has been substantially documented in situ and in vitro (for example, (Dempsher, Larrabee et al. 1955; Kiraly and Dolivo 1982; McCarthy, Tank et al. 2009)). In vitro characterizations of this phenomenon with PRV and HSV in dissociated neuronal cultures have suggested a mechanism involving fusion, since the phenotype is dependent on the fusion protein gB and the process is blocked by tetrodotoxin but not by cadmium, a calcium channel blocker (Mayer, James et al. 1985; McCarthy, Tank et al. 2009). Therefore the formations of viral-induced excitatory synaptic connections that allow for continuous propagation of action potentials do not reflect normal chemical transmission at conventional synapses, but rather an abnormal coupling that requires viral DNA replication, and can be strain dependent (Mayer, James et al. 1985; Mayer, James et al. 1986).
In non-disease models of peripheral neuropathies, spontaneous abnormal electrogensis and repetitive firing are known consequences of peripheral nerve insult, and result in tactile allodynia (Devor 2006). For example, axotomy has been shown to increase the excitability of DRG cells, which respond to the insult by firing spontaneously and synchronously, termed ectopic discharge; the spontaneous activity can arise at the axon terminals, midaxonally, or in the soma, and these effects have been associated with consequent pain (Kajander and Bennett 1992; Zhang, Donnelly et al. 1997; Liu, Wall et al. 2000). Infected nerves may respond to PRV invasion in a similar way to injury in relation to neuropathic pain, and therefore understanding the different models that exist to explain outbursts of pain and sensory spread may shed light on how PRV alters neuronal physiology. For example, a common form of PNS cross-excitation is known as ephaptic, or electrical, crosstalk, in which ionic current from one fiber will directly excite neighboring fibers if there is sufficient surface area of close membrane apposition between adjacent neurons in the absence of the normal glial insulation (Devor 2006). This coupling of fibers would result in instantaneous signal amplification, and data from this thesis and other publications (Mayer, James et al. 1986) suggests this type of mechanism is occurring during alphaherpesvirus infection. However, there is another very different type of non-ephaptic cross-excitation among injured neurons termed “crossed-afterdischarge” which appears more relevant to certain injuries of the DRG, in which the spread of excitation from active to passive neurons is due to chemical mediators such as K⁺ ions and neurotransmitters that are released into the extracellular space and then diffuse to access and excite all neighboring neurons; as concentrations of this signaling mediator increase, so does the discharge rate of non-stimulated neighbors, and the DRG then erupts into self-sustained firing (Devor 2006). Clearly this pathway deserves more consideration in the context of PRV infection.
Glial Cells and Immune Cells during Nerve Injury

A relatively unexplored territory is the possible involvement of satellite glial cells (SGCs) and Schwann cells in the symptomology of sensory neuropathic pain during PRV infection. In sensory and autonomic ganglia, each neuron is ensheathed by several SCGs, and the neuron with its surrounding SGC lamina is considered a distinct morphological and functional unit; these specialized glial cells contain various receptors and form a barrier around neurons to potential hazards (Hanani 2005). In the context of HSV infection, SGCs have typically been regarded as acting like barriers to lateral spread. In support of this, HSV-1 replication was shown to be restricted in clonally derived cultures of SGCs in vitro (Wilkinson, Leaver et al. 1999). Similarly, SCGs have been suggested to have a protective role, since during DRG infection of HSV, only SGCs became apoptotic, whereas glial and neuronal cells in the spinal cord were apoptotic (Ozaki, Sugiura et al. 1997). In addition, there is evidence that reactivation of HSV from latency in trigeminal ganglia using UV irradiation may modulate cytokine production by SCGs, with a notable increase in TNF-α and IL-6, suggesting their involvement in viral clearance (Shimeld, Easty et al. 1999).

However, SGCs have other roles besides simply protective and supportive, and in a non-disease models, SGCs have been demonstrated to participate in neuropathic pain states. Following axotomy or inflammation, SGCs proliferate and are highly plastic (Hanani 2005). In the DRG, it was shown that following sciatic nerve neuritis, new gap junctions were formed between SGCs of different neurons, which were completely absent in controls, and this was suggested to contribute to chronic pain (Ledda, Blum et al. 2009). Likewise, Schwann cells, which normally provide trophic support to peripheral axons, also undergo phenotypic modulation after injury and are key factors in Wallerian degeneration; they release pro-inflammatory cytokines that sensitize nociceptors, but also produce factors to counterbalance (Campana 2007). In infection
of the SMG, we did observe infection of SGCs surrounding the neurons, though whether this was productive infection was not explored, nor did we investigate the potential importance or contribution of these satellite cells to the observed phenotypes. Recent data from the Enquist lab has also demonstrated a link between nerve injury signaling pathways and PRV initial invasion (Koynuku et al, in press). This promises to be an exciting avenue for further exploration.

When looking at viral infections in vivo versus in vitro, one obvious added complexity that often confounds data interpretation is the involvement of the host immune system. This is a very broad subject matter that is dealt with in depth in other reviews and textbooks; nonetheless, for the purpose of putting the described spontaneous activity and peripheral neuropathy during viral infection or axotomy into a larger context, one topic that deserves more attention is the contribution of immune cells to pathological pain states. Immune cells are capable of direct attack to peripheral nerves, and direct assault, or even activation of the immune system near peripheral nerves, is sufficient to create increases in peripheral nerve hyperexcitability and/or damage, which is considered a significant contributor to neuropathic pain; the proinflammatory cytokines appear to be particularly important in creating this pain state (Watkins and Maier 2002). Microarray studies of host gene expression after PRV infection of the CNS found significant increase in nitric oxide synthase (NOS) (Paulus, Sollars et al. 2006), consistent with previous histology observations (Serrano, Enquist et al. 2002; Marcaccini, Lopez-Pena et al. 2007), and these could be involved in enhanced pain transmitter release in sensory neurons (Watkins and Maier 2002). In addition, the finding that cyclooxygenase (COX) is required for production of infectious PRV (Ray, Bisher et al. 2004) is interesting because COX-2 is involved in pain production (for example (Miyamoto, Saura et al. 2000)), and therefore this is another potential link that can be further investigated in relating PRV to the body of literature dealing with pain pathways and abnormal sensory experiences.
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Pseudorabies (Aujeszky's disease) and its eradication a review of the U.S. experience,


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References


References


APPENDIX

List of Publications and Scientific Conference Presentations of Data from this Dissertation

Publications


External Poster Presentation