Neuronal Survival Following Rna Virus Infection Facilitates Viral Persistence, Reactivation, And Pathogenesis

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Neuronal Survival Following Rna Virus Infection Facilitates Viral Persistence, Reactivation, And Pathogenesis

Abstract
Many RNA viruses, following entry into the central nervous system (CNS), remain detectable within the brain long after acute infection has resolved, typically in the absence of clinical signs of CNS disease. In many of these infections, (including measles virus (MV), West Nile virus, sindbis virus, rabies virus, and influenza virus), detection of viral protein or RNAs within the CNS long after the acute phase did not correlate with recovery of infectious virus in infected animals. This led many to consider these residual RNAs as inert viral remnants in neurons that had survived both viral infection and the antiviral host response. In this dissertation, I demonstrate that long-term viral persistence following infection with a neurotropic RNA virus can be reactivated, and that such recrudescence leads to a novel neuropathogenic outcome. In these studies, I characterized the persistence of MV in neurons of the CNS in immunocompetent mice and demonstrate that reactivation of viral transcription and protein synthesis is associated with a loss of adaptive immunity and the onset of severe CNS disease and motor dysfunction in mice that had presumably cleared the acute infection. The cerebellum/brain stem is the primary site of long-term maintenance of viral RNA, and loss of cell mediated viral control induces gait and motor problems consistent with cerebellar ataxia. Finally, using primary neuronal cultures I characterized the role of BST2 during neuronal viral infection and demonstrate, opposite to its well-characterized contributions in restricting viral particle release, that neuronal BST2 promotes viral spread. Together, these results show that persistent viral RNAs in the CNS are not inert, but can result in pathogenic host consequences distinct from those seen during an acute viral infection. Further, my findings underscore the cell-type specific differences of viral infection on ISG functions and immunity, highlighting the ramifications of such viral control mechanisms and their ability to promote the maintenance of viruses in the CNS that can ultimately lead to host pathogenesis.

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NEURONAL SURVIVAL FOLLOWING RNA VIRUS INFECTION FACILITATES VIRAL
PERSISTENCE, REACTIVATION, AND PATHOGENESIS

Katelyn Dyan Miller

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DEDICATION

I dedicate this Ph. D. dissertation to my family, friends, and mentors, without your support I would not be here today.

“The darkest places in hell are reserved for those who maintain their neutrality in times of moral crisis.” ~Dante Alighieri
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ABSTRACT

NEURONAL SURVIVAL FOLLOWING RNA VIRUS INFECTION FACILITATES VIRAL PERSISTENCE, REACTIVATION, AND PATHOGENESIS

Katelyn D. Miller
Glenn F. Rall, Ph.D.

Many RNA viruses, following entry into the central nervous system (CNS), remain detectable within the brain long after acute infection has resolved, typically in the absence of clinical signs of CNS disease. In many of these infections, (including measles virus (MV), West Nile virus, sindbis virus, rabies virus, and influenza virus), detection of viral protein or RNAs within the CNS long after the acute phase did not correlate with recovery of infectious virus in infected animals. This led many to consider these residual RNAs as inert viral remnants in neurons that had survived both viral infection and the antiviral host response. In this dissertation, I demonstrate that long-term viral persistence following infection with a neurotropic RNA virus can be reactivated, and that such recrudescence leads to a novel neuropathogenic outcome. In these studies, I characterized the persistence of MV in neurons of the CNS in immunocompetent mice and demonstrate that reactivation of viral transcription and protein synthesis is associated with a loss of adaptive immunity and the onset of severe CNS disease and motor dysfunction in mice that had presumably cleared the acute infection. The cerebellum/brain stem is the primary site of long-term maintenance of viral RNA, and loss of cell mediated viral control induces gait and motor problems consistent with cerebellar ataxia. Finally, using primary neuronal cultures I characterized the role of BST2 during neuronal viral infection and demonstrate, opposite to its well-characterized
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CHAPTER 1: Keeping it in check: chronic viral infection and antiviral immunity in the brain

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I. Preface

It is becoming clear that the manner by which the immune response resolves or contains infection by a pathogen varies based on the affected tissue. Unlike many peripheral cell types, central nervous system (CNS) neurons are generally non-renewable. Thus, cytolytic and inflammatory strategies that are effective in controlling infections in the periphery could be damaging if deployed in the CNS. Perhaps for this reason, the immune response to some CNS viral infections favors maintenance of neuronal integrity and non-neurolytic viral control. This modified immune response — when combined with the unique anatomy and physiology of the CNS — provides an ideal environment for the maintenance of viral genomes, including those of RNA viruses. It is therefore possible that such viruses can reactivate long after initial viral exposure, contributing to CNS disease. My thesis work uses a mouse model of neuroviral infection to provide mechanistic insights into the maintenance of RNA viral genomes within the CNS, and how reactivation of viral replication can result in novel diseases long after the primary infection.
II. Introduction

An oversimplification that is promoted in much of the scientific literature is that extracellular, receptor-binding ligands — including viruses, cytokines, and interferons (IFNs) — transduce invariant signaling pathways, independent of the activated cell type. Such generalizations limit our ability to fully appreciate the complexity and diversity of the cellular response to pathogens and potent pathogen-fighting proteins. There are also clinical ramifications of this myopic view: for example, ignoring the possibility that a particular cell population may behave uniquely upon cytokine encounter could limit drug efficacy or hinder the development of therapeutics. Here, I discuss some recently defined neuron-specific immune responses that broaden our view of how CNS infections, especially those caused by RNA viruses, are controlled.

Intuitively, the notion that neurons differ immunologically from other cell types makes sense: we cannot tolerate the loss of these generally nonrenewable cells as we can the lysis of more easily replaced epithelial cells. For example, herpes simplex virus (HSV) infection of epithelial cells results in massive immune- and virus-mediated cell death (Paludan et al. 2011; Jones et al. 2003; Braaten et al. 2005); however, lost cells are readily replaced, as observed in the healing that follows a cold sore. If lysis of irreplaceable neurons occurred in the same manner, neural circuits could become compromised, and, depending on the magnitude of damage, could permanently impair the host. Thus, the immune response to a viral challenge must be tailored to promote survival of infected neurons while destroying infected epithelial or endothelial cells. However, such neuronal sparing might result in long-term consequences that are spatially or temporally separated from acute infection.
In this introductory chapter, I will integrate insights from the fields of virology, neurobiology and immunology to provide an overview of how both RNA viruses and DNA viruses access the restricted environment of the CNS, and how the host contends with such infections. I will particularly focus on a developing literature that elucidates cell-specific immunity and the consequences of non-lytic viral clearance within the brain. These unique attributes of immune-mediated viral control in the brain may set the stage for persistence of viruses in neurons, rather than sterile clearance, the focus of my research. Thus, this chapter then focuses on unique observations gained from a model of measles virus infection of the CNS, which are particularly pertinent to the studies presented throughout my dissertation. I conclude with a forward-looking hypothesis: that non-lytic clearance of neuronal infections may allow for persistence of RNA viruses that induce pathogenesis long after primary exposure.

III. Viral Entry and Spread into the CNS

Viral entry

The brain is shielded from external threats at both macro- and microscopic levels: it is encased in bone to prevent physical injury and separated from peripheral tissues and blood via highly specialized barriers. Although such characteristics may limit infections of CNS-resident cells, these barriers can be breached. Three major routes of viral entry into the brain have been identified: direct infection of the cells that comprise the blood–brain barrier (BBB) and blood–cerebrospinal fluid (BCSF) barrier (with consequent release of viral particles into the parenchyma), infection of cells that are able
to cross these barriers, and trans-neuronal migration across synapses from the peripheral nervous system (PNS) into the CNS (Figure 1.1 and 1.2).

Within the CNS, the BBB and BCSF barriers restrict the migration and diffusion of cells, pathogens, antibodies, and macromolecules into the brain parenchyma. Neurotropic RNA viruses, including poliovirus (PV), measles virus (MV), and some flaviviruses, can circumvent these barriers by directly infecting the tightly associated endothelial or epithelial cells that comprise them (Koyuncu et al. 2013). Viral particles can then be released from the basolateral membrane into the parenchyma. For example, following MV infection of human brain microvascular endothelial cells (HBMEC), release of viral particles occurs from both the apical and basolateral HBMEC membranes, without disrupting cell polarity or barrier integrity, allowing MV to spread into the parenchyma (Dittmar et al. 2008). Alternatively, barrier integrity may be compromised when the tight junctions between these cells loosen due to inflammation and cytokine exposure, allowing free viral particles to diffuse directly from the blood or CSF into the brain. For example, peripheral West Nile virus (WNV) infection acts through the engagement of Toll-like receptor 3 (TLR3) to induce the synthesis of cytokines — including tumor necrosis factor-alpha (TNFα) — by circulating antigen presenting cells (T. Wang et al. 2004). In turn, TNFα reduces BBB integrity by loosening tight junctions (Libbey & Fujinami 2014), allowing for WNV migration through the less-restrictive BBB. In reality, immune modulation of barrier integrity is not as simple as this description implies. The balance of different cytokines can determine the extent to which the BBB is perturbed or stabilized. For example, IFNs, which are also produced in infected hosts, help to keep the barrier intact (Daniels et al. 2014); thus, the relative type and ratios of cytokines synthesized in response to various infections will differentially affect barrier integrity (Daniels & Klein 2015).
Viruses may also passively access resident CNS cells by infecting lymphocytes or monocytes that can be transported across a cellular barrier. This strategy is often referred to as the “Trojan horse” approach, because viral particles are released once the leukocyte gains access to the parenchyma. A classic example of this mode of invasion is human immunodeficiency virus type 1 (HIV-1), in which CD16+ monocytes, permissive for HIV-1, traffic across the BBB and release virions that can then infect CNS resident microglia (McGavern & Kang 2011; Kramer-Hämmerle et al. 2005).

A third mode of CNS entry is trans-neuronal migration, a strategy adopted by pseudorabies virus (PRV), rabies virus (RV), and many herpesviruses. Intracellular trafficking in PNS neurons, which is necessary to shuttle cellular components to and from the synapse, can be commandeered to facilitate viral travel within and among synaptically-connected neurons. The best-characterized examples of this type of spread are members of the herpesviruses, including herpes simplex virus type 1 (HSV-1), and the closely related PRV (Koyuncu et al. 2013; Kramer & Enquist 2013). Following infection of epithelial cells in the oral mucosa, HSV-1 spreads to sensory and autonomic ganglia, establishing lifelong latency. Reactivation of virus from latency — due to decreases in immune monitoring, other infections, or stress — leads to an active infection in PNS neurons, in which viral membrane proteins (including US9, glycoprotein E (gE) and glycoprotein I (gI)) can direct movement of newly replicated viral particles from neurons to the epithelium, establishing a new lytic infection (Howard et al. 2012). During transport, viral components are shuttled along axons via microtubule tracks and in association with their dynein and kinesin motors (Kramer et al. 2012; Zaichick et al. 2013). Beyond the value of these studies to understand how neurotropic viruses are propagated, viruses which spread across synapses (which also include RV and MV) have provided a valuable method to trace neural circuits in vivo (Granstedt et al. 2013;
Hagendorf & Conzelmann 2015) using recombinant viruses encoding fluorescent proteins. These unique virological tools may also inform the development of strategies to deliver therapeutic payloads from the periphery to the CNS.

**Figure 1.1:** Viral entry into the CNS. Three modes of viral entry into the brain are shown.

Viruses may directly infect the cells comprising the blood–brain barrier (BBB), followed by release into the parenchymal space (left panel). Alternatively, viruses may diffuse across permeable regions of the BBB (middle panel). Of note, BBB permeability can be influenced by cytokines, such as tumor necrosis factor (TNF) and various interferons (IFNβ, IFNγ and IFNλ), which can loosen or reinforce the barrier integrity. In the ‘Trojan horse’ approach (right panel), infected lymphocytes or monocytes (including macrophages) traffic across the BBB or blood–cerebrospinal fluid barrier, releasing virus once in the brain parenchyma. Miller KD, Schnell MJ, Rall GF. Keeping it in check: chronic viral infection and antiviral immunity in the brain. Nature Reviews Neuroscience. November 2016:1-13.
Trans-synaptic spread of viral particles involves the transport of viral genomes and associated proteins via microtubules and molecular motors. The left panel shows the movement of rabies virus (RV) from the muscle, across the neuromuscular junction, and the dynein-mediated retrograde transport of this virus into the CNS. In the right panel, the transport of viruses (including herpes simplex virus (HSV), varicella zoster virus (VZV) and pseudorabies virus (PRV)) occurs across the epithelial or endothelial–neuron junction. In these neurons, retrograde transport brings the virus to the neuronal soma, and anterograde transport delivers the virus to the peripheral nervous system (PNS)–CNS synaptic junction. IFNAR, IFN α/β receptor; IFNGR, IFNγ receptor; HIV-1, human immunodeficiency virus type 1; MV, measles virus; PV, poliovirus; TNFR, TNF receptor; WNV, West Nile virus. Miller KD, Schnell MJ, Rall GF. Keeping it in check: chronic viral infection and antiviral immunity in the brain. Nature Reviews Neuroscience. November 2016:1-13.
Viral spread

Once a virus has infected a neuron, there are two primary modes of subsequent spread to other cells: release of infectious viral particles that can infect distant permissive cells, or transfer of viral nucleic acid, subviral particles, or infectious virions between an infected and uninfected cell that are in direct contact. The former mechanism requires release of viral particles through the neuronal membrane (chiefly via budding out of the infected cell), whereas the latter is primarily dependent on viral proteins that mimic or co-opt cellular processes to direct insertion of viral fusion proteins into a host cell membrane or to direct the spread of viral capsids, as seen with HSV (Kramer & Enquist 2013). Both modes of viral spread occur in neurons; however, in many instances, viral transfer to adjacent neurons happens in the absence of syncytia formation, and little or no extracellular infectious virus can be detected, suggesting that neurons facilitate a distinct mode of spread for many viruses (Koyuncu et al. 2013). Interestingly, trans-synaptic spread of MV within primary mouse hippocampal neurons occurs independently of known MV receptors, which are critical for syncytia formation in non-neuronal cells (Lawrence et al. 2000; Makhortova et al. 2007). The paucity of viral particles in the extracellular space may protect the neuron from plasma membrane damage via budding, and facilitate viral evasion of antibody detection. Although many neurotropic infections spread by direct contact at the pre- and post-synaptic junction, alternative modes of transport may also be used (Chapter 3 and Appendix). For example, although RV primarily spreads trans-synaptically in a retrograde manner, an electron microscopy study showed the presence of viral particles in the extracellular neuronal space, accompanied by direct neuronal budding (Iwasaki et al. 1975).
Syncytia formation and trans-synaptic spread

Viruses gain entry into permissive cells through an interaction between virally-encoded glycoproteins, expressed on the outer surface of the virus particle, and cellular receptors. Entry can be achieved through endocytosis into vesicles or via membrane fusion (Zhong et al. 2013). For fusogenic viruses, exit from the cell occurs either through the budding of virus particles through the plasma membrane or by fusion of an infected cell with an adjacent, uninfected cell (Zhong et al. 2013). The latter process results in the formation of multinucleated cells, or syncytia. The formation of syncytia may support further viral production but irrevocably leads to the death of these fused cells. Similarly, release of infectious particles by budding often leads to infected cell death (Watanabe et al. 2015).

However, viruses that are considered cytopathic in renewable cell types — including MV, RV and PRV — can switch to a non-productive, non-syncytia forming mode of spread when infecting neurons, promoting neuronal survival (Lawrence et al. 2000; Makhortova et al. 2007; Kramer & Enquist 2013; Taylor et al. 2012; Lancaster & Pfeiffer 2010). Often, this is correlated with the absence of detectable extracellular viral particles. Spread of these viruses within neurons is primarily trans-synaptic, although the neuronal processes that enable a switch from viral budding and syncytia formation to non-cytolytic, trans-synaptic spread are not yet defined.

At least two possibilities might explain viral movement across the synapse. In one scenario, spread of viral particles between neurons requires ligand-receptor interactions, similar to infection in non-neuronal cells. Directed transport to the synapse and focal fusion at the synaptic cleft might be required for a virus to migrate across the synapse: thus, the process that occurs in non-neuronal cells might also be operative in
neurons. Trans-synaptic spread might require the same cellular and viral proteins that allow for fusion of non-neuronal cells, or may be unique to the pre-post synaptic interface (Appendix). For example, in MV neuronal infection, expression of the primary receptors that are utilized in non-neuronal cell infection are not required; however, a fusion event still is critical for spread to occur, perhaps by forming a “pore” through which the viral ribonucleic acid is transported (Sattentau 2008).

Alternatively, the close approximation between the pre- and post-synaptic membrane, coupled with the unique attributes of the synaptic junction, may allow for the passive transport of viruses that have trafficked or assembled there (discussed in more detail in the appendix to this dissertation). The release of neurotransmitters and uptake of their receptors make the synaptic interface particularly fluid, which may make it uniquely able to support receptor-independent trafficking.

Defining long-lasting neuronal infections

One outcome of viral neuroinvasion is that the viral genome, proteins, and/or complete virus particles may remain in the brain long after initial exposure. To describe the myriad ways by which viruses establish enduring interactions with host neurons, numerous descriptors have been employed, including “prolonged”, “persistent”, “latent”, “smoldering”, “quiescent” and “chronic” (Wherry & Ahmed 2004; Oldstone 2009; Koyuncu et al. 2013); however, their use is not consistent. Variables including detection threshold, target organs, and cell-specific influences on the viral life cycle collectively contribute to the challenge of establishing an agreed-upon nomenclature. Moreover, some viruses can reactivate to cause the same disease as the acute infection (such as herpes simplex), while others manifest differently upon reactivation (such as varicella
zoster virus (VZV), which causes chicken pox as a primary infection, but typically causes shingles upon reactivation). Others result in pathogenesis only after protracted infection (such as tumor-causing viruses).

To provide some clarity to this ambiguous list of descriptors, I propose three classifications. **Latent infections** are defined as those in which the virus establishes a non-lytic state during which host-to-host transmission is not possible unless the virus reactivates to produce infectious virions. **Chronic transmissible infections** are characterized by the continuous production of infectious progeny, and their ability to be transferred to new hosts. **Chronic non-transmissible infections** are those in which consistent detection of viral nucleic acid over extended periods of time is observed, but in which transmission to new hosts does not occur.

Latency is most frequently attributed to herpesvirus infections, such as HSV-1, HSV-2 and VZV. After initial infection of epithelial cells, these viruses become non-lytic within PNS neurons and viral nucleic acid is maintained in a heterochrominated episomal state with negligible transcription (Kramer & Enquist 2013). A small number of viral transcripts are synthesized during latency and are termed latency-associated transcripts (LATs). These RNA species do not encode functional proteins, but are thought to prevent neuronal apoptosis and to disrupt both innate and adaptive immune signaling, through mechanisms that include inhibition of caspase activity and granzyme B-mediated killing (Kinchington et al. 2012; Jiang et al. 2011). VZV also produces various proteins, including ORF63, that prevent neuronal apoptosis (Kinchington et al. 2012). The term “latent” accurately conveys the status of these viruses: hidden, incapable of transmission, but able to fully reactivate, spread, and be transmitted to a new host. Another type of latency, which is not typically seen in neurons, occurs after
viral nucleic acid is reverse transcribed from RNA to DNA and then integrated into the host genome. This process is unique to the retroviruses, such as HIV-1 (Mohammadi et al. 2015). In this type of latency, integrated viral genomic DNA becomes indistinguishable from host DNA, and viral genes can be epigenetically silenced or activated throughout the cell’s lifetime and passed on to daughter cells.

In a chronic transmissible infection, infectious virus can be continuously recovered from the host and can be disseminated to new hosts, as in hepatitis B and C. A well-characterized mouse model of a chronic transmissible CNS infection is lymphocytic choriomeningitis virus (LCMV). LCMV infection of newborn mice leads to a noncytopathic chronic infection in almost every tissue. Infectious LCMV particles can be recovered from multiple organs throughout life and can be shed in the feces or transmitted vertically to offspring (Oldstone 2009; Oldstone 2006; Traub 1936). Although most strains of mice survive LCMV infection with no overt pathogenic consequences, some studies reported learning and memory deficits in these chronically infected animals (Brot et al. 1997), underscoring the potentially subtle effects of long-term infection on CNS function.

Chronic non-transmissible infections are also characterized by sustained viral replication or consistent detection of viral nucleic acid over extended periods of time, but in the absence of further host dissemination. One example may be rare cases of CNS infection with measles. Acute infection can, in some instances, lead to the development of neuropathogenic diseases, including subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE). These uncommon neurological diseases often present months to years after viral exposure, and are characterized either by negligible viral replication or persistence of replication-competent nucleic acid in the
CNS (Norrby & Kristensson 1997; Gutierrez et al. 2010; Griffin 2014). In both SSPE and MIBE, no viral dissemination to uninfected hosts has been reported. Determining whether the state of the virus that causes these sequelae is “latent” or “chronic non-transmissible” is difficult, due both to the small number of clinical specimens available, and a lack of small animal models that mimic SSPE disease (Griffin et al. 2012; Hayashi et al. 2002; Gutierrez et al. 2010). In humans, it may be that neurological symptoms appear only once viral replication reaches a critical threshold or that the virus has infected a key site within the brain, exceeding the host’s capacity to control the infection. Alternatively, non-replicating MV genomes may be maintained for prolonged periods, to be reactivated later. Either way, the MV genome remains intact, in some form, long after control of the acute infection is achieved, in the absence of further viral dissemination.

A final point of clarification: not all neurotropic infections lead to long-lasting associations. Some, such as reovirus, can induce neuronal apoptosis via induction of pro-apoptotic proteins such as BAX (Clarke et al. 2009; Berens & Tyler 2011). Why some infections lead to neuronal suicide while others lead to a long (potentially unhappy) marriage is a major focus in the field of neurovirology: answering this question may lead to the discovery of virus-specific therapies to prevent or minimize infection-triggered neuropathology.
IV. Immune Clearance of Neuronal Infection

The various permutations of neurotropic viral infections pose unique challenges for the host, including how to detect antigens within the CNS, how to enable T lymphocytes to engage with neurons that express negligible levels of proteins typically present on target cells, and how to mitigate the risks of neuroinflammation and widespread loss of generally nonrenewable neurons.

Type I interferon signaling

The early response to an infection begins with engagement of pathogenic motifs by pattern recognition receptors (PRRs), which are expressed on (or in) virtually all cells. The binding of these receptors to conserved motifs, such as double-stranded RNA, lipopolysaccharides, or glycoproteins, propagates signals that culminate in the production of type I IFNs, chiefly IFNα and IFNβ. These IFNs are secreted from the infected cell, and act in both a paracrine and autocrine fashion by binding to the type I IFN receptor (IFNAR), a heterotetramer with phosphorylatable cytoplasmic domains. This engagement leads to the phosphorylation of tyrosine kinases (including Janus-associated kinases) and the receptor itself, and is followed by tyrosine phosphorylation of cytoplasmic signal transducer and activator of transcription 1 (STAT1) and STAT2, which are usually abundant but inactive within the cytoplasm. Activated STAT1–STAT2 heterodimers couple with interferon regulatory factor 9 (IRF9) to form the complex ISGF3, which translocates to the nucleus to bind IFN stimulated response elements (ISRE) within the promoters of IFN stimulated genes (ISGs). These genes encode proteins that eliminate infected cells or aid in viral clearance. Type I IFN also binds to
adjacent, uninfected cells to shield them from infection. Although this pathway is operative in many cells, alternative IFN-triggered pathways that limit viral spread, but do not depend on induction of the “usual suspect” ISGs can be induced in some cell types, including neurons (Goodbourn et al. 2000).

Neurons also secrete type I IFNs, which can act in an autocrine or paracrine manner on neurons or neighboring parenchymal cell types (Delhaye et al. 2006). RV, which infects muscle cells and peripheral neurons following a bite from an infected animal, induces copious IFN early after infection in vivo and in vitro (Faul et al. 2010). By contrast, IFN-induced STAT phosphorylation in primary hippocampal neurons is delayed, with maximal activation occurring only after ~24 hours (Rose et al. 2007; Cavanaugh et al. 2015). Delayed STAT activation coincides with delayed expression of traditional ISGs (Cavanaugh et al. 2015). The protracted interval between receptor binding and STAT activation may be due to a greatly reduced basal expression of STAT in these hippocampal neurons, as compared to other cell types (Rose et al. 2007; Podolsky et al. 2012; Cavanaugh et al. 2015). Interestingly, lower homeostatic STAT expression is not unique to neurons, but has also been observed in another non-renewable cell type, cardiac myocytes (Zurney et al. 2007). Like neurons, cardiac myocytes have high basal IFNβ expression, which may protect them from infection (Cavanaugh et al. 2015; Zurney et al. 2007). Perhaps the disparity between expression of IFNs and the signal transduction molecules that they induce may skew towards protection from infection, rather than induction of a potentially cytotoxic response. Surprisingly, synthesis of ISGs can differ within a single neuron: IFN-β induces a noncanonical, local antiviral response in axons that is not observed in the neuronal soma (Rosato & Leib 2015; Song et al. 2016). The startling implication of this work is that
neurons, especially those with long processes as in the PNS, may “compartmentalize” the response to extracellular immune mediators.

Although much of this introductory chapter focuses on neuronal responses to infections and antiviral cytokines, it is important to underscore that differential responses to, and production of, type I IFN have been demonstrated in other parenchymal cell populations as well, and may influence the neuronal response. For example, when comparing microglia and oligodendroglia collected after infection with a neurotropic strain of mouse hepatitis virus (MHV), it was shown that microglia are better producers of type I IFN and downstream interferon-stimulated gene products (Kapil et al. 2012). Further studies using MHV have shown that viral tropism is determined after viral entry events and dependent on downstream type I IFN responses (Zhao et al. 2011, Zhao et al. 2013). Overall, the fact that different cell types show distinct homeostatic expression of key signal transducers and their downstream gene targets underscores the cellular diversity that can follow cytokine engagement.

Perhaps predictably, for many neurotropic RNA viruses, including MV, Theiler’s murine encephalomyelitis virus (TMEV), Murray Valley encephalitis virus (MVEV), WNV, and others, experimentally-induced loss of type I interferon signaling results in pathogenesis, altered viral tropism (generally enhanced neurovirulence), and an inability to control viral spread both in vivo and in vitro (Paul et al. 2007; Holmgren, Miller, Cavanaugh & Rall 2015a; Ireland et al. 2007; Cavanaugh et al. 2015; Weber et al. 2014; Fensterl et al. 2012; Samuel & Diamond 2005; Lobigs 2003; Nayak et al. 2013). Although most of these studies were performed using type I IFN receptor knockout mice (IFNAR KO) lacking receptor expression on all cells, selective disruption of neuronal IFN signaling (using neuron-specific knockouts of IFNAR) also results in death following VSV
infection (Detje et al. 2009). Moreover, infection of olfactory neurons and mucosa with either a neurotropic RNA virus (VSV) or a neurotropic DNA virus (cytomegalovirus) leads to a robust type I IFN response deep within the brain, preventing viral spread and attendant disease (van den Pol et al. 2014). Thus, infection of cells in direct contact with the environment (including sensory olfactory neurons) can trigger a long-distance warning (production of type I IFN) that ultimately limits or precludes viral spread to remote regions of the brain.

Antigen presentation and generation of CNS immunity

For some time, it was known that the primary cell populations of the adaptive immune system, T cells and B cells, contributed to viral control within the brain; however, the apparent absence of a CNS lymphatic drainage system left it unclear how antigens could exit the parenchyma to promote the activation and proliferation of naïve antigen specific T cells (Libbey & Fujinami 2014). Recent findings have begun to resolve this mystery: these include the identification of lymphatic drainage portals from the CNS into deep cervical lymph nodes and the presence of a fluid gradient that flushes the brain of extracellular proteins (termed “glymphatics” because of the crucial role of glia in this process) (Aspelund et al. 2015; Jessen et al. 2015; Louveau et al. 2015). CSF moves toward the perivascular space, where it is transported into the dense brain parenchyma via aquaporin 4 water channels expressed on cortical astrocytes. The CSF movement drives interstitial fluid (ISF) toward perivenous spaces, where it then drains toward the newly identified meningeal or dura matter lymphatic vessels, and ultimately to the deep cervical lymph nodes, where T cell activation and proliferation can occur (Aspelund et al. 2015; Louveau et al. 2015; Iliff et al. 2012). These studies illuminate how antigens and
Professional antigen presenting cells can exit the CNS to alert naive T cells in the lymph nodes.

**T cell mediated pathogen clearance**

After T cells mature in lymphoid tissues, they enter the bloodstream where they can interact with adhesion molecules expressed on the surface of blood vessel endothelia within infected tissues. Mature T cells chiefly engage with selectins (and later, integrins) on the surface of the BBB or BCSF barrier. The expression of these adhesion molecules is induced by chemokines produced within the parenchyma by infected neurons and adjacent glia. This results in migration of T cells across the barrier (diapedesis). Although it was previously believed that neurons do not express major histocompatibility complex (MHC) class I molecules (and thus could not be recognized, at least in the canonical manner, by CD8⁺ T cells), we now know that some neuronal populations constitutively synthesize these cell surface proteins and that others can induce them following injury or infection (Neumann et al. 1995; Neumann et al. 1997; Cebrián et al. 2014). Even so, most neurons do not express typical levels of class I MHC antigens under noninflammatory conditions (Joly et al. 1991), and thus T cell effector functions, including cytokine production, may not be triggered by the infected cell (the neuron) directly but rather by adjacent class I MHC-expressing cells (usually glia) that can display antigenic peptides via cross-presentation (Calzascia et al. 2003). Although cross-presenting glia may not be directly infected, this strategy allows for elaboration of antiviral processes. Resident CNS cells may not only be invisible to immune cells due to reduced expression of MHC recognition molecules, but may also express immunomodulatory molecules, such as programmed death 1 ligand 1 (PD-L1) (Jeon, St...
Leger, Cherpes, Sheridan & Hendricks 2013a), that downmodulate T effector function. Remarkably, the class I MHC expression system that is key to T cell recognition likely has other functions in neurons as well, including neurodevelopment and neuronal plasticity (Huh et al. 2000; Cebrián et al. 2014; Goddard et al. 2007).

One of the major strategies used by activated T cells to combat neuronal viral infections is the production of IFNγ. Similar to the type I IFNs, IFNγ transduces a signal via receptor binding, leading to STAT1 activation and homodimerization. Activated STAT1 homodimers translocate to the nucleus, bind to gamma activated sequences (GAS) in the promoters of approximately 100 genes (which overlap with, but are generally distinct from, the ISGs induced by type I IFNs), promoting their transcription and translation (Figure 1.3). These gene products, similar to ISG proteins, combat viral infection or induce apoptosis of the infected cell (Goodbourn et al. 2000).

STAT1 can be activated in neurons after IFNγ exposure, but the kinetics of induction are markedly slower than those observed in treated mouse embryonic fibroblasts (MEFs), similar to the delayed response seen following type I IFN exposure (Rose et al. 2007). In addition, IFNγ induces the transcription of both traditional genes (that is, those that are typically expressed in response to IFNγ in other cellular populations) and non-traditional genes in primary hippocampal neurons after exposure (O’Donnell et al. 2012). This diverse profile of changes in gene expression may affect the cellular outcome: although IFNγ can induce necroptosis, in neurons the virus is controlled in a non-cytolytic manner (presumably due to the paucity of STAT1 and non-traditional GAS gene induction) (Burdeinick-Kerr et al. 2009; Patterson, Lawrence, et al. 2002) (Figure 1.3). This is not unique to neurons: IFNγ is also essential for controlling MHV infection of oligodendrocytes via non-cytolytic pathways (Parra et al. 1999;
González et al. 2006). How are genes activated when basal levels of available STAT1 are low in resting neurons? Interestingly, the majority of STAT1 KO mice challenged with a neuron-restricted MV infection survive. By contrast, IFNγ KO mice all show severe signs of chronic disease, with approximately 50% succumbing to infection (O'Donnell et al. 2012; Patterson, Lawrence, et al. 2002), suggesting that the requirement for IFNγ is decoupled from the main transducer through which it signals. This observation led to the identification of an IFNγ-dependent, STAT1-independent activation of anti-viral and pro-survival genes (O'Donnell et al. 2012; O'Donnell et al. 2015), which might be facilitated by access of other signaling factors — including ERK1/2 and AKT — to the activated IFNγ receptor when STAT1 is absent or not abundant (Figure 1.3 B/C).
In cells with abundant levels of signal transducer and activator of transcription 1 (STAT1) signaling proteins, engagement of the interferon-γ (IFNγ) receptor (IFNGR) by its ligand transduces a primarily STAT1-driven cellular response, leading to activation of gene products that are chiefly antiviral (part a). By contrast, when a particular cell population (such as hippocampal neurons) expresses reduced homeostatic levels of STAT1 (part b) or when STAT1 is removed by genetic deletion (part c), alternative signaling molecules with an affinity to the IFNGR may bind to this receptor, transducing unique cellular responses. In the case of neurons, this includes activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), which then can result in the induction of genes encoding pro-survival proteins. JAK1, Janus kinase 1; KO, knockout; MEF, mouse embryonic fibroblast. Miller KD, Schnell MJ, Rall GF. Keeping it in check: chronic viral infection and antiviral immunity in the brain. Nature Reviews Neuroscience. November 2016:1-13.

**Figure 1.3:** The receptor-occupancy hypothesis.
IFNγ is critical for the control of multiple neurotropic viral infections in both mice and primary neuronal cultures. Recently, IFNγ was identified as a key suppressor of HSV and VZV reactivation in the trigeminal ganglion of both humans and mice (St Leger & Hendricks 2011; T. Liu et al. 2000; Theil et al. 2014; T. Liu et al. 2001; Kinchington et al. 2012). What makes these studies particularly intriguing is the type of T cell shown to be constitutively secreting IFNγ: T resident memory cells (T\(_{\text{rm}}\)) (Theil et al. 2014; St Leger & Hendricks 2011; T. Liu et al. 2000; T. Liu et al. 2001; T. Liu et al. 1996; Wakim et al. 2010). T\(_{\text{rm}}\) (defined by CD103 and CD69 expression) are in direct proximity to latently infected PNS neurons and do not reenter circulation. Furthermore, these brain-resident lymphocytes have a unique molecular signature that distinguishes them from other types of cytotoxic T cells or from memory T cells (Wakim et al. 2012; Wakim et al. 2008). T\(_{\text{rm}}\) populations expand and contract in their resident tissue, acting as a first line of defense against reinfection (Park & Kupper 2015). Moreover, as suggested below, these cells may be crucial sentinels that keep chronic neuronal infections at bay; their loss may therefore also contribute to viral reactivation.

In addition to cytokine secretion, some T lymphocytes kill infected cells through perforin and/or granzyme-mediated mechanisms. Perforins, found in the lytic granules of CD8\(^+\) cytotoxic T cells, effectively punch holes in the membrane of infected target cells, allowing for the delivery of granzymes leading to lysis of the infected cell. Granzymes are serine proteases that induce caspase cleavage and activation of pro-apoptotic cellular proteins, such as BID. This mode of T cell killing, which efficiently eliminates “viral factories” has been primarily studied in rapidly dividing cells. Interestingly, in some neuronal infections, the secretion of granzyme does not lead to lysis, but rather aids in preventing viral reactivation and replication, while sparing the infected neuron (Knickelbein et al. 2008). In addition to their ability to kill cells, granzymes can directly
cleave eukaryotic translation initiation factor 4 gamma 3 (eIF4G3; a cellular protein that is important for host and viral translation) and ICP4 (a herpesvirus-specific protein needed for transcription of early and late viral genes) (Marcet-Palacios et al. 2011; Knickelbein et al. 2008). By cleaving eIF4G3, granzymes thus block viral translation but fail to induce neuronal apoptosis, further preventing viral dissemination within the host and sparing the infected neuron. Cleavage of ICP4 by granzymes directly prevents reactivation of latent HSV from infected neurons. In these instances, granzymes are acting on proteins other than their traditional targets to induce an alternative neuronal response. It has also been speculated that virus-encoded RNAs and proteins can contribute to non-lytic outcomes. For instance, HSV LATs inhibit the action and expression of various caspase proteins, key mediators of the cell death process. Despite these fail-safes, bystander immune-mediated neuronal death may occur. For example, TMEV infection of mice results in hippocampal neuron death through a mechanism that is dependent on inflammatory monocyte infiltration and activation (Howe et al. 2012).

Humoral responses within the CNS

The notable absence of B cells in brains of virus-infected mice, coupled with non-cytolytic trans-synaptic viral spread, led to the misperception that B cells and the antibodies they secrete play minor roles in viral control. In fact, numerous human CNS infections, including those caused by MV, PV, VZV, HSV, and flaviviruses, among others, are characterized by the presence of intrathecal antibodies (Ab) in the CSF (Phares, Stohlman & Bergmann 2013a; Skoldenberg et al. 1981; S et al. 1985). Humoral responses appear to be associated with protective rather than pathogenic functions, as seen for Japanese Encephalitis virus and some neurotropic retroviruses (Phares,
Stohlman & Bergmann 2013a). Antibodies may be particularly beneficial for those infections that result in extracellular infectious virus production.

V. Neuronal Subtypes and Infection

A central theme up to now has been the notion that infected cells, such as neurons, respond to immune effectors in cell-specific ways. However, the existence of many sub-populations of neurons, segregated by location and function, raises the issue of whether responses may differ within these neuronal subsets. Recent studies have shown that cerebellar granule neurons and cortical neurons pretreated with type I IFN vary in their ability to control a WNV infection (Cho et al. 2013). Moreover, IFN treatment had a much greater impact on the spread of infection in cerebellar granule neurons than it did in cortical neurons (100 vs. 15 fold reduction) and this difference correlated with discrete patterns of ISG induction (Cho et al. 2013). Animal model studies have also shown differences in the propensity for a virus to infect individual neuronal subpopulations and regions of the brain (Figure 1.4); for example, the hippocampus is heavily infected by RABV, whereas MV is more often found in the midbrain (Jehmlich et al. 2013; Liebert et al. 1986; Backzo et al. 1988; Zerboni & Arvin 2015; Lucas et al. 2015; Gomme et al. 2012). Whether these distinctions can be attributed to differences in viral tropism or intrinsic variations in the neuronal response to soluble immune effector proteins (or perhaps, even, how the virus gains access to the brain) is unknown. Answering this question will require further studies that, necessarily, must integrate virology, immunology and neurobiology.
**Figure 1.4:** Tropism of neurotropic RNA viruses for distinct brain regions and neuronal subpopulations.

The schematics show a simplified sagittal view of the mouse brain with the regions known to be infected by various viruses indicated in red. The symbol ‘>’ indicates higher propensity for a virus to infect a certain cell type or region of the brain than another cell type or region. MHV, mouse hepatitis virus; MV, measles virus; RABV, rabies virus; WNV, West Nile virus. Miller KD, Schnell MJ, Rall GF. Keeping it in check: chronic viral infection and antiviral immunity in the brain. Nature Reviews Neuroscience. November 2016:1-13.
VI. Measles Virus Infection of the Central Nervous System

Introduction

Before the introduction of widespread vaccination programs, according to the World Health Organization (WHO), MV infection was responsible for approximately 2.6 million deaths each year before 1980. Despite the availability of a safe and relatively inexpensive vaccine, there were 134,200 MV-associated deaths in 2015, with rates of MV infection resurging due to decreases in the vaccinated public (Campbell 2016). The majority of these fatalities occurred as a result of viral immunosuppression, leading to opportunistic secondary infections and host death. However, CNS complications subsequent to MV infection, as seen with the human diseases SSPE and MIBE, can occur after uncomplicated MV infection. Perhaps predictably, outcomes of such sequelae are poor: SSPE is invariably fatal (Griffin et al. 2012; Gutierrez et al. 2010; Ludlow et al. 2014; Norrby & Kristensson 1997). SSPE can present months to years after a clinically typical MV infection, and is characterized by unrestricted MV replication in the CNS (Griffin et al. 2012; Gutierrez et al. 2010; Ludlow et al. 2014; Norrby & Kristensson 1997). Clear indicators predicting the development of SSPE have not yet been identified, but age at time of infection can increase susceptibility to SSPE; children infected with MV before 2 years of age have an increased likelihood of developing SSPE (Gutierrez et al. 2010).

MV is a negative sense single stranded RNA virus of the genus Morbillivirus within the family Paramyxoviridae. The timing to development of SSPE (in some cases, as long as a decade) is surprising, as RNA viruses are not thought to persist long-term in the CNS. The non-segmented MV genome encodes 8 proteins: 6 structural and 2 non-structural. The structural genes consist of the MV nucleoprotein (N), fusion (F),
hemagglutinin (H), matrix (M), phosphoprotein (P), and the RNA-dependent RNA polymerase (L; “large”). F, H, and M comprise the viral envelope, and P, L, and N associate with genomic RNA to form the ribonucleoprotein (RNP) (Figure 1.5). The non-structural MV proteins, V and C, delay apoptosis and inhibit interferon production respectively. Many groups have long speculated that mutations in varying viral genes (specifically, M, P, and F) result in enhanced neuropathogenesis, promoting decreased viral budding, and increased ability to “hide” from the immune system (Millar et al. 2015; Cattaneo et al. 1987; Haase, Swoveland, et al. 1981; E. M. Jurgens et al. 2015; Knut et al. 1986; Liebert et al. 1986; Kweder et al. 2015). However, these studies failed to identify consistent gene mutations amongst groups, or fully take into account the ability of MV to switch from lytic budding to non-cytolytic trans-synaptic spread when infecting neuronal populations.
Figure 1.5: Measles virus structure and encoded genes

Measles virus spread in neurons of the central nervous system

The inability to recover extracellular infectious virus in brains of SSPE patients led our laboratory to investigate the mechanism by which MV spreads in neurons. To do so, we employed a novel transgenic mouse model, as mice are not normally permissive to MV infection. These transgenic mice express CD46, one of three human MV receptors identified to date (Rall et al. 1997; Naniche et al. 1993; Dorig et al. 1993), under the control of the neuron specific enolase promoter (NSE-CD46+ mice) (Rall et al. 1997; Naniche et al. 1993; Dorig et al. 1993). Using this model system, neurons of the CNS can be exclusively infected, both in vivo and ex vivo.

Typically, MV infection of non-neuronal cells leads to the formation of giant multinucleated cells (syncytia; Figure 1.6). As MV proteins H and F are inserted into the lipid bilayer of the infected host cell, these molecules can interact with receptors on adjacent uninfected cells, inducing membrane fusion of the infected and uninfected cell. Formation of syncytia correlates with massive amounts of detectable extracellular virus and ultimately results in cell death by 3-4 days post infection. In contrast, and similar to what is seen in SSPE, MV infection of primary neurons does not result in syncytia formation, detectable extracellular virus, or cell death (Figure 1.6) (Lawrence et al. 2000). Moreover, spread of MV in primary neurons occurs in the absence of MV receptor expression, but is dependent on cell-cell contact. Further studies by our lab have shown that MV spread in neurons relies heavily of MV F and neurokinin-1 (NK-1), a receptor for the neurotransmitter substance P expressed on CNS neurons. It is hypothesized that NK-1 and F interactions possibly induce the formation of a micro-pore through which MV genomic RNA and associated N, P, and L proteins can spread trans-synaptically in the absence of extracellular virus release (Figure 1.7) (Makhortova et al. 2007). While this
hypothesis remains to be tested, MV infection of neurons is non-lytic, as compared to the highly lytic infection that occurs in non-neuronal cells.

**Figure 1.6: MV spread differs among cell types.**

Cells infected with MV Edmonston at an MOI=1. 72 hours post infection cells were stained with a polyclonal MV antibody. Left: MV infected Vero cells (African monkey kidney epithelial cells). Right: MV infected primary NSE-CD46+ neurons. Arrow indicates synaptically connected neurons.
Figure 1.7: Schematic for hypothetical micro-pore formation in MV infected neurons.

Neurokinin-1 (blue) and the MV fusion protein (red) interact at the synaptic junction facilitating the fusion of pre and post-synaptic neurons, thus allowing the MV ribonucleoprotein (green) to spread to uninfected cells without budding, and releasing extracellular virus.

**Immune mediated clearance of measles virus in the central nervous system**

Trans-synaptic spread of MV could facilitate “masking” of MV from the immune system: i.e., anti-viral antibodies in the brain parenchyma would be unable to access MV antigens because they are entirely intracellular. However, using NSE-CD46+ mice backcrossed to varying immune knockout backgrounds, it is clear that adaptive immune responses—chiefly T cells-- are critical in combatting neuronal MV infection of the CNS.

Specifically, infection of NSE-CD46+/RAG2 KO adult mice, deficient in mature B and T cells, results in 100% animal death in 3-4 weeks (Lawrence et al. 1999; Holmgren,
Miller, Cavanaugh & Rall 2015b; Solomos et al. 2016; Cavanaugh et al. 2015; O'Donnell et al. 2015). To further elucidate the role of individual adaptive immune cell types in controlling MV infection, we employed combinations of immune knockout NSE-CD46$^+$ animals and antibody mediated immune depletions. In this way we identified CD4$^+$ T cells as key effectors of neuronal MV control, as all infected NSE-CD46$^+$ CD4$^+$ T cell depleted mice succumb to viral challenge. However, CD4$^+$ T cells alone are insufficient to control viral replication, as these cells need interaction with either B cells or CD8$^+$ T cells in order to promote animal survival, as evidenced by animal death in infected NSE-CD46$^+$/B cell KO mice depleted of CD8$^+$ T cells (Solomos et al. 2016; Tishon, Lewicki, Andaya, McGavern, Martin & Oldstone 2006a). Aside from the role of individual immune cell types themselves, we have identified type I and II interferons (IFN$\alpha$, IFN$\beta$, and IFN$\gamma$) as key effectors of innate and adaptive immunity required for neuronal MV control, as a proportion of infected animals deficient in these signaling molecules ultimately succumb to viral challenge (Patterson, Lawrence, et al. 2002; O'Donnell et al. 2015; Cavanaugh et al. 2015; Holmgren, Miller, Cavanaugh & Rall 2015b). Importantly, while both innate and adaptive immune components are essential in promoting mouse survival following neurotropic MV infection, immune-mediated viral control is noncytopathic: NSE-CD46$^+$/perforin KO mice survive viral challenge, and no evidence of neuronal loss is observed in brains of mice that controlled a MV challenge. Thus, neither the infection nor the host response result in neuronal death, paving the way for long-term persistence of viral RNAs in the brain, the topic of this dissertation.
**Altered neuronal immune signaling**

Neuronal survival may not simply be a consequence of avoiding cell death events, but may be due in part to induction of pro-survival proteins (Cavanaugh et al. 2015; O'Donnell et al. 2015; Rose et al. 2007; Podolsky et al. 2012). For instance, exposure of mouse embryonic fibroblasts to type II IFN results in robust STAT activation (phosphorylation) and downstream induction of interferon stimulated genes (ISGs), while the same IFN exposure in neurons results in delayed kinetics of STAT activation and induction of STAT independent ISGs (O'Donnell et al. 2015). This delayed STAT activation and induction of alternative ISGs may steer neuronal cells away from a lytic fate toward a pro-survival signaling profile. One such alternatively induced ISG is bone marrow stromal antigen 2 (BST2, aka tetherin), primarily known for its ability to physically tether budding virions to the host cell membrane (Holmgren, Miller, Cavanaugh & Rall 2015b; Hammonds et al. 2010). BST2 is much more highly induced upon neuronal exposure to interferon than that observed in non-neuronal MEFs (Chapter 3). However it is clear that BST2 does not play a role in restricting MV release from neurons, its well characterized traditional role, but may actually facilitate the formation of the micro-pore in neurons allowing MV to spread trans-synaptically (Appendix).

**Conclusions**

Non-cytolytic immune control, trans-synaptic spread, and differential uses of ISGs highlight the unique outcomes in neurons when infected with MV, possibly enabling this RNA virus to escape sterile immune mediated clearance and persist in the brain long-term, to possibly reactivate later in life (Chapter 2). To this end, MV mRNAs have been identified in the brains of humans who died of natural causes, in the absence of overt clinical CNS disease, decades after an acute MV infection (Katayama et al. 1998;
Katayama et al. 1995), making it clear that long-term MV persistence is not unique to SSPE. Further, MV has been postulated to be an etiological trigger for a variety of CNS and peripheral diseases, including multiple sclerosis, Alzheimers, and otosclerosis; however, no data have yet been obtained that fulfill Koch’s postulates allowing for a clear designation of etiology (Richard et al. 2015; B. E. Cohen et al. 2014; Kawashima et al. 1996; Schubert 2006; Doi et al. 2016; Riddell et al. 2007; Jarius et al. 2016). In sum, much more research will need to be conducted in order to elucidate the consequences of long-term RNA viral persistence (Chapter 2).

VII. Emerging Principles in Neurovirology

Preservation of virus-challenged neurons from immune-mediated lysis seems advantageous to the host, but this leaves open the possibility of long-term viral maintenance in surviving neurons (Table 1.1). Previously, many believed that neurotropic RNA viruses were sterilely cleared from the CNS. Indeed, unlike DNA viruses or retroviruses, which can establish latent infections through episome formation or integration, RNA viruses have no clear means to “survive” within a host cell. This is especially relevant given the lability of naked RNA within the cytoplasm, which arises due to the inherently unstable ribose subunit and the susceptibility of the 2’ hydroxyl group to deprotonation. On the other hand, RNA viral genomes are unlikely to persist in the cytoplasm as naked RNA; ribonucleoprotein complexes would provide some protection and viral RNAs (like other cellular RNAs) may also be sequestered in stress granules. Thus, mechanisms must exist to protect RNA viral genomes, allowing for their long-term stability in the cytoplasm.
Do these long-term infections have pathogenic potential? A set of studies from the late 1980s showed that MV RNA can persist for decades after acute resolution in human brains without causing neurological symptoms (Katayama et al. 1998; Nakayama et al. 1995; Kawashima et al. 1996; Haase et al. 1985; Katayama et al. 1995); in these studies, organs from individuals who had died of non-viral, non-CNS related causes were screened, and a high proportion of brain tissues were found to be MV RNA-positive. In addition, some have argued that MV entry into the human CNS can occur pursuant to acute MV infection (Hanninen et al. 2014), although only a small fraction of acutely infected persons will manifest neurological consequences. Further studies using macaques demonstrated that MV viral RNA is detectable in peripheral tissues long after acute infection has resolved (Lin et al. 2012). Accordingly, viral RNAs were generally considered “fossils” that were unlikely to contribute to human disease. Surprisingly, autopsy studies performed on brains of patients that succumbed to SSPE have shown regions of the brain with no detectable MV proteins expressed, despite the presence of MV RNA, suggesting that RNA, even with its inherent instability, can be maintained in a translationally silent state (Allen et al. 1996).

The long-term persistence of viral RNA in the CNS is not unique to MV. For example, infection of mice with MHV A59, used to study the demyelinating disease multiple sclerosis, leads to encephalitis and hepatitis. The infectious virus is cleared from the liver and CNS within 20 days; however the mice develop a progressive, immune-mediated demyelinating disease (Matthews et al. 2001), in which viral nucleic acid persists (Lavi et al. 1984). The potential importance of viral nucleic acid persistence in demyelination has been subordinated by the prevailing view that long-term disease is caused by an over-activation of the host response toward myelin proteins. Other neurotropic RNA viruses known to persist within the mouse brain (sometimes longer
than one year post exposure) in the absence of detectable antigen or infectious progeny include sindbis virus (SV), Sendai virus, and RV (Koch et al. 1984; Gomme et al. 2012; Griffin & Levine 1992). However, the lack of recoverable infectious virus does not preclude the possibility that these viruses are actively suppressed in the CNS, similar to the control of neuronal herpesvirus infections by T<sub>rm</sub>. Could decreases in the magnitude or quality of the host response (for example with aging or following immunosuppressive therapy) lead to loss of resident memory cells and reactivation of viral replication, temporally separated from an initial infection?

The short answer is that we do not yet know. However, it was recently shown that an endogenous retrovirus, integrated into the host genome millions of years ago, could contribute to human neurological disease. Amyotrophic lateral sclerosis (ALS) is a progressive neurological disease of poorly understood etiology, but consistent inflammatory and immune mediated pathogenesis. The expression of human endogenous retrovirus (HERV-K), specifically the envelope protein, was proposed as a possible cause for the neuropathology seen in ALS (Li et al. 2015).
VIII. Perspectives

Limits of detection, reproducibility, consistency in brain regions that are analyzed, and patient-to-patient variability all contribute to the challenges and dangers of ascribing neurotropic infections as etiologic causes of poorly understood CNS diseases. Moreover, the association of “new” viruses with CNS disease, including the flavivirus, Zika, and its link to microcephaly (Brasil et al. 2016; Rasmussen et al. 2016), or the emergence of more neurovirulent influenza strains (Wiley et al. 2015) are reminders that our understanding of the pathogenic consequences of CNS infections remains quite primitive. Translational studies have provided insights into the links between infections and disease, but are not without controversy. For example, the prevalence of human cytomegalovirus (CMV) in glioblastoma has been hotly debated, though anti-CMV treatments lead to reduction in tumor burden in some patients (Söderberg-Nauclér 2015). Furthermore, losses in host immune status due to age or chemotherapy are well known to provoke disease, as seen with JC virus infection and progressive multifocal leukoencephalopathy (Sudhakar et al. 2015).

Whether CNS virus infections play a larger role in human diseases of unknown etiology remains controversial. In support of this notion, CNS neurons may be an ideal harbor for long-term infections: nonlytic immune mechanisms spare neuronal loss while providing an avenue for a noncytopathic virus to persist. Moreover, trans-synaptic spread likely enables viral escape from antibody recognition or phagocytosis by antigen presenting cells. From an evolutionary perspective, neuronal survival is paramount; thus, sparing infected neurons a lytic fate may promote survival early on, but could potentially open the door for viral reactivation later in life. We do not know if there are viruses that are typically found in CNS tissues of overtly healthy individuals; with the advent of
RNAseq technology, such studies might shed light on the potential “virome” within the brain in both asymptomatic individuals as well as those with neurological conditions.

One final point worth noting concerns the utility of mouse models (on which many of the studies cited in this introductory chapter were based) to study human CNS diseases. Scientists often make the mistake of assuming that mouse survival is equivalent to an absence of disease. This may mean that the long-term ramifications of acute virus infections, especially those of RNA viruses that are not generally considered life-long, may be overlooked. However, we are increasingly becoming aware that the presence of viral fragments or latent viruses that can reactivate might evoke non-lethal pathogenic consequences, due either to viral replication and cell damage, or to immune responses directed against viral antigens. Such diseases, as seen with the learning defects in LCMV-infected mice, may be subtle. Consequently, the parallel development of more precise tools to assess CNS disease in mice, including impacts on learning, behavior and memory, should refine how we describe neuropathogenesis in the many valuable mouse models currently in use. Finally, determining whether or not persistent viral nucleic acids detected within the brain are replication competent, and how these viruses evade complete clearance (the focus of Chapter 2), could herald novel antiviral therapies to treat or prevent devastating and prevalent human neurological and neurodegenerative diseases (Gomme et al. 2012; Maehlen et al. 1991).
CHAPTER 2: RNA viral persistence, reactivation, and pathogenesis in the central nervous system

This chapter is in preparation for publication

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I. Summary

It is well established that viral RNA from many neurotropic RNA viruses (e.g., measles virus, West Nile virus, sindbis virus, rabies virus, influenza virus) remains detectable in the brain parenchyma long after acute infection has resolved. Moreover, the presence of these RNAs in the absence of overt central nervous system (CNS) disease has led many to believe that these are viral remnants with little or no potential to reactivate. Here, we show that long-term viral persistence occurs following infection with a neurotropic RNA virus and that viral reactivation can trigger neuropathogenesis months after acute control. Recrudescence of viral transcription and protein synthesis occurs after experimental depletion of adaptive immunity and is associated with a loss of T resident memory (T<sub>rm</sub>) immune lymphocytes. Viral reactivation coincides with the onset of severe CNS disease and motor dysfunction in mice that had presumably cleared the infection. Viral replication and T<sub>rm</sub> are localized within the cerebellum/brain stem, and pathogenesis associated with viral reactivation is consistent with gait and motor problems, similar to cerebellar ataxia in humans. Our results illuminate the potential consequences of non-lytic viral control in the CNS and demonstrate that what were once considered “resolved” RNA viral infections can induce diseases distinct from those that accompany acute infection.
II. Introduction

Many CNS disorders of unknown etiology have long been speculated to have viral triggers, such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Jarius et al. 2016; Hottenrott et al. 2017; Li et al. 2015). However, the inability to recover infectious virus or consistently detect viral genomes in patient tissue has cast doubt on viral etiologies for these prevalent and devastating CNS diseases. The inability to recover infectious virus from affected brain tissues, however, does not preclude the possibility that earlier infection may have triggered neuronal dysfunction or autoimmune activation, ultimately resulting in disease in the absence of direct viral reproduction (Jurgens et al. 2012; Brot et al. 1997; Gomme et al. 2012). The mechanism by which viruses may contribute to the development of inflammatory CNS diseases is not known, but in this report, we show that neurons of the CNS can harbor long-term persistent infections that can reactivate and trigger neuropathology that is temporally and phenotypically distinct from what occurs during the acute infection.

Most neurons are post-mitotic, making them a generally nonrenewable cell population; thus, the host immune response has developed strategies to control neurotropic infections that do not depend on neuronal lysis. The abundance of neurotropic viruses that are controlled by the immune response in the absence of neuron loss, including rabies virus (RV), sindbis virus (SV), measles virus (MV), West Nile virus (WNV), herpes simplex virus (HSV), and varicella zoster virus (VZV)) (Kinchington et al. 2012; Katayama et al. 1998; Griffin 2010; Graham et al. 2016), highlights that non-cytolytic immune-mediated control is a general host strategy aimed at preserving the affected neuronal population. However, such non-lytic viral control may allow neurons to harbor noncytopathic viruses, allowing for the incomplete eradication of viruses from the
The host and cellular mechanisms that govern viral persistence and reactivation are reasonably well understood for large DNA viruses, such as HSV and VZV. For example, recent studies using VZV and HSV have shown that cells of the adaptive immune system are crucial for suppressing viral reactivation from latency in the CNS and peripheral nervous system (PNS) (St Leger & Hendricks 2011; Jeon, St Leger, Cherpes, Sheridan & Hendricks 2013b; Divito et al. 2006; Himmelman et al. 2011; Khanna et al. 2003; T. Liu et al. 1996; T. Liu et al. 2000; Wakim et al. 2012; Theil et al. 2014; Knickelbein et al. 2008). CD8$^+$ T cells are in direct contact with trigeminal ganglionic neurons latently infected with HSV. Moreover, the presence of these cells was essential to prevent reactivation of latent virus (T. Liu et al. 2000; Khanna et al. 2003; Knickelbein et al. 2008; T. Liu et al. 2001; T. Liu et al. 1996). Furthermore, these CD8$^+$ T cells prevented viral reactivation in a non-cytolytic manner through interferon gamma (IFNγ), granzyme B, and lytic granule mediated mechanisms (Divito et al. 2006; St Leger & Hendricks 2011; Jeon, St Leger, Cherpes, Sheridan & Hendricks 2013b; Knickelbein et al. 2008). The CD8$^+$ T cells responsible for maintenance of viral persistence in the absence of reactivation are T resident memory (T$_{rm}$) lymphocytes, which permanently reside, expand, and contract within the immediate vicinity of latent viral infections, distinct from memory T cells of the circulating lymph (Wakim et al. 2010; Wakim et al. 2008; Wakim et al. 2012).

For many neurotropic viruses of both mice and humans, infectious virus cannot be recovered from brains after the acute phase of infection, despite the presence of detectable RNA in these tissues (Katayama et al. 1998; Katayama et al. 1995; Griffin &
Levine 1992). This observation led many virologists to suggest that the detectable viral RNA was a fossil, incapable of reactivation and thus having little impact on host biology. However, it is clear that persistent RNA viral infections of the CNS can evoke devastating host diseases as evidenced by fatal CNS diseases such as subacute sclerosing panencephalitis (SSPE). SSPE can occur months to years after acute infection with MV (Anlar et al. 2002), and the incidence of SSPE, once thought to occur at a rate of 1:1700-10,000 acute MV infections, has been reevaluated based on new data to be 1:600 in individuals infected before 1 year of age. This re-evaluation highlights that neuropathogenic consequences following persistence of an RNA virus may be more common than once thought (Ludlow et al. 2014; Campbell 2016; Gutierrez et al. 2010). Furthermore, the disease presentation following SSPE development (motor dysfunction, cognitive defects, and ultimately death) is wholly different from that seen during acute MV infection (maculopapular rash, fever, conjunctivitis), suggesting that the clinical manifestations following persistence and reactivation of a previously cleared RNA viral infection may be distinct. The long-term presence of viral (non-self) antigens in the CNS may evoke a chronic inflammatory response, even in the absence of production of infectious viral particles.

In this chapter of my dissertation, I demonstrate long-term persistence of MV in the CNS of immunocompetent mice in the absence of clinical signs of CNS disease. Viral replication in the CNS is most likely prevented by $T_{rm}$ and loss of these adaptive immune cell populations in the brains of persistently infected mice results in pathogenesis including gross motor dysfunction (spastic paraparesis with dyscoordination of the hind limbs) and weight loss. Viral persistence and reactivation occurs in the cerebellum/brain stem regions of the CNS, resulting in symptoms similar to cerebellar ataxia in humans. Together our data highlight the ability of an RNA virus to
persist in neurons of the CNS and, when the host is faced with immunodeficiency, cause
disease distinct from that seen during acute infection.

III. Results

_CD46 mice survive neuronal MV challenge, but fail to clear viral RNA_

Our laboratory previously showed that intracranial inoculation of MV into
immunocompetent, adult NSE-CD46<sup>+</sup> mice, in which the MV receptor CD46 is restricted
to CNS neurons, results in 100% survival with no signs of disease or weight loss at any
time point post-infection. In contrast, immunodeficient NSE-CD46<sup>+</sup>/RAG2 KO mice
progressively lose weight and succumb to the viral challenge within 2-4 weeks post-
infection, likely due to unrestricted viral replication in neurons (Figure 2.1 A/B)
(Patterson, Lawrence, et al. 2002; Lawrence et al. 1999; Rall et al. 1997; Naniche et al.
1993; Dorig et al. 1993). Interestingly, while these mice succumb to viral infection,
careful analysis of brains from moribund NSE-CD46<sup>+</sup>/RAG2 KO mice reveals no
neuronal loss, suggesting that disease may be more attributable to neuronal dysfunction
rather than frank neuron death. In order to further characterize the possible prolonged
CNS consequences following infection of immunocompetent mice, we analyzed viral
RNA (with random hexamer priming to generate cDNA) and mRNA (with oligo dT
priming to generate cDNA) levels at varying times post infection (Figure 2.1C/D). As
expected, immunocompetent NSE-CD46<sup>+</sup> mice controlled viral replication, reflected as
significant decreases in detectable viral RNA from 7-14 days post-infection (dpi). Surprisingly,
however, viral RNA and mRNA were still readily detected in brains long
after presumptive clearance (e.g., 90 dpi), in the absence of clinical symptoms of CNS
disease.
Figure 2.1: NSE-CD46+ mice survive MV challenge but fail to clear viral RNA.

A. NSE-CD46+ (n=10) or NSE-CD46+/RAG-2 KO (n=5) mice were challenged i.c. with 1 x 10^4 pfu of MV-Edmonston and monitored daily for survival. B. Baseline weights of all mice from (A) were obtained and compared to weights taken throughout the infection time course. Percent weight gain or loss was then calculated. C. NSE-CD46+ mice were challenged i.c. with 1 x 10^5 pfu of MV-Ed and were then sacrificed at the indicated dpi. RNA was purified from collected brains, and subsequently analyzed by qRT-PCR (using random hexamers to make cDNA). D. MV nucleoprotein (N) mRNA levels in NSE-CD46+ mice challenged i.c. with MV-Ed were detected using the approach outlined above, substituting oligo dT primers for random hexamers to generate cDNA. Data are represented using the ΔΔCT method. Results are representative of at least 3 independent experiments with an n>5-10 mice per group. * P< 0.05 Mann Whitney U Test.
Resident memory T cells are the most abundant effector cells in the brain during persistent infection

Recent studies using VZV and HSV have shown that cells of the adaptive immune system (T<sub>rm</sub>) are crucial for maintaining DNA viral infections in the CNS and PNS in a latent state (St Leger & Hendricks 2011; Jeon, St Leger, Cherpes, Sheridan & Hendricks 2013b; Divito et al. 2006; Himmelstein et al. 2011; Khanna et al. 2003; T. Liu et al. 1996; T. Liu et al. 2000; Wakim et al. 2012; Theil et al. 2014; Knickelbein et al. 2008). Since CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells are paramount in controlling acute MV infection of the CNS (Solomos et al. 2016; Tishon, Lewicki, Andaya, McGavern, Martin & Oldstone 2006b) coupled with the knowledge that T<sub>rm</sub> are essential in preventing viral reactivation of DNA viruses in neurons, we next characterized the cell populations present in brains of mice long after viral challenge, when viral RNA can still be detected (Tishon, Lewicki, Andaya, McGavern, Martin & Oldstone 2006a; Weidinger et al. 2001; Weidinger et al. 2000; Lawrence et al. 1999; Reuter et al. 2012; Solomos et al. 2016). Lymphocytes purified from whole brains of infected mice at varying time points were subjected to flow cytometry analysis. No significant differences in the total number of B cells, T cells, or NK cells were observed in mice that were persistently infected (data not shown). However, a significant increase in the proportion of CD8<sup>+</sup> T cells expressing the resident memory markers CD103 and CD69 was detected, indicative of a T<sub>rm</sub> phenotype (Figure 2.2A and Figure 2.3). We then confirmed that the long-term presence of these cells in the brain is predicated on an active infection, rather than inflammation induced during the act of intracranial viral inoculation. Indeed, T<sub>rm</sub> cells were detected after inoculation with replicating MV; T<sub>rm</sub> were not found in either mock infected controls (PBS IC) or animals infected with a replication incompetent MV (UV MV) (Figure 2.2B).
Figure 2.2: CD8$^+$ T resident memory cells are highly abundant in the CNS during persistent infection.

A. Lymphocytes purified from whole brain tissue of perfused mice at indicated times post infection subjected to flow cytometry analysis. Black bars: total number of CD3$^+$/CD8$^+$ T cells. White: total number of CD3$^+$/CD8$^+$/CD103$^+$ T cells as a proportion of total CD3$^+$/CD8$^+$ T cells. n=5/group. Statistical analysis comparing the proportion of CD103$^+$/CD8$^+$/CD3$^+$ T cells during acute and persistent infection. B. Lymphocytes purified from whole brain tissue of mock infected mice (PBS), mice inoculated with inactivated virus (UV MV), or mice infected with replication competent MV, collected at 90dpi and subjected to FACS analysis. CD103$^+$/CD3$^+$/CD8$^+$ shown as a percent of total CD3$^+$/CD8$^+$ T cells. n=6/group. */# P< 0.05 Mann Whitney U Test.
Figure 2.3: T<sub>rm</sub> express CD69.

Lymphocytes purified from whole brains of mice infected for >90 days. Percent CD69<sup>+</sup> of CD3<sup>+</sup>CD8<sup>+</sup>CD103<sup>+</sup> T cells in the brains of uninfected or persistently infected NSE-CD46<sup>+</sup> mice. * P< 0.05 Mann Whitney U Test. N=6-18/group. Representative of at least 2 independent experiments.

The presence of T<sub>rm</sub> in the brain does not indicate that these cells have a functional consequence; therefore, we went on to analyze the effector function of these T<sub>rm</sub> using intracellular cytokine staining coupled with flow cytometry analysis. Coincident with the increase in T<sub>rm</sub> during persistent infection (Figure 2.4A), levels of the effector molecules granzyme B and interferon gamma (IFN<sub>γ</sub>) were also elevated, as compared to the remaining CD8<sup>+</sup> T cells in the brain (Figure 2.4B/C). Thus, T<sub>rm</sub> present during persistent infection maintain an effector phenotype months after viral challenge.
Figure 2.4: CD8⁺ T<sub>r</sub>m cells maintain an effector phenotype during persistent infection.

Lymphocytes purified from whole brains of mice infected for 7 or >90 days. A. Percent CD3⁺CD8⁺CD103⁺ (T<sub>r</sub>m) of CD3⁺CD8⁺T cells in the brains of mice infected for indicated time. B. Left – Percent IFNγ⁺ T<sub>r</sub>m of CD3⁺CD8⁺CD103⁺ T cells. Right - Percent IFNγ⁺CD3⁺CD8⁺CD103⁻ T cells. C. Left – Percent GranzymeB⁺ T<sub>r</sub>m of CD3⁺CD8⁺CD103⁺ T cells. Right – Percent GranzymeB⁺ T<sub>r</sub>m⁺ of CD3⁺CD8⁺CD103⁻ T cells. * P< 0.05 Mann Whitney U Test. N=5-8/group purified CNS lymphocytes. Representative of at least 2 independent experiments.
Transient depletion of the adaptive immune response leads to viral reactivation

This effector phenotype of $T_m$ in the brain during persistent infection, coupled with our observation of the critical role of adaptive immunity in controlling acute CNS neuronal MV infection (Figure 2.1A), led us to ask whether the persistent MV RNA observed in our model system was capable of reactivation when $T_m$ are depleted. Because $T_m$ do not recirculate (Mueller & Mackay 2015), and are present within the brains of NSE-CD46$^+$ mice, antibody mediated depletions were not feasible due to the restricted access of blood-borne molecules (e.g., antibodies) across the blood brain barrier (BBB). In order to circumvent BBB restrictions and assess the role of adaptive immunity in suppressing viral replication long-term, we used sub-lethal irradiation to transiently deplete adaptive immune cells in persistently infected mice. After irradiation we assessed relative amounts of detectable viral RNA and mRNA with time post immune depletion. We observed significant increases in viral RNA and mRNA present within the brains of persistently infected mice after immunosuppression (Figure 2.5 A/B and Figure 2.6). Further, we began to detect expression of viral protein in the brains of immunosuppressed mice, not seen in persistently infected immunocompetent mice (Figure 2.5C). Thus, persistent MV RNA in the brain can reactivate and synthesize viral proteins upon transient immunosuppression.
Figure 2.5: Sub-lethal irradiation leads to increased detection of MV RNA, mRNA, and protein.

NSE-CD46\textsuperscript{+} mice challenged i.c. with 1x10\textsuperscript{4} PFU MV-Edmonston for at least 90 days were sub-lethally irradiated (6.5gy). RNA expression levels were determined by RT-qPCR using random hexamer or oligo dT priming for cDNA generation followed by qPCR with primers specific for the MV nucleoprotein and cyclophilin B as a standard. Data analyzed using the \( \Delta \Delta CT \) method. n=8-12/group from at least 2 independent experiments. * P < 0.05 Mann Whitney U Test. A. cDNA generated using random hexamer priming. B. cDNA generated using oligo dT priming. C. Western blot analysis of protein purified from whole brain tissue probed for MV Fusion protein and GAPDH. Arrow indicates MV Fusion protein.
Figure 2.6: Sub-lethal irradiation results in increased detection of RNA for varying MV genes.

NSE-CD46+ mice challenged i.c. with 1x10^4 PFU MV-Edmonston for at least 90 days were sub-lethally irradiated (6.5gy). RNA expression levels were determined by RT-qPCR using random hexamer or oligo dT priming for cDNA generation followed by qPCR with primers specific for the MV fusion protein, hemagglutinin protein, or matrix protein, and cyclophilin B as a standard. Data analyzed using the ΔΔCT method. n=8-12/group from at least 2 independent experiments. * P < 0.05 Mann Whitney U Test. A-C. cDNA generated using random hexamer priming. D-F. cDNA generated using oligo dT priming.
Complete ablation of the adaptive immune response leads to viral reactivation and pathogenesis

Sub-lethal irradiation only transiently depletes adaptive immune responses; thus, reactivated virus can be re-controlled as immunity rebounds. To further investigate the possible pathogenic outcomes of viral reactivation under conditions of permanent immune cell loss or reduced potency, we ablated adaptive immunity in persistently infected mice, and subsequently assessed pathogenesis in these mice. Bone marrow chimaeras (BMCs) were established, using persistently infected mice as recipients and bone marrow from donor immunocompetent (WT) or immunodeficient (RAG2 KO) mice. Using this approach, we could permanently deplete the adaptive immune response from persistently infected mice and monitor for viral reactivation and disease (Figure 2.7A and Figure 2.8).

A proportion of reconstituted persistently infected immunodeficient (RAG2 KO) animals displayed weight loss and kyphosis, not seen in mice reconstituted with wild type bone marrow (Figure 2.7 B/C). Coincident with these signs of illness, mice began to display spastic paraparesis with dyscoordination of the hind limbs (Figure 2.7C), requiring euthanasia of ~30% of persistently infected immunodeficient recipients (Figure 2.7B). Surprisingly, analysis of whole brain tissue from immunodeficient mice experiencing viral reactivation, compared to immunocompetent controls, did not reveal extraordinary amounts of viral RNA (Figure 2.7D). While a clear trend towards increasing viral RNA was observed in immunodeficient animals, detectable viral RNA was well outside of the range observed in moribund NSE-CD46+/RAG2 KO mice (~10^5-10^7 fold change of MV nucleoprotein RNA over uninfected) or mice acutely challenged with MV 1 day post bone marrow reconstitution (Figure 2.9 A/B). Further, the
pathogenic phenotype observed in persistently infected immunodeficient animals did not mirror that seen in moribund NSE-CD46\textsuperscript{−}/RAG2 KO animals (kyphosis, labored breathing, and weight loss with death in ~3 weeks post infection (Figure 2.1A/B), but no paraparesis or motor dysfunction). Thus viral RNA levels in persistently infected, immunodeficient bone marrow recipients did not explain the observed pathogenic phenotype.
Figure 2.7: Ablation of the adaptive immune response after resolution of acute infection leads to motor dysfunction and pathogenesis.

Bone marrow chimaeras were generated using NSE-CD46\textsuperscript{+} mice that had been challenged with 1x10\textsuperscript{4} PFU MV-Edmonston for >90 days. Infected mice were reconstituted with the indicated bone marrow (WT or RAG2 KO, introduced via the retro-orbital sinus) and monitored daily. A. Schematic of bone marrow chimaera generation. B. Survival of reconstituted mice. n=14-18/group. Log-rank (Mantel-Cox) test for significance. C. Baseline weights of all mice from (B) were obtained and compared to weights taken throughout the reconstitution time course. Data are presented with animals grouped into WT mice, no disease RAG2 KOs, and diseased (sick) RAG2 KOs. n=14-18/group. D. RT-qPCR analysis of whole brain tissue collected from reconstituted mice at indicated time post reconstitution. N=3-6/group. p values determined using the Mann-Whitney U test. Data are representative of 2 independent experiments.
Figure 2.8: Bone marrow chimaeras are depleted of lymphocytes in the brain.

Lymphocytes purified from whole brains of persistently infected mice or persistently infected mice 6 weeks post bone marrow reconstitution with RAG2 KO or WT bone marrow. A. Percent CD4\(^+\)CD8\(^+\) and CD19\(^+\) cells sorted per brain. B. Total CD8\(^+\)/CD3\(^+\)/CD103\(^+\) cells/brain. C. Total CD19\(^+\) cells per brain. N=2-4 mice per group.
Figure 2.9: Viral RNA levels in moribund animals.

NSE-CD46+ mice challenged ic with 1x10⁴ PFU MV-Edmonston. RT-qPCR analysis of whole brain tissue collected from indicated animals. A. NSE-CD46+/RAG2 KO animals at indicated time post infection. B. Uninfected NSE-CD46+ mice irradiated and reconstituted with WT or RAG2 KO bone marrow 1 day prior to infection. N=2-10/group.
Viral persistence and reactivation occurs in the cerebellum/brain stem region of the CNS

Neurotropic viruses, including WNV and MV, show differential infectivity throughout brain regions based on histological analysis of post-mortem brain tissue (Hussmann et al. 2013; Cho et al. 2013; Reuter et al. 2012; Schubert 2006; Haase, Stowring, et al. 1981; Allen et al. 1996; Omalu et al. 2003). Models of continually replicating MV infection in the mouse CNS, as well as encephalitic models in rats, show progression of MV through different regions of the brain over the course of infection, as well as regions of the brain that do not become infected (Niewiesk et al. 1993; Jehmlich et al. 2013). Furthermore, studies using WNV in mice have shown that there are basal differences in the ability of WNV to infect cortical vs. granular neurons. Viral infection of these distinct neuronal populations correlated with intrinsic differences in the levels of innate immune effectors (interferon stimulated genes) expressed within these cell types (Cho et al. 2013). A model of MV-induced encephalitis in rats also showed that GABAergic and glutamatergic neurons were the predominant populations infected, and that fewer cholinergic neurons, catecholaminergic neurons, astrocytes, microglia, and endothelial cells became infected (Jehmlich et al. 2013). These studies collectively demonstrate inherent differences in the ability of different viruses to infect distinct regions of the brain.

Based on these data, we decided to further explore the regional distribution of MV during persistent infection and reactivation, with the hypothesis that MV reactivation in a particular brain region might explain the observed pathogenic phenotype and limited detection of viral RNA during reactivation in our BMC model. Collection of RNA from individual brain regions and spinal cords in persistently infected mice showed a significant increase in detectable viral RNA in the cerebellum/brain stem region when
compared to cerebral hemispheres and spinal cords of the same animal (Figure 2.10A). Analysis of cerebellar tissue and cerebral hemispheres obtained from persistently infected mice 14 days post sub-lethal irradiation (14dpr) showed a significant increase in viral replication in the cerebellum (Figure 2.10B). Further, immunohistochemical analysis of sectioned brain tissue from persistently infected mice 14dpr revealed MV proteins primarily in the cerebellum (Figure 2.11F), and analysis of serial sections from persistently infected mice 14dpr showed both CD3+ and CD8+ cells entering the brain through the 4th ventricle in direct proximity to the cerebellum, indicating that as the immune response rebounds after irradiation, effector cells are recruited to the sites of viral reactivation in the cerebellum (Figure 2.12 and Figure 2.13). Together, these data show that the cerebellum/brain stem is the primary site of long-term MV persistence and reactivation. We posit that viral reactivation in this site, the motor coordinator of the brain, is the cause of the unique pathogenic phenotype observed with MV reactivation in our BMC model system.
**Figure 2.10:** The cerebellum is the predominant site of viral persistence and reactivation.

A. RT-qPCR analysis of cerebellar tissue compared to cerebral hemispheres and spinal cords collected from individual NSE-CD46+ mice infected for at least 90 days. Data are representative of 2 independent experiments. n=6/group. B. RT-qPCR analysis of cerebellar tissue compared to cerebral hemispheres and spinal cords collected from individual NSE-CD46+ mice infected for at least 90 days followed by 14 days of sublethal (6.5gy) irradiation. cDNA generated using random hexamer priming. Primers specific for the MV nucleoprotein and cyclophillin B were used and data analyzed using the ΔΔCT method. n=6/group. * P < 0.05 Mann Whitney U Test.
**Figure 2.11:** The cerebellum is the predominant site of viral reactivation.

NSE-CD46⁺ mice challenged i.c. with $1 \times 10^4$ PFU MV-Edmonston subjected to immunohistochemical analysis of sectioned whole brain tissue, stained with a polyclonal MV antibody. A-C. Moribund NSE-CD46⁺/RAG2 KO animal. D-F. Persistently infected NSE-CD46⁺ animal subjected to sub-lethal irradiation, 14 days post irradiation. Arrows indicate positive MV staining.
Figure 2.12: CD3$^+$ and CD8$^+$ cells are predominately found in the cerebellum during viral reactivation.

Serial sections obtained from NSE-CD46$^+$ mice challenged i.c. with $1 \times 10^4$ PFU MV-Edmonston 90 days post infection, 14 days post sub-lethal irradiation, subjected to immunohistochemical analysis of sectioned whole brain tissue A-C. Staining for CD3$^+$ cells. D-F. Staining for CD8$^+$ cells. Arrows indicate infiltrating antibody positive cells at the 4$^{th}$ ventricle in the CNS.
Figure 2.13: $T_m$ are enriched in the cerebellum.

Lymphocytes purified from whole brains of mice infected for >90 days. Percent CD103$^+$ of CD8$^+$CD103$^+$ T cells purified from cerebral hemispheres or the cerebellum of NSE-CD46$^+$ mice challenged i.c. with $1 \times 10^4$ PFU MV-Edmonston 90 days post infection. * $P < 0.05$ Mann Whitney U Test. NS – not significant. N=9/group. Representative of at least 2 independent experiments.
IV. Discussion

Before the introduction of widespread vaccination programs, MV infection was responsible for approximately 2.6 million deaths annually, according to the World Health Organization (WHO). Despite the availability of a safe and relatively inexpensive vaccine, there were 134,200 MV-associated deaths in 2015, with rates of MV infection resurging due to decreases in the vaccinated public (Campbell 2016). The majority of these fatalities occur as a result of viral immunosuppression, leading to opportunistic secondary infections. However, CNS complications subsequent to MV infection, including SSPE and measles inclusion body encephalitis (MIBE), can occur after uncomplicated MV infection. For the majority of affected individuals, these consequences are invariably fatal (Griffin et al. 2012; Gutierrez et al. 2010; Ludlow et al. 2014; Norrby & Kristensson 1997). SSPE can present months to years after a clinically typical MV infection, and is characterized by unrestricted MV replication in the CNS (Griffin et al. 2012; Gutierrez et al. 2010; Ludlow et al. 2014; Norrby & Kristensson 1997). Clear indicators predicting the development of SSPE have not yet been identified, but age at time of infection can increase susceptibility to SSPE; children infected with measles virus before 2 years of age have a greatly increased likelihood of developing SSPE (Gutierrez et al. 2010). The timing to development of SSPE (in some cases, as long as a decade) is surprising, as RNA viruses are not thought to persist long-term in the CNS. Many groups have long speculated that mutations in varying viral genes (M, P, and F) result in enhanced neuropathogenesis, promoting decreased viral budding, and increased ability to “hide” from the immune system, resulting in the development of SSPE (Millar et al. 2015; Cattaneo et al. 1987; Haase, Swoffeland, et al. 1981; E. M. Jurgens et al. 2015; Knut et al. 1986; Liebert et al. 1986; Kweder et al. 2015). However, these studies failed to identify consistent gene mutations amongst groups, or fully take
into account the ability of MV to switch from lytic budding to non-cytolytic trans-synaptic spread when infecting neuronal populations.

Our findings highlight the ability of a contained, but not cleared, neuronal MV infection to cause pathogenesis distinct from, and temporally separated from, an acute infection. As mentioned earlier, the long-term presence of detectable viral RNA in the CNS is not unique to MV infection; this outcome is also observed with influenza virus, Sindbis virus, Sendai virus, West Nile virus, and rabies virus (Aronsson et al. 2002; Griffin & Levine 1992; Koch et al. 1984; Graham et al. 2016; Gomme et al. 2012). However, the presence of viral RNAs has not been shown to cause CNS disease or pathology, leading many to believe that residual RNAs were remnants of the original infection, unable to reactivate.

The fact that MV RNA persists in the CNS long-term may not be all that surprising, as clearance and control of MV from CNS neurons is mediated in a non-cytolytic fashion (Patterson, Lawrence, et al. 2002). Such non-cytolytic clearance of viral pathogens preserves the infected neuron, while sparing the host the devastating consequences of neuronal loss, creating a “harbor” for many viruses to persist. However, if the immune response allows for long-term presence of non-self viral RNAs, it must have mechanisms in place to prevent viral reactivation and the ensuing host damage as a result of such an event. This is especially clear in the case of HSV infection of peripheral neurons. Latently infected trigeminal ganglion neurons are the predominant site of HSV latency and persistence, but \( T_m \) lymphocytes, which continuously reside next to infected neurons, keep the virus in check. These \( T_m \) secrete interferons and lytic granules that directly prevent HSV reactivation from latency, while maintaining the integrity of the host neuron (Wakim et al. 2010; Wakim et al. 2012; St
Here, we show that T<sub>r</sub><sub>m</sub> are highly enriched in the MV-infected brain long after inoculation, and maintain an effector phenotype throughout MV persistence in the CNS. Gross depletion of these cells and adaptive immunity enables MV to reactivate within the CNS, causing disease similar to cerebellar ataxia (gait and motor coordination abnormalities) and highlighting the ability of adaptive immunity to control long-term subclinical infections of the CNS.

Finally, as stated previously many CNS diseases of unknown etiology have long been speculated to have a viral trigger, but the inability to consistently detect viral antigens or recover infectious virus from host tissue has cast doubt on such claims. Regardless of the inability to detect viral components, in the case of MS, a positive MRZ reaction (intrathecal antibody directed against 2 of 3 viruses; MV, rubella virus, or varicella zoster virus) is an efficient prognostic indicator of MS (although not used clinically), suggesting some relation to a viral infection acquired earlier in life and disease development. Further, persistent MV infection has also been speculated as a causative agent of otosclerosis (localized bone displacement resulting in hearing loss) (Potocka-Baklažec et al. 2014; B. E. Cohen et al. 2014; Rudic et al. 2015) suggesting again that an infection acquired earlier in life can contribute to distinct diseases that are not typically attributed to a viral etiology. In fact, studies have identified a link between cytomegalovirus (CMV) and glioblastoma, again suggesting a viral etiology of disease in the CNS, as anti-CMV treatments lead to a reduction in glioblastoma tumor burden (Söderberg-Nauclér 2015). Together, chronic non-transmissible RNA viral infections can have severe consequences to the host. Future studies are underway to determine exactly how adaptive immunity (most likely T<sub>r</sub><sub>m</sub>) prevent MV reactivation over time and determine the neuronal consequences of continued cytokine and viral exposure.
V. Conclusion

Non-cytolytic RNA viruses, after entry into the central nervous system (CNS), can establish long-term residence in the brain, in part due to non-cytolytic immune mediated viral clearance. The complex architecture and distinct cell populations within the CNS contribute to the challenging task of defining how neurotropic RNA viral infections may alter CNS function following infection. The development of novel animal models, in conjunction with more precise tools and technologies will greatly accelerate our understanding of how persisting viruses can cause CNS disease long after initial exposure. In this chapter, I have shown that an RNA viral infection can result in CNS pathogenesis long after the acute infection has resolved. Further studies are needed explore how the host response restricts viral replication, and how reactivation of virus leads to a novel disease outcome.
CHAPTER 3: BST2/Tetherin is induced in neurons by type I interferon and viral infection but is dispensable for protection against neurotropic viral challenge

This chapter is adapted from


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I. Summary

In permissive mouse central nervous system (CNS) neurons, measles virus (MV) spreads in the absence of hallmark viral budding or neuronal death, with transmission occurring efficiently and exclusively via the synapse. MV infection also initiates a robust type I interferon (Ifn) response, resulting in the synthesis of a large number of genes, including bone marrow stromal antigen-2 (Bst2)/tetherin/CD317. Bst2 restricts the release of some enveloped viruses, but to date, its role in viral infection of neurons has not been assessed. Consequently, we investigated how Bst2 was induced, and what role it played in MV neuronal infection. The magnitude of induction of neuronal Bst2 RNA and protein following Ifn exposure and viral infection was notably higher than in similarly treated mouse embryo fibroblasts (MEFs). Bst2 synthesis was both Ifn- and Stat1-dependent. Although Bst2 prevented MV release from non-neuronal cells, its deletion had no effect on viral pathogenesis in MV-challenged mice. Our findings underscore cell type-specific differences in Bst2 function, and its impact on viral infection and pathogenesis.
II. Introduction

Many of the foundational principles in immunology have resulted from basic observations of virus-cell interactions, including the induction and antiviral function of both type I and type II Ifns (reviewed in (Goodbourn et al. 2000; Sorgeloos et al. 2013; Snell & D. G. Brooks 2015)). Viral infection of a cell typically results in cellular production and secretion of type I Ifns, which can then bind to cell surface receptors in a paracrine and autocrine fashion, leading to synthesis of interferon stimulated genes (ISGs). These ISGs, in turn, aid in clearing the viral infection by directly cleaving viral nucleic acids, triggering cellular apoptosis, inducing autophagy, upregulating MHC class I expression to aid in CD8^+-mediated cytotoxicity, and preventing viral egress. Moreover, the induction of type I Ifn contributes to the recruitment of adaptive immune effectors to infected sites, which further promotes viral clearance.

One ISG that is highly induced following infection by many viruses, and in response to both type I and II Ifn signaling, is bone marrow stromal antigen-2 (Bst2; also known as HM1.24, tetherin, and CD317) (Blasius et al. 2006). Bst2 was first discovered as a marker expressed on terminally differentiated B cells and in certain human hematopoietic malignancies (Goto et al. 1994). The discovery that Bst2 could directly prevent human immunodeficiency virus type 1 (HIV-1) virion release by tethering virus particles to an infected host cell ignited interest in this novel antiviral protein. The subsequent finding that the HIV-1 vpu protein directly antagonizes Bst2 underscored that viruses have evolved to modulate the antiviral role of this cellular protein (Neil et al. 2008; Fitzpatrick et al. 2010; Perez-Caballero et al. 2009; Van Damme et al. 2008; Hammonds et al. 2010). It has been shown that Bst2 can restrict budding of a variety of
enveloped viruses including Ebola, vesicular stomatitis virus (VSV), and herpes simplex
virus (HSV)-2 (Kaletsky et al. 2009; Weidner et al. 2010; Y. Liu et al. 2015).

Bst2 is a transmembrane protein thought to directly tether the viral membrane to
the host cell membrane (reviewed in (Hotter et al. 2013; Martin-Serrano & Neil 2011;
Evans et al. 2010)). Bst2 has also been shown to activate the NF-κB pathway,
presumably further amplifying cellular stress signals (Cocka & Bates 2012). While the
functional roles of Bst2 in uninfected cells are just emerging, it is clear that many viruses
encode proteins that subvert Bst2’s antiviral activities, often by altering clathrin-mediated
endocytic pathways (Jia et al. 2014; Serra-Moreno et al. 2013; Masuyama et al. 2009).
To date, studies aimed at understanding Bst2’s role in preventing viral infection have
been limited to immune cells and rapidly dividing cell types such as fibroblasts (Sarojini
et al. 2011). Although Bst2 is induced in primary neurons following virus infection,
whether it modulates the viral life cycle in these cells has not been explored (Cho et al.

One of our laboratory’s primary interests is to understand MV infection and
spread within CNS neurons. To do so, we use transgenic mice that express CD46, the
first identified human MV receptor, under the control of the neuron-specific enolase
promoter (NSE-CD46+ mice) (Naniche et al. 1993; Dorig et al. 1993; Rall et al. 1997).
Because mice are not normally permissive to MV infection, we can use this model to
restrict viral replication to CNS neurons. MV spread within primary neurons that are
obtained from these mice occurs through synaptic connections in the absence of
extracellular viral release, distinct from the productive and lytic infection it causes in non-
neuronal cells (Makhortova et al. 2007; Lawrence et al. 2000; O’Donnell et al. 2012).
Functional clearance of MV from the CNS of NSE-CD46+ mice is noncytolytic and
dependent on both T cells and Ifnγ (Lawrence et al. 1999; Patterson, Lawrence, et al. 2002). Interestingly, neurons differ fundamentally in the basal levels of key signaling molecules that are required for ISG induction when compared to mouse embryonic fibroblasts (MEFs) (Rose et al. 2007). During the course of previous studies, we identified numerous genes that were synthesized in primary hippocampal neurons and MEFs following Ifnγ treatment. From this list, Bst2 was among the top 5 induced genes (O'Donnell et al. 2012). Here, we investigate the induction and contribution of Bst2 in MV infection of CNS neurons both in vivo and ex vivo.

Using knockout mice and primary neuronal cell cultures, we show that induction of Bst2 is dependent on Stat1 signaling induced by type I Ifn, and that Bst2 expression can restrict MV cellular egress in a rapidly dividing, non-neuronal cell type. Moreover, Bst2 is induced by Ifn in neurons to a much greater extent than that observed in MEFs. Given this induction, we were surprised to find that the genetic absence of Bst2 had no apparent effect on neuronal viral pathogenesis following infection of NSE-CD46+ mice. Our data support a growing literature showing that neurons combat and control viral infections in fundamentally different ways than rapidly dividing cell types.

III. Results

_Bst2 is induced following interferon exposure in neurons and MEFs_

In a previous study, we identified Bst2 as among the most highly induced neuronal genes following MV infection (O'Donnell et al. 2012). To quantify this induction more fully, we cultured primary hippocampal neurons and fibroblasts from day (d) 15 embryonic mice, as previously described (Lawrence et al. 2000; Rall et al. 1997). Five d
post-plating, primary neurons and fibroblasts were exposed to 100 U/ml of murine recombinant type I Ifn (Ifnβ) or type II Ifn (Ifnγ). As expected, Ifn-treated neurons and MEFs showed a significant and rapid synthesis of Bst2 RNA relative to untreated cells (Figure 3.1 A/B). Treatment of both neurons and MEFs with Ifnβ resulted in significantly greater induction of Bst2 RNA than treatment with Ifnγ. Interestingly, increases in Bst2 induction were up to 50-fold greater in neurons than in MEFs.

To confirm that the changes in gene expression correlated with protein accumulation, we examined Bst2 protein expression after Ifn treatment by western blot and immunofluorescence microscopy. Little basal Bst2 was detected in neurons in the absence of Ifn, but increased appreciably following Ifn exposure. No increase in protein accumulation was observed in MEFs (Figure 3.1 C). When examining Bst2 protein expression at the single cell level using immunofluorescence microscopy, similar results were observed: there was an appreciable increase in detectable Bst2 protein expression in neurons after Ifn treatment, but not MEFs (Figure 3.1 D). These data indicate that Bst2 gene induction and protein accumulation occur in both neurons and MEFs, but that the magnitude of induction is greater in neurons.
Figure 3.1: Bst2 RNA and protein is induced in both primary neurons and MEFs in response to interferon, but to a higher extent in neurons.

Primary neurons spiked with mouse recombinant type 1 interferon (Ifnβ) or type 2 interferon (Ifnγ), at a concentration of 100U/ml, were assayed for changes in Bst2 RNA by RT-qPCR. Data are represented as fold-change compared to untreated cells using the ΔΔCT method. N=5 per group. Unpaired T test with equal standard deviation samples compared to 0 hour of same cell type. ** p<0.005 * p<0.05. Results of at least 3
independent experiments are presented. Error bars represent the standard deviation among groups. A) Bst2 RNA abundance in primary neurons. B) Bst2 RNA abundance in primary mouse embryo fibroblasts. C) Western blot for Bst2 and Gapdh protein from primary neurons and MEFs following Ifn exposure at a dose of 100U/ml. Image captured using LI-COR Odyssey. D) Coverslips of primary neurons and MEFs that were spiked with 100U/ml of Ifn for 48h or left untreated were stained for Bst2 (green), Hoescht (blue), and β-actin (red). Each sample set is shown as Bst2 and Hoescht staining (left), or as a merged image of Bst2, Hoescht, and β-actin (right). Primary neurons without (top) or with (bottom) Ifn treatment (left panels). Primary MEFs without (top) or with (bottom) Ifn treatment (right panels).

Bst2 blocks MV egress in non-neuronal cells

The observation that Bst2 is induced in response to Ifn exposure led us to assess the role of Bst2 in limiting MV release. We infected 293T cells that had been engineered to stably contain a tetracycline-inducible Bst2 gene, using the FLP-IN 293T-Rex system (Invitrogen) (hereafter 293T-Bst2 cells). Bst2 is not expressed in these cells in the absence of tetracycline (Weidner et al. 2010). 293T-Bst2 cells expressing or not expressing Bst2 were infected with MV, and supernatants were collected at various times post-infection and titers determined (Figure 3.2). As expected from studies of other enveloped viruses in dividing cells, Bst2 expression resulted in a >50-fold decrease in infectious MV released into the supernatant. This confirms that MV, like Ebola, HIV-1, and VSV, is susceptible to Bst2-mediated restriction.
Figure 3.2: Bst2 expression reduces MV egress in non-neuronal cells.

Supernatants were taken from MV-infected 293T-Bst2 cells with or without tetracycline at 24, 48 and 72hpi. Results of at least 3 independent experiments are presented. Error bars are used to indicate the standard deviation among groups. The number of infectious virus particles released was determined by plaque assay. Data are considered significant as determined by ANOVA. * p<0.02. N=3.

Bst2 expression is significantly induced after viral infection in primary neurons

The observation that Bst2 can limit MV release coupled with the knowledge that Bst2 is induced more abundantly in neuronal cell populations than dividing MEFs, led us to further explore the contribution of Bst2 in preventing MV spread and pathogenesis in neuronal cells. For these experiments we use a transgenic mouse model in which CD46, one of 3 identified MV receptors, and the primary receptor for vaccine strains such as MV-Edmonston, is constitutively expressed under the transcriptional control of the neuron specific enolase promoter (NSE-CD46+ mice), allowing for exclusively neuronal infection (Naniche et al. 1993; Dorig et al. 1993; Rall et al. 1997). Primary hippocampal neurons explanted from NSE-CD46+ mice were challenged with MV-Edmonston.
Expression of Bst2 RNA was significantly elevated in MV-infected neurons, increasing as the virus spread throughout the culture (Figure 3.3). Induction was dependent on replicating virus, as UV-inactivated MV did not appreciably alter Bst2 expression levels. To confirm that Bst2’s induction in response to viral infection was not unique to MV, we infected primary neurons with lymphocytic choriomeningitis virus (LCMV), another neurotropic, enveloped, RNA virus. Again, a significant increase in Bst2 RNA expression was observed, correlating with time post infection (data not shown).

Figure 3.3: Viral infection induces Bst2 RNA synthesis in neurons

Primary neurons infected with MV (MOI=1) or challenged with the same dose of UV-inactivated MV were assayed for Bst2 RNA levels by RT-qPCR. Data are represented as fold-change over untreated using the ΔΔCT method. N=3 per group. Results of at least 3 independent experiments are presented. Error bars are used to indicate the standard deviation among groups. Unpaired T test with equal standard deviation samples compared to uninfected control. ** p<0.005 * p< 0.05.
To define how Bst2 is induced following MV infection, we utilized primary hippocampal neurons obtained from several knockout mice lacking key elements of the Ifn response pathway. When CD46⁺/Ifnγ knockout primary neurons were challenged with MV, Bst2 RNA was present at similar levels to control neurons at 24 and 48 h post-infection (Figure 3.4 A). Because neurons can produce type I Ifn, we next infected CD46⁺/Ifnar KO neurons (Delhaye et al. 2006), reasoning that, though these neurons could synthesize type I Ifns in response to infection, the lack of a functional receptor would preclude them from mounting a transcriptional response to secreted Ifns. Indeed, the absence of a functional type I Ifn receptor ablated the virus-induced synthesis of Bst2 at both 24 and 48 h post-infection (Figure 3.4 B). Even at 72 h post-infection, when MV had spread extensively through the culture, as assessed by increased levels of MV nucleoprotein RNA (Figure 3.4 C), Bst2 RNA levels remained unchanged. This finding was further supported when primary neurons isolated from mice lacking Stat1, a central signaling molecule in the type I Ifn cascade, were also unable to induce Bst2 during virus infection (Figure 3.4 B). From these data, we conclude that primary neurons infected with MV synthesize Bst2 RNA through a Stat1-mediated signaling pathway triggered by type I Ifn.
**Figure 3.4:** Bst2 induction in neurons is dependent on type I Ifn signaling.

A) Primary CD46\(^+\) and CD46\(^-/\)Ifn\(^\gamma\) KO neurons were infected with MV for the indicated times and assayed for Bst2 RNA expression by RT-qPCR. B) Primary NSE-CD46\(^+\), NSE-CD46\(^+\)/Stat1 KO, and NSE-CD46\(^+\)/Ifnar KO neurons were infected with MV for the indicated period and assayed for Bst2 RNA expression. C) Relative levels of MV nucleoprotein RNA in indicated genotypes of primary neurons. Data represented as fold change over uninfected using the \(\Delta\Delta CT\) method. Results of at least 3 independent experiments are presented. Error bars represent the standard deviation among groups. N=3-4 per group. Unpaired T test with equal standard deviation samples compared to uninfected control. * \(p<0.05\).
Bst2 expression is induced in vivo after MV infection via type I Ifn signaling

To assess the role of type I Ifn signaling in inducing Bst2 expression in vivo, we infected NSE-CD46+ and NSE-CD46+/Ifnar KO mice intracranially with $1 \times 10^4$ plaque forming units of MV-Edmonston. All mice were monitored daily for signs of disease. At three days post infection (dpi), animals were sacrificed and whole brains were examined for Bst2 induction via RT-qPCR. Mice that lacked the type I Ifn receptor, and thus Ifn signaling, were unable to induce Bst2 expression, in contrast to those with an intact Ifn signaling pathway (Figure 3.5). These in vivo data support the results obtained from our primary neuronal cultures and confirm type I Ifn as a critical inducer of Bst2 synthesis in neurons after viral challenge.

Figure 3.5: Bst2 expression is induced in vivo after MV infection via type I Ifn signaling.

Mice of the indicated genotypes were infected intracranially with $1 \times 10^4$ PFU of the MV-Edmonston. Bst2 RNA expression in whole brains was assessed 3 dpi. Data represented as fold change over uninfected using the $\Delta \Delta CT$ method. N=3-4 per group. Error bars represent the standard deviation among groups. Unpaired T test with equal standard deviation samples compared to uninfected control. ** $p<0.005$. 

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Bst2 is dispensable in vivo for survival after neuronal MV challenge

We hypothesized that the strong induction of Bst2 in neurons after Ifn exposure or viral challenge, as well as the reduction in MV release from Bst2 expressing 293T-Bst2 cells, might imply a critical role for this molecule in maintaining neuronal health by either contributing to an anti-viral state, or by skewing the virus toward a mechanism of inter-neuronal spread. This was assessed following infection of various knockout mice. Bst2 KO mice and Ifnar KO mice were backcrossed to NSE-CD46+ mice; all F2 and F3 progeny were screened for CD46 and Bst2 or Ifnar expression to ensure genotypes (data not shown). Adults were then challenged with MV. Surprisingly, the absence of Bst2 did not appreciably affect pathogenesis in vivo, while the absence of the type I Ifn receptor (Ifnar), as well as its downstream signaling molecule Stat1, resulted in early mortality after infection for approximately 10-30% of infected mice (Figure 3.6 and (Cavanaugh et al. 2015; O'Donnell et al. 2012)). All NSE-CD46+/Bst2 KO mice survived infection, whereas control NSE-CD46+/Rag2 KO mice, deficient in mature B and T cells, died as a consequence of unrestricted viral spread, as previously reported (Figure 3.6 A) (Tishon, Lewicki, Andaya, McGavern, Martin & Oldstone 2006a; Patterson, Lawrence, et al. 2002). Moreover, both NSE-CD46+ and NSE-CD46+/Bst2 KO mice maintained their weight throughout the experiment, whereas NSE-CD46+/Rag2 KO mice progressively wasted until they were euthanized (data not shown). Taken together, these data show that Bst2 is significantly induced in neurons and highly dependent on type I Ifn signaling, but is ultimately dispensable for survival after neuronal viral challenge in vivo.
**Figure 3.6:** Bst2 is dispensable in vivo for survival after neuronal MV challenge.

Mice of the indicated genotypes were infected intracranially with $1 \times 10^4$ PFU of MV-Edmonston. Percent survival with days post-infection.

**IV. Discussion**

Our findings underscore the importance of considering cell type specificity when evaluating antiviral responses. While both primary neurons and fibroblasts significantly upregulate Bst2 RNA and protein in response to viral infection and Ifn exposure, primary mouse hippocampal neurons do so to a much greater extent. Although these neurons have low basal expression of many ISGs, their induction is appreciable after viral infection. The role or evolutionary advantage of lower homeostatic expression of key signaling molecules in neurons remains to be clearly defined (Rose et al. 2007; Podolsky et al. 2012). While the well-studied ISG, Bst2, was significantly induced in primary neurons and in vivo, this protein appears dispensable for survival after neurotropic
infection. Induced Bst2 expression was capable of suppressing MV release in 293T-Bst2 cells, making the lack of an effect of Bst2 following CNS viral challenge that much more surprising.

Previous studies from our laboratory, using mice deficient in the type I Ifn receptor, Ifnar, or its downstream signaling molecule Stat1, showed that functional type I Ifn signaling is important for the ability to survive a neuronal MV challenge \textit{in vivo}, with >25% of mice succumbing to infection, and all surviving animals showing lasting signs of infection and neuropathology (Cavanaugh et al. 2015; O’Donnell et al. 2012). While the Edmonston strain of MV fully activates type I Ifn signaling during infection, as opposed to WT strains, it is clear that loss of type I Ifn signaling results in CNS pathogenesis. The confirmation of Bst2 as one of the most highly induced Ifn ISGs in neurons led us to hypothesize that this gene may be a key effector in preventing MV induced death and CNS disease, though our subsequent experiments indicated that this is not the case. It has been previously shown that loss of Bst2 may hinder the ability of certain viruses to gain entry to a host cell, specifically influenza virus, vesicular stomatitis virus, and human cytomegalovirus (Viswanathan et al. 2011; Swiecki et al. 2012). Bst2 can modulate the actin network through a Rich2 complex, maintaining microvilli integrity (Rollason et al. 2009). As MV spreads trans-synaptically in CNS neurons, these functions of Bst2 might help explain why Bst2 KO mice show no pathogenic consequences after MV infection, and may implicate Bst2 as a promoter of neuronal MV transmission (Appendix).

It is increasingly clear that neuronal viral infections are resolved by the host immune response differently from infections of renewable cells. Although perforin-mediated cell killing is a primary strategy to eliminate virus from rapidly dividing and
renewable cell types, neurons are generally non-renewable, and thus it has been proposed that these same cytolytic strategies may be more harmful than beneficial within the brain. For a number of viral model systems, it has been shown that cytokine-mediated, non-cytolytic strategies control many neurotropic infections (Burdeinick-Kerr et al. 2009; Griffin 2010; Patterson, Daley, et al. 2002; Griffin & Metcalf 2011; St Leger & Hendricks 2011; T. Liu et al. 2000; Wakim et al. 2012; Wakim et al. 2010). These data suggest that the mechanisms by which viral infections are resolved are exquisitely cell type-specific, and may reflect evolutionary pressures to restrict an infection while minimizing cytopathology and tissue damage. Even more, differences among neuronal subpopulations may further stratify the response to a viral infection, as was recently shown for West Nile virus (Cho et al. 2013). Our studies underscore the need to consider cell type differences when defining antiviral mechanisms; doing so may lead to more precisely tailored therapeutic strategies to resolve life-threatening viral encephalitic diseases.
V. Conclusion

Viral infections of the central nervous system can lead to debilitating disease and death. Moreover, it is becoming increasingly clear that non-renewable cells, including most central nervous system neurons, combat neurotropic viral infections in fundamentally different ways than other rapidly dividing and renewable cell populations. Here we identify type I interferon signaling as a key inducer of a known anti-viral protein (Bst2) in neurons. Unexpectedly, this gene is dispensable for clearance of neurotropic viral infection despite its well-defined contribution to limiting the spread of enveloped viruses in proliferating cells. A deeper appreciation of the importance of cell-type heterogeneity in antiviral immunity will aid in the identification of unique therapeutic targets for life-threatening viral infections.
CHAPTER 4: Discussion and Future Considerations

Portions of this chapter are adapted from:

Miller KD, Rall GF. What Kaplan-Meier survival curves don't tell us about CNS disease. 


We gratefully acknowledge Dr. Christine Matullo and the rest of the Rall lab for their contributions in discussing this manuscript as well as our funding sources from the National Institute of Health: F31 NS092307 and T32 NS007180-32 (KDM).
I. Summary

Central nervous system (CNS) consequences of viral infections are rare, but when they do occur, they are often serious and clinically challenging to manage. Our awareness of the perils of neuroinvasion by viruses is growing: the recently appreciated impact of Ebola and Zika virus infections on CNS integrity, decreases in vaccination coverage for potentially neurotropic viruses such as measles, and increased neurovirulence of some influenza strains collectively highlight the need for a better understanding of the viral-neural interaction. Many such infections cause severe acute disease, but the potential for neurotropic viruses to cause long-lasting damage has not been fully explored. In this chapter, I discuss some of the unique observations presented in this dissertation and integrate our data with a growing literature to provide some early insights into how CNS neurons, when faced with a viral infection that spreads trans-synaptically, are spared from lysis, thus creating a harbor for long-term persistence and potential pathogenic consequences later in life. At the conclusion of this chapter, I will draw attention to an often overlooked aspect of neuropathogenesis research: that lack of overt disease, often equated with survival post-infection, likely only scratches the surface of the myriad ways by which neurotropic infections can impair CNS function.
II. Introduction

All living things, from bacteria to humans, are potential hosts for viral infections. Though readers of this dissertation have so far survived these challenges, viral infections are not without impact or lasting consequence. Pathogens cause a wide range of outcomes, from short-lived inconveniences such as congestion, body aches, and lethargy, to more serious manifestations, including diarrhea, high fever, liver cirrhosis, and encephalitis. The identification of measles virus (MV) RNA in the CNS of immunocompetent mice long after viral challenge was the starting point for one of the major studies presented in this dissertation (Chapter 2), and begged the question: why would a host allow a possibly deadly encephalitic virus to be maintained long-term? There were a variety of directions that this project could have gone, including defining the “state” of persisting virus, establishing how viral RNA is maintained long-term, profiling the immunological consequences that may arise from the long-term presence of “non-self”, and describing the host consequences when faced with viral reactivation. Ultimately, I made the decision to understand the pathogenic consequences following RNA viral reactivation in the brain, reasoning that such an event would illuminate both the immunological (how is viral replication inhibited?) and virological (can maintained viral RNA reactivate?) mechanisms of this phenomenon.

The discovery that persistent viral RNA can reactivate and lead to host pathogenesis was intriguing, as, to date, RNA viruses are generally not thought to persist in a replication competent state for extended periods of time. Further, the site of viral persistence and reactivation made these findings even more exciting as they lend credence to the hypothesis that both brain region and neuronal subtype play active roles in dictating the course of a neuroviral infection. Defining the mechanism by which the host immune response actively suppresses viral replication in the absence of neuronal
cell death, and establishing the state of persisting virus in the neuronal cytoplasm will be extremely interesting avenues to explore in the future. Further, when considering the role of brain region and neuronal subtype, the observations presented in Chapters 3 and 4 concerning BST2 expression and function highlight the nuances that may arise in individual cell types when faced with a viral challenge. Together, these data underscore the need to pay closer attention to cell-type specific differences that occur after viral infection.

III. Host Immunity Actively Suppresses Viral Replication in Neurons without Causing Neuronal Loss

Although non-lytic viral control may allow neurons to survive an antiviral immune assault, a consequence of such a strategy is that non-cytolytic viruses are incompletely eradicated from the brain, as observed in NSE-CD46+ mice. Following acute viral control, immunocompetent MV infected mice maintain viral RNA for months to years in a replication competent form without clinical manifestations of CNS disease (Chapter 2 and data not shown). Detection of viral RNA long after infection is not a new finding: many viruses, including those with RNA genomes, can be maintained months after infectious virus falls below detection limits, and in the absence of overt signs of CNS disease (Schubert 2006; Graham et al. 2016; Griffin & Levine 1992). But for many of these studies, it has been assumed or proposed that these RNAs are not replication-competent, but rather inert “fossils” marking a previous infection. We set out to test a different hypothesis: that such long-lasting infections, while not lethal to the host, could nevertheless impair neuronal function and host cell metabolism if reactivated (Sun et al. 2016; Gomme et al. 2012). Moreover, the CNS diseases associated with chronic viral reactivation may not be the same as those seen in acute infection, and may in fact be
subtler. For example, infection of neonatal mice with lymphocytic choriomeningitis virus (LCMV) leads to a life-long infection associated with learning and memory defects (Brot et al. 1997). In these mice, viral persistence is associated with decreased neurotransmitter release (somatostatin) (Lipkin et al. 1988) without overt neuronal loss or other signs of neuropathology. Thus, non-cytolytic viral control (the continued clearance of virus from infected cells in the absence of cell death) in neurons may be double-edged, enabling survival of crucial and generally irreplaceable cells, but providing a haven for persistence of non-cytopathic viruses.

The plethora of neurotropic viruses that are known to be controlled in a non-cytolytic manner (e.g. rabies virus, sindbis virus, measles virus, West Nile virus, herpes simplex virus, varicella virus) (Kinchington et al. 2012; Katayama et al. 1998; Griffin 2010; Graham et al. 2016) highlights that non-cytolytic immune-mediated control is not necessarily virus-specific, but may reflect a general host strategy to retain these crucial and generally irreplaceable cells. Non-cytolyic immune control of viral infection is not wholly limited to neurons within the CNS, as clearance of mouse hepatitis virus infection from oligodendrocytes also occurs in a non-cytolytic manner (Parra et al. 1999). Thus there appear to be conserved mechanisms that modulate or prevent immune mediated cytotoxicity after viral infection of the CNS.

The mechanisms by which the immune response hinders viral replication while sparing the infected cell is not clearly defined in all of examples noted above, especially for those viruses with an RNA genome. In chapter 2, I have made the case that T resident memory cells (T<sub>rm</sub>) are a key immune component necessary for preventing viral reactivation within the CNS. However, it is worth noting that only approximately 50% of T cells present in the brain during viral persistence express the hallmark T<sub>rm</sub> CD
antigens, CD103 and CD69. This leads to the question of the function of the remaining 50% of brain CD8\(^+\) T cells: is it possible that such cells are merely “passing through” and playing no active role in suppressing viral reactivation, or can these T cells contribute to suppressing viral replication via a secondary or alternative mechanism?

The use of antibody-mediated immune depletions to define the functions of individual immune cells are unlikely to be helpful in answering these questions, as their access to the brain is restricted by the blood-brain barrier. Therefore, new mouse models and drugs that can directly target and deplete individual cell types from both the periphery and CNS will be required to understand the function of specific CNS-resident immune populations during persistent infection. One approach that could be extremely useful is the targeted expression of the diphtheria toxin receptor (DTR) on specific cell types. In this approach, administration of diphtheria toxin (DT), which efficiently crosses the BBB, leads to the eradication of cells expressing DTR (Reuter et al. 2012). Thus, specific cell types could be selectively depleted if they are engineered to express DTR, such as CD103\(^+\) or interferon gamma-expressing cells. Using this model, one can accurately assess the contribution of individual cell types in suppressing viral reactivation.

While most of the studies presented in Chapter 2 focused on viral reactivation in the absence of adaptive immunity, it is clear that innate immune signaling may also play a critical role in the clearance and control of persisting virus, as evidenced by significant increases in detectable viral RNA after long-term infection of NSE-CD46\(^+\)/IFNAR KO mice (data not shown). These data suggest that type I interferons may aid in controlling persistent neuronal infection, and may account for the observed lack of 100% penetrance in pathogenesis after viral reactivation; e.g. loss of adaptive immunity alone.
may not be wholly sufficient to lead to CNS pathogenesis following viral reactivation, as type I and III interferons can still exert their anti-viral functions. Future studies using persistently infected NSE-CD46+/IFNAR KO mice that are transformed, through bone marrow chimaera strategies, to lack both adaptive immune cells and type I interferons (NSE-CD46+/IFNAR KO/RAG2 KO mice) would be informative for the elucidation of the role of interferons in controlling viral reactivation.

The importance of type I interferon signaling in controlling neurotropic infections is clear, evidenced by the differential ability of West Nile virus to infect varying neuronal populations due to differences in type I interferon signaling (varying profiles of interferon stimulated genes (ISG) amongst sub-populations) (Cho et al. 2013). Interestingly, the mechanisms by which interferon stimulated genes control neuronal infections may not be identical to their traditionally described modes of action in non-neuronal cells (Chapters 3 and 4). Our studies with BST2 in neurons underscore this point. The inability to detect infectious MV during neuronal infection led us to hypothesize that BST2 may prevent MV release by tethering viral particles to the cell membrane, as observed for other enveloped viruses in non-neuronal cells (Y. Liu et al. 2015; Perez-Caballero et al. 2009). To my surprise, my analysis determined that BST2 was dispensable for MV control in neurons (Chapter 3). Even more, further studies showed that BST2 appears to enhance MV spread in neurons of the CNS, demonstrating a “pro-viral” role of this ISG (Chapter 4). Pro-viral roles of BST2 have recently been demonstrated for other viruses, including vesicular stomatitis virus, cytomegalovirus, and HIV (Viswanathan et al. 2011; Swiecki et al. 2012; Londrigan et al. 2015), though not in neurons. However, the exact mechanism by which BST2 exerts pro-viral actions has yet to be clearly defined and remains an active area of investigation. Some have speculated that the ability of BST2 to tether emerging virions from an infected cell creates a physical
linkage that enables a budding viral particle to maintain close proximity to adjacent uninfected cells, perhaps facilitating cell-to-cell transfer, while preventing extracellular viral release. Thus, BST2 may aid in “bridging the gap” between cells that are in close proximity to one another. In the case of CNS neurons, our early data on the cellular localization of this ISG suggests that this may not be the case, as MV spreads predominantly via synaptic junctions and BST2 is not found in purified synaptic preparations. Therefore, BST2 may be playing another, role other than physically bridging the gap between synaptically connected neurons. Understanding the normal cellular processes carried out by BST2 will be critical in better understanding its function during viral infection.

One caveat to consider when discussing the experiments presented in this dissertation concerns the use of primary hippocampal neurons. While primary neuronal cultures have been vital to many of our understandings of neuronal function and signaling, and are clearly superior to neuroblastoma cell lines that do not recapitulate many attributes of neurons in vivo, they only offer a limited picture to the complexity of the brain. Oligodendrocytes, astrocytes, and microglia contribute to the intricate meshwork and communication networks essential for a fully functional neuronal network, and their absence from a powerful but simplistic primary neuron culture may not allow a direct correlation between in vitro cultures and viral spread and pathogenesis within the fully intact CNS. How these interactions may contribute to ISG induction and function has yet to be fully realized. Further, the use of hippocampal neuron cultures alone can only offer insight into the mechanisms of viral clearance in this individual neuronal sub-type. As Diamond and colleagues showed, differences among neuronal sub-populations do exist, and thus future studies will determine if parallel pathways are being utilized in other cellular and neuronal sub-types (Cho et al. 2013).
In the future, to easily and readily deduce the contributions of individual neuronal populations, brain regions, and interferon responses during MV persistence, one can use Gt(Rosa)26Sor Im4(ACTB-tdTomato-EGFP) J mice (hereafter, ROSA) (Gomme et al. 2012). ROSA mice constitutively express tdTomato in all cells. After exposure to Cre recombinase, the TdTomato, flanked by two loxp sites, is excised, allowing for expression of a downstream EGFP (Muzumdar et al. 2007). We have backcrossed ROSA mice to NSE-CD46+ mice generating NSE-CD46+/ROSA mice that allow for neuronal infection with MV. In parallel, we have engineered a recombinant Edmonston vaccine strain of MV expressing Cre recombinase (Cre-MV). Infection of NSE-CD46+/ROSA mice with Cre-MV leads to the Cre-induced irreversible change from tdTomato expression in infected cells to EGFP expression. Thus, this system allows for a straightforward identification of cells that have been infected with MV, via a permanent somatic change. Providing these cells survive infection, as we know that they do (via non-cytolytic viral control and absence of virus-mediated neuronal loss), we can now analyze, using FACS and laser capture microdissection techniques, the unique signatures and profiles of persistently infected neurons, as well as neurons that were once infected but have fully resolved the infection (and are no longer viral RNA-positive). This technique has previously allowed for the identification of neuronal impairment following rabies virus infection of CNS neurons (Gomme et al. 2012). Together, these studies will further define the role of adaptive and innate immunity in controlling persistent infections in the CNS and add to the growing literature aimed at understanding the distinctive outcomes following neurotropic infection.
IV. Viral Promotion of Persistent Infection

The recent identification of “arbitrium” signaling (Latin for “decision” signaling) in directing lysis-lysogeny decisions made by bacteriophage during the course of an infection has highlighted the novel ability of viruses to communicate with their progeny (Erez et al. 2017). In these studies, the authors demonstrate that during the course of bacteriophage infection of *Bacillus subtilis*, a phage-derived small peptide fragment (AimP) accumulates to high levels in culture medium as the bacteriophage population surges through lytic viral replication. High concentrations of AimP can then promote its internalization by uninfected bacterium. Upon phage infection of these AimP-carrying bacterium, a lysogenic (vs. lytic) program is promoted via AimP’s interaction with the phage genome and other phage-derived proteins. In sum, a peptide derived from parent virus can “instruct” progeny virus to maintain a non-lytic lysogenic state. As levels of AimP fall due to decreased lytic viral replication in a culture (and increased host bacterium replication), lysogenized phage can then transition back to a lytic state and resume producing more phage progeny. Thus, the idea that a virus is an inert replication machine intent on producing the maximal number of infectious particles may be limited, as viral replication appears to be far more regulated. The direct signaling of a progenitor virus with its progeny in directing a lysogenic fate, as opposed to lytic viral life cycle in which many thousand more progeny viruses are created, is a means by which a virus can preserve itself in the absence of replication. While this phenomenon has thus far been limited to bacteriophage, it is interesting to consider if parallel mechanisms could influence the lytic to non-lytic transition seen in eukaryotic viral infections of neurons.

Aside from virally directed promotion of persistence (e.g., lysogeny, in the case of bacteriophage, during which viral DNA is indistinguishable from host DNA) it is unlikely that persistent viral RNAs are maintained in a naked state within a host cell. More likely,
persistent MV RNA can directly interact with RNA binding proteins within a cell (which are abundant in neurons) and be maintained in stress/neuronal granules (Protter & Parker 2017). The physical architecture of neurons in which the cell body, containing the genomic information required for survival, is highly separated by the axon from the dynamic signaling networks seen at the synapse helps to explain the importance of RNA granules and localized transcription seen in neurons. For a cell to respond quickly to external stimuli (e.g. interferons and growth factors) it must be able to quickly change its transcriptional and translation profiles. If RNAs were not readily available at the functional synapse, a neuron would instead need to rely on transmitting external stimuli from the synapse through the axon to the nucleus, followed by a reverse journey in which the required transcripts and proteins then traffic back from the cell body to the synapse to exert their function. To circumvent this time-consuming process and allow neurons to quickly respond to localized stimuli, neurons have evolved to maintain RNAs and translational machinery at the site of need: the synapse (Luchelli et al. 2015; Buchan 2014; Protter & Parker 2017). Therefore, it is possible that cytoplasmic MV RNA can be maintained and stabilized by neuronal RNA binding proteins. Alternatively, the stability provided to MV RNA through generation of the ribonucleocapsid may enable MV to withstand cellular RNAse activity, allowing for the long-term maintenance of viral RNAs in the absence of binding neuronal proteins. Defining the physical state of viral RNA during persistent infection (and where, within the neuron, the RNA is sequestered) will be paramount to understanding how inherently labile RNA molecules are maintained in the neuronal cytoplasm long-term, and whether viral proteins aid in directing this long-term stability.

Defective viral genomes (DVGs), or viral genomes incapable of replicating without helper virus, have been speculated as a mode by which RNA viruses can persist
in the absence of host clearance, as virus is not actively replicating, viral RNAs fall below limits of detection in a host cell (Lopez 2014). DVG formation after MV infection has been shown previously (Shingai et al. 2007). However, it has also been demonstrated that non-defective (replication competent) MV can induce a persistent infection in cultured cells via an auto-regulation mechanism; as high levels of the MV nucleocapsid protein (N) accumulate within an infected cell, viral replication is slowed, presumably due to the inability of the MV polymerase to access viral RNA tightly bound to N (Doi et al. 2016). While both the presence of MV DVGs and MV auto-regulation appear to be plausible hypotheses to explain the ability of a cytoplasmic RNA virus to persist long-term, the latter mechanism is more likely. The hypothesis that persistent MV RNA is maintained as a DVG, unable to replicate in the absence of helper virus, is unlikely, considering the ability of MV to fully reactivate after long-term persistence. Identifying the genomic sequence of persistent MV RNA will generate a better understanding as to possible genomic mutations, or lack there of, that enable MV to persist.

V. Clinical Consequences of Viral Persistence

While the clinical manifestations of long-term RNA viral persistence have yet to be fully realized, many CNS disorders of unknown etiology have long been speculated to have viral triggers, such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Jarius et al. 2016; Hottenrott et al. 2017; Li et al. 2015). However, in many instances, the inability to recover infectious virus or consistently detect viral genomes in patient tissue has cast doubt on viral etiologies of these inflammatory CNS diseases. The inability to reproducibly identify viral RNAs, proteins, or particles in host tissues may
be due to technical challenges (e.g., limits of detection or inconsistencies amongst tissues sampled between groups). Regardless of the inability to detect viral components, in the case of MS, a positive MRZ reaction (intrathecal antibody directed against 2 of 3 viruses; MV, rubella virus, or varicella zoster virus) is an efficient prognostic indicator of MS (however, this is not used clinically), suggesting some relation to a viral infection acquired earlier in life and disease development. Further, persistent MV infection has also been speculated as a causative agent of otosclerosis (localized bone displacement resulting in hearing loss) (Potocka-Bakłażec et al. 2014; B. E. Cohen et al. 2014; Rudic et al. 2015) suggesting again that an infection acquired earlier in life can contribute to distinct diseases that are not typically attributed to a viral etiology.

While viral genomes or infectious virus may not be consistently recovered from these patients, it does not preclude the possibility that earlier infection may have triggered neuronal dysfunction or autoimmune activation, ultimately resulting in disease (H. A. Jurgens et al. 2012; Brot et al. 1997; Gomme et al. 2012). The long-term presence of non-self antigens maintained through persistent infection, even in the absence of viral replication, may evoke immune responses if foreign peptides are expressed at the surface of an infected cell, long after the initial active infection has cleared. Further, the role of antibody secreting cells (ASCs) in the promotion of clinical CNS diseases during persistent viral infections must be considered. During mouse hepatitis virus and Theiler’s murine encephalomyelitis virus persistent infections of the CNS, ASCs predominantly secrete antibody directed against viral antigens, but a subset of ASCs can begin to secrete antibodies with the ability to cross react with components of the myelin sheath surrounding neurons, contributing to clinical CNS disease (Phares, Stohlman & Bergmann 2013b). Regardless of the mechanism by which persistent or earlier acquired infections contribute to CNS disease, it is clear that infections of the CNS can lead to
sequelae different than those seen during an acute infection (Chapter 2). The ability of persistent infection to cause disease different from that observed during the acute infection is further evidenced when considering varicella zoster virus; initial infection results in chicken pox while reactivation of persistent infection results in the development of shingles (Kinchington et al. 2012). The CNS appears readily primed to harbor persistent viral infections, due to non-cytolytic viral control as well as possible viral mutations accumulating during replication; therefore viral causes of disease must be reconsidered in the context of inflammatory CNS diseases of unknown etiology.

VI. The Mouse Physical Exam

While there is little doubt that murine models of neuropathogenesis have proven extraordinarily valuable in understanding and defining how viral infections can cause CNS disease, simply reporting outcomes in a binary way (death or survival) is likely ignoring lessons that can be learned from these models. The 100% survival of NSE-CD46\(^+\) and NSE-CD46\(^-\)/BST2 KO mice after MV infection clearly highlight this point, as NSE-CD46\(^+\) mice maintain viral RNA long after initial clearance and NSE-CD46\(^-\)/BST2 KO mice maintain reduced levels of viral RNA after infection (Chapters 2 and 4). Had viral RNAs not been analyzed in these infected animals over time we would have overlooked the subtle observations that led to the novel discoveries presented in this dissertation; MV can persist and reactivate in the CNS long after acute infection and BST2 enhances neuronal MV spread.

How might we better assess non-fatal consequences of neurotropic infections, aside from simply monitoring viral loads in the brain? I propose three additions or alterations to survival studies often used in mouse pathogenesis efforts. First, basic vital
functions should be collected on infected mice throughout the course of infection, including weight changes, blood pressure, temperature, and pulse oxygen saturation. A quick Google search uncovers multiple companies that offer equipment to reliably and quickly collect these data from mice, in some instances simultaneously. Inclusion of these data may aid in defining dis-regulated regions of the brain during infection or ongoing neurological disease (for example: the hypothalamus for temperature, and the brainstem and medulla oblongata for breathing). Second, while detailed memory and behavior tests in mice require dedicated equipment and laboratories, many motor and cognitive tests to assess a mouse’s balance and general awareness/cognition are easily done with limited time, expertise, and expense, even for investigators without access to highly sophisticated tools. For example, a cylinder test can quickly identify unilateral motor deficits. In this test, a mouse is placed into a clear cylinder and the number of load bearing contacts the animal makes with its limbs when rearing is quantified. A normal mouse will make an equal number of load bearing contacts with left and right forelimbs; in contrast, favoring one limb over another may indicate damage in specific hemispheres of the brain leading to a loss of motor control, as seen in human stroke patients (Mani et al. 2013; S. P. Brooks & Dunnett 2009). Simple motor tests have identified motor and rearing abnormalities associated with hippocampal dysfunction after infection with both influenza and dengue virus (Amaral et al. 2011; H. A. Jurgens et al. 2012). Another straightforward motor phenotype analysis is to perform a footprint analysis, in which the mouse’s front and rear paws are dipped in a dye that leaves footprints as the mouse walks across absorbent paper. By measuring the length and width of strides, gait disturbances can be identified and quantified (S. P. Brooks & Dunnett 2009). Moreover, open field and grooming tests can also identify cognitive and processing phenotypes. In the open field test, a mouse is placed into an open space
surrounded by walls; thereafter, the time spent in center quadrants is measured. A typical healthy mouse will immediately proceed to the wall of the field and explore around its edges until it feels comfortable to explore more open areas. Mice experiencing anxiety or motor deficits will show other behaviors, which can include a lack of movement from the open area, or a lack of curiosity about the new space (S. P. Brooks & Dunnett 2009). Similarly, the grooming test is performed by observing a mouse in its home cage, and quantifying the time spent grooming over a 10 minute period. Healthy animals spend approximately 10% of their time grooming, while animals exhibiting repetitive behaviors may spend increased time on this task (Tsai et al. 2012).

A more complete explanation of the potential benefits (and pitfalls) of using these approaches to identify motor and cognitive defects in mouse models was published in an excellent 2009 review by Brooks and Dunnett (S. P. Brooks & Dunnett 2009). Finally, I propose that instead of photographs to document a particular morbidity, investigators provide links to videos in their publications allowing the reader to have a more complete understanding of observed disease phenotypes; the ubiquitous presence of cell phones with high-quality video capacity makes this once-challenging task straightforward.

Certainly, many investigators have adopted strategies such as these in evaluating cognitive or motor deficits, but most of these studies are found within the neurobiology literature, and are less prevalent in the neurovirology/neuroimmunology fields. I make the simple case here that such efforts could be time well spent, and could accelerate new discoveries or deeper insights into how neurotropic viruses might impact host biology, especially when the diseases that persisting viruses may cause are more subtle than those observed during acute challenge.
VII. Conclusion

The studies presented in this dissertation add to a growing literature aimed at elucidating cell type specific differences following viral infection. I demonstrated the unique ability of a cytoplasmic RNA virus to persist in CNS neurons for extensive periods (months-years) in the absence of clinical symptoms of disease, presumably as a result of trans-synaptic viral spread and non-cytolytic immune control of viral infection. I then showed that virus RNA replication, transcription, and protein synthesis can re-initiate and cause host pathogenesis in response to losses of host adaptive immunity. Further, studies aimed at understanding the role of interferon stimulated genes (ISGs) during neurotropic viral infection highlight the ability of BST2, often characterized as an antiviral ISG, may in fact promote viral replication in primary hippocampal neurons. In sum, this dissertation urges the reader to consider the cell type-specific differences in viral replication, host immunity, and individual protein functions, particularly within the CNS.

Finally, in this discussion I have made the case for a more thorough "exam" of mouse physiology after viral infection, though a reasonable counterpoint is that these suggested ancillary tests could become time-consuming. To this point, we are reminded of Stanley Cohen’s Nobel Prize lecture in 1986, in which he credited his discovery of epidermal growth factor to the “side effects” of precocious eyelid opening and tooth eruption (S. Cohen 1986). In his lecture, Cohen notes that, had he not paused to watch his mice, this important but subtle clue would have gone unnoticed, and would have likely postponed the paradigm-shifting discoveries that followed. Similarly, had I not assessed MV RNA loads in apparently healthy mice long after viral inoculation, I would have missed the novel discoveries presented in this dissertation.
CHAPTER 5: Materials and Methods

I. Ethics Statement

These studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were reviewed and approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee (Office of Laboratory Animal Welfare assurance number A3285-01).

II. Cell Culture, Interferon Treatment and Virus Infections

Primary hippocampal neurons were obtained from day 14-16 mouse embryos and cultured in neurobasal media (Gibco) supplemented with L-glutamine in the absence of an astrocyte feeder layer, as described previously (Rall et al. 1997; Lawrence et al. 2000; Makhortova et al. 2007). Neurons were plated on 15-mm glass coverslips or in 12-well plates coated with poly-L-lysine (Sigma-Aldrich) at a density of $2 \times 10^5$ cells/well, unless otherwise noted. Neuron cultures were frequently quality-controlled, and were routinely >95% Map2-positive. Neurons were plated and incubated for 5 days (d) prior to Ifn treatment or infection to allow for full differentiation. Primary mouse embryonic fibroblasts (MEF) were isolated from the same embryos and maintained in complete DMEM (DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin). All cells were maintained at 37°C, 5% CO$_2$ in a humidified incubator. For cells treated with Ifns, recombinant murine Ifnβ or Ifnγ (Millipore) was diluted in B27-free Neurobasal media, added to the cultures (100 U/ml final), and incubated for the indicated times prior to collection.

MV-Edmonston (vaccine strain) was purchased from American Type Culture...
Collection and passaged and titered in Vero cells, (obtained originally from African green monkey kidney epithelium). Passages 2 or 3 of the MV stock were used for all infections. LCMV Armstrong (LCMV-Arm; ATCC) was passaged and plaque purified in BHK-21 cells (baby hamster kidney fibroblasts), and titers were determined on Vero fibroblasts. All infections of primary cultures were carried out at a multiplicity of infection (MOI)=1. Briefly, conditioned media was removed and cells were inoculated with 500µl of virus diluted in unconditioned Neurobasal medium or DMEM for 1 hour (h) at 37°C. After infection, cells were gently washed and conditioned medium replaced until indicated time of harvest. 293T-Rex cells (Invitrogen) were modified to inducibly express Bst2 (293T-Bst2 cells) upon tetracycline exposure, (1ug/ml), and were a gift from Dr. Ju-Tao Guo (Drexel University). These cells were maintained as described in the original report (Weidner et al. 2010). To induce Bst2 expression, cells were treated with tetracycline for 24h, and were then infected with MV at a multiplicity of infection (MOI) of 1.0 for 1h. Virus was then removed and replaced with fresh media, with or without tetracycline.

**Synaptosome Purification**

Cells were washed with cold PBS and scraped into cold 0.32M sucrose/PBS. This was followed by slow freezing (in a Styrofoam box) at -80°C. Samples were then thawed briefly in a 37°C water bath, followed by 30 seconds of homogenization with a handheld motorized pestle mixer. Samples were then fractionated at 1.2xg for 20 min at 4°C. Supernatants were transferred to a new tube and spun at 12.4xg for 10 min at 4°C. Supernatants (non-synaptic fraction) and pellets (synaptic fraction) were re-suspended in Illustra Triple Prep kit (GE Healthcare) buffer, and then RNA and protein where purified according to manufacturer’s instructions.
III. RNA and Protein Analysis

*Reverse transcriptase quantitative real-time PCR (RT-qPCR)*

RNA was purified from whole cell lysates using the RNeasy Mini kit (Qiagen). RNA was quantified using a Nanodrop spectrophotometer. RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random hexamer priming (total RNA) or oligo dT (mRNA enriched) priming. Gene-specific primers were used in combination with probes designed using the Universal Probe Library algorithm (Roche) and Universal Master mix (Roche); all reactions were run on a Mastercycler Realplex2 system (Eppendorf). Cycling conditions were 50°C, 2 min; 95°C, 10 min; followed by 40 (2-step) cycles (95°C, 15 sec; 60°C, 60 sec). Relative quantification to the control (cyclophilin B) was done using the comparative ΔΔCT method. Individual sample PCR reactions were performed in triplicate. The following gene specific primers (Integrated DNA Technologies) were used: Cyclophilin B Forward – 5’ – TTC TTC ATA ACC ACA GTC AAG ACC - 3’, Cyclophilin B Reverse – 5’ – ACC TTC CGT ACC ACA TCC AT - 3’, UPL 20; Bst2 Forward- 5’ - GAA GTC ACG AAG CTG AAC CA - 3’, Bst2 Reverse- 5’ – CCT GCA CTG TGC TAG AAG TCT C - 3’, UPL 78; MV nucleoprotein Forward - 5’ – GGA AAC TGC ACC CTG TAG TGC TAG AAG TCT C - 3’, UPL 80. MV fusion protein Forward - 5’ - GGT CCG ATG ACT CCA ACA AT - 3’, UPL 88. MV matrix protein Forward - 5’ - AAT TCA GAT CGG TCA ATG CAG - 3’, MV matrix protein Reverse- 5’ - CCT ATC GCC TTG TCA ATC CT - 3’, UPL 62. MV hemagglutinin protein Forward - 5’ - AAA TTG GAT TAT GAT CAA TAC TGT GC - 3’, MV hemagglutinin protein Reverse- 5’ - CAG TAG AGT TGA GTT CAC CAA TG - 3”, UPL 5.
Western Blot Analysis

Whole cell lysates for western blot analysis were collected by scraping cells in 1X protein solubilization buffer (containing SDS and EDTA) or protein was obtained from homogenized brain tissue using TRIreagent (Sigma) as per manufacturers instructions and stored at -80°C until analysis. Protein was run into a 10% Bis-Tris gel (Life Technologies) in MES running buffer (Life Technologies) and transferred to an Immobilon membrane (Millipore). Membranes were blocked with blocking buffer (Odyssey) and immunoblotted for Gapdh (Millipore AB2302; 1:10,000), Bst2 (Sigma PRS4661; 1:1000), human polyclonal MV (a gift from Michael B. Oldstone 1:3000), or MV fusion protein (Bioss; 1:1000). Secondary antibodies were obtained from LI-COR (IRDye® 680RD Donkey anti-Chicken IgG (H + L); IRDye® 800CW Donkey anti-Rabbit IgG (H + L)). Images were captured with LI-COR Odyssey Classic Infared Imager.

Immunofluorescence

Primary neurons and MEFs were plated on coverslips and treated as previously described. Coverslips were fixed using 4% paraformaldehyde/4% sucrose in phosphate buffered saline, followed by further fixation and permeabilization with 100% methanol and then with 0.2% Triton in PBS. Coverslips were blocked with 10% goat serum, 10% fetal bovine serum in PBS. Primary antibodies for Bst2 (Sigma; 1:200) and mouse β-actin (Sigma; 1:2000) were applied. Directly conjugated secondary antibodies were used (Hoescht; 1:1000, Donkey anti-rabbit AF488; 1:5000, Goat anti-mouse AF555; 1:5000). Coverslips were mounted to slides using Citifluor AF1 (Electron Microscopy Sciences) and sealed. Images were captured at 40x using an inverted TE2000 Nikon C1 confocal microscope.
IV. *In vivo Infection, Irradiation, and Reconstitution*

Homozygous NSE-CD46⁺ transgenic mice (line 18; H-2ᵇ) (Rall et al. 1997) were maintained in the closed breeding colony of the Fox Chase Cancer Center. Homozygous NSE-CD46⁺ and haplotype-matched homozygous immune knockout (KO) mice were intercrossed for three or more generations to obtain NSE-CD46⁺ mice on the desired KO background. STAT1 KO mice were obtained from Jackson Laboratory (Meraz et al. 1996). IFNAR KO (Muller et al. 1994) mice were obtained from Luis Sigal (Fox Chase Cancer Center, Philadelphia PA) RAG2 KO (Shinkai et al. 1992) mice were a gift from F. W. Alt (Howard Hughes Medical Institute, Boston, Mass). The genotypes of perforin KO (Kagi et al. 1994), IFNγ KO (Dalton et al. 1993) along with all animals used in these studies was confirmed by PCR analysis of tail biopsy DNA.

Isoflurane-anesthetized mice were infected with MV-Edmonston via intracranial inoculation of $1\times10^4$ PFU in a volume of 30 µl, delivered along the midline using a 27g needle. Mice were monitored daily post-infection for signs of illness, including weight loss, ruffled fur, ataxia, and seizures. Moribund mice were euthanized in accordance with IACUC guidelines. RNA was isolated from individual mice at indicated times post infection using TriReagent (Sigma) and subjected to analysis as described above.
Irradiation and Bone Marrow Chimaeras

**Sub-lethal irradiation:** Mice infected for at least 90 dpi were subjected to sub-lethal (6.5gy) panoramic gamma irradiation using a Shepherd Model 81-14R Cs-137 panoramic irradiator. Animals were monitored daily for signs of illness until indicated time of collection. Transient immune depletion was verified by flow cytometric analysis of peripheral blood lymphocytes obtained from the retro-orbital sinus of isoflurane anesthetized mice.

**Bone marrow chimaera generation:** Mice infected for at least 90 dpi were subjected to lethal (2 doses of 5.5 gy separated by 4 hours) panoramic gamma irradiation using a Shepherd Model 81-14R Cs-137 panoramic irradiator and immediately placed onto a medicated diet (Uniprim chow). In parallel, donor animals were euthanized and bone marrow extracted from the femur and tibia, which was then pooled from individual donor animals and passed through a 70 µm cell strainer. Red blood cells (RBC) were depleted using ACK lysis buffer and cells were washed twice with sterile PBS. The isolated cells were counted using a Countess® cell counter. 10 x 10^6 cells were suspended in 100-200µl of PBS, and administered to irradiated recipient mice via retro-orbital sinus 18 hours after the final irradiation of the recipients. Reconstituted animals were observed daily for signs of illness. Depletion and reconstitution was confirmed by flow cytometry analysis of splenic and brain lymphocytes. Further confirmation was performed by staining stored splenic tissue from individual animals.
**Flow cytometric analysis of purified lymphocytes**

Brains and spleens were removed and pressed through a 70 µm nylon mesh cell strainer in PBS. Dissociated tissue was run over a 70% Percoll gradient for 20 m at 4°C. Mononuclear cells (MNCs) were recovered from the pellet, washed with PBS, treated with 0.84% ammonium chloride to remove contaminating red blood cells (RBCs) and washed again. Collected MNCs were counted and plated into a v-bottom 96-well plate for subsequent antibody staining and analysis by multi-color flow cytometry. The following antibodies (purchased from eBioscience and Biolegend) were used: APC-eFluor 780 anti-mouse CD4, PE anti-mouse CD3e, PerCP-Cyanine 5.5 anti-mouse CD8, APC anti-mouse CD103, Alexa Fluor 700 anti-mouse CD69, PE/Cy5 anti-mouse CD19, Pacific Blue anti-mouse IFNγ, PE-Cyanine 7 anti-mouse granzyme B. Cells were incubated with antibody for 1 h at 4°C and then washed following the incubation period.

For detection of intracellular proteins, cells were collected and stimulated overnight with 100 ng/ml mouse recombinant IL-2 (R&D Systems) in a 37°C 5% CO₂ humidified incubator, followed by 4 h of brefeldin A exposure (to plug the Golgi apparatus) using before initial extracellular staining. After extracellular staining as described above, cells were fixed overnight in 0.5% Paraformaldehyde, followed by intracellular staining for 1 h at 4°C. Pelleted, stained cells were re-suspended and read in a BD LSR II system. Percentages obtained from flow cytometry were combined with cell counts in order to calculate total cell numbers.
**Immunohistochemistry**

10 µm transverse sections were obtained from flash frozen brains (dry ice/isopentane) in tissue embedding compound. Tissue sections were fixed with 95% ETOH and rehydrated in PBS, followed by blocking with goat serum, avidin, and biotin (Vector Laboratories, Burlingame California). Primary antibodies (Human polyclonal anti-MV serum – a gift from Michael B. A. Oldstone; Mouse anti-CD3e – BD, Mouse anti-CD8a -eBioscience) were applied for 1 h at room temperature. Secondary antibodies (biotinylated goat anti-human HRP - Vector; biotinylated goat anti-Armenian hamster – Santa Cruz; biotinylated goat anti-rat – Santa Cruz) were applied followed by avidin-biotin peroxidase. Antibody-positive cells were visualized using diaminobenzadine (DAB). Tissues were counterstained using hematoxylin and mounted in an aqueous medium. Tissue was visualized using a Nikon SMZ stereo dissecting microscope equipped with epiflorescence optics.

**V. Statistical Analysis and Figure Preparation**

Data representation and statistical analysis were performed using Prism GraphPad. Statistical analysis was performed as indicated in the figure legend. Figures represent the results of at least 3 independent experiments, unless otherwise noted in the figure legend. Samples were compared to an uninfected or untreated control. #/*: p< 0.05. Error bars represent the standard error of the mean.
Appendix: Murine BST2/tetherin promotes measles virus infection in neurons *in vivo* and *ex vivo*

This chapter is in preparation for publication

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I. Summary

BST2/tetherin is an anti-viral protein induced in response to cellular exposure to both type I and type II interferon. BST2, a transmembrane cell surface protein, is generally considered an antiviral protein, because it can restrict the release of budding virus particles and subsequent infection of other cells by tethering them to the host cell membrane. We previously showed that murine BST2 is induced in primary neurons following measles virus (MV) infection or type I interferon exposure; however, BST2 was dispensable for protection against challenge with neuron-restricted MV. As this was the first exploration of BST2 in neurons, we further investigated MV spread and clearance from neurons of mice lacking BST2. Surprisingly, and in contrast to its antiviral role in non-neuronal cells, we found that murine BST2 promotes neuronal MV spread both in and ex vivo. BST2 is enriched in the neuronal cell body during infection and may aid in facilitating intra-neuronal MV spread. These studies highlight a cell-type dependent role of a well-characterized antiviral protein in enhancing neuron-specific viral infection.
II. Introduction

Bone marrow stromal antigen 2 (BST2; also known as tetherin, HM1.24, and CD317 (Blasius et al. 2006)) was first identified as a marker of terminally differentiated B cells (Goto et al. 1994). Little attention was paid to this protein until the discovery that it could prevent the release of human immunodeficiency type I (HIV-1) virus particles from infected cells by physically tethering budding HIV-1 particles to the cell membrane (Neil et al. 2008; Fitzpatrick et al. 2010; Perez-Caballero et al. 2009; Van Damme et al. 2008; Hammonds et al. 2010). This finding was not limited to HIV-1; it has since been shown that BST2 can restrict the release of many enveloped viruses, including herpes simplex virus type 2, vesicular stomatitis virus, and Ebola virus (Y. Liu et al. 2015; Weidner et al. 2010; Kaletsky et al. 2009). The importance of BST2 in controlling viral infections is highlighted by the fact that many viruses, including HIV-1, encode proteins that directly antagonize BST2’s antiviral activity (Van Damme et al. 2008; Kaletsky et al. 2009).

While the contribution of BST2 in restricting viral budding continues to be extensively studied, its role in normal cellular processes is still being defined. Currently, it is known that BST2 is a transmembrane protein with multiple isoforms, in which differing isoforms have alternative signaling capabilities (e.g. certain BST2 isoforms have the ability to activate the NF-κB pathway while others can not) (Cocka & Bates 2012; Evans et al. 2010; Hotter et al. 2013). BST2 has also been implicated in modulating clathrin-mediated endocytosis (Cocka & Bates 2012; Martin-Serrano & Neil 2011; Masuyama et al. 2009), and can contribute to organization of the actin cytoskeleton (Rollason et al. 2009). Further, BST2 is induced in response to cellular type I and II interferon (IFN) in many cell types (Blasius et al. 2006), consistent with its role in antiviral defense. We previously showed that BST2 is highly induced in primary mouse central nervous system (CNS) hippocampal neurons in response to both IFN exposure and MV
infection, but was dispensable for immune-mediated control of MV in infected mice (Holmgren, Miller, Cavanaugh & Rall 2015a; O'Donnell et al. 2012).

MV is not a natural mouse pathogen; in order to enable infection of CNS neurons, our laboratory uses a transgenic mouse model that expresses the measles vaccine strain receptor, CD46, under the transcriptional control of the neuron specific enolase (NSE) promoter (NSE-CD46\(^+\) mice) (Rall et al. 1997). This allows for exclusive infection of mouse CNS neurons with the MV-Edmonston vaccine strain (Naniche et al. 1993; Dorig et al. 1993; Rall et al. 1997). Using this model system, we have shown that MV infection of neurons, both \textit{in} and \textit{ex vivo}, does not result in neuronal death (Patterson, Lawrence, et al. 2002) and MV spread between neurons occurs in the absence of viral budding (Makhortova et al. 2007; Lawrence et al. 2000). Thus, the mechanism by which MV is transmitted from infected to uninfected neurons is almost exclusively trans-synaptic. In these early studies, we provide evidence that, contrary to its more well-defined contributions in restricting viral spread in non-neuronal cells, in neurons, BST2 expression enhances viral replication, and its loss results in more rapid clearance of viral RNA \textit{in vivo}. Furthermore, BST2 expression is not enriched at the synapse following MV infection of primary CD46\(^+\) neurons, suggesting that it exerts its pro-viral role upstream of the neuronal synapse (the site of MV spread between synaptically connected infected and uninfected neurons) (Lawrence et al. 2000; Makhortova et al. 2007).
III. Results

*Reduced viral RNA in infected brains of mice lacking BST2*

After reporting the observation that loss of BST2 had no pathogenic consequence following neuronal MV infection (Holmgren, Miller, Cavanaugh & Rall 2015b), we next sought to determine if BST2 expression had any impact on viral replication or spread in neurons over time. Viral RNA was therefore monitored in whole brains of NSE-CD46⁺ and NSE-CD46⁺/BST2 KO mice throughout a two-week course. Surprisingly, we observed a trend towards less viral RNA in the brains of NSE-CD46⁺/BST2 KO mice early during infection (Supplementary Figure S1), reaching statistical significance by 14 dpi. These data were the first indication that BST2 may facilitate MV replication or spread in CNS neurons in this transgenic mouse model.

![Figure S1](image_url)

**Figure S1:** Loss of BST2 leads to decreased detection of MV RNA in vivo.

NSE-CD46⁺ and NSE-CD46⁺/BST2 KO mice were infected intracranially with $1 \times 10^4$ PFU MV-Edmonston. Whole brains were collected and analyzed for the MV nucleoprotein RNA using RT-qPCR at 3, 7 and 14 days post infection. $n = 10-12$ group. Data are represented using the $\Delta\Delta$CT method. * $p < 0.05$ Mann-Whitney U Test.
Reduced measles virus RNA in infected BST2 deficient neurons

To further characterize the role of BST2 in neuronal MV infection, we purified primary hippocampal neurons derived from E14 embryos of the indicated genotype, generating primary neuronal cultures that are non-dividing and synaptically connected. Using these cultures, we assessed the kinetics of MV replication in primary NSE-CD46+ and NSE-CD46+/BST2 KO cultures by analyzing the MV nucleoprotein RNA levels present at varying times post infection (Supplementary Figure S2A). Similar to the results obtained from NSE-CD46+/BST2 KO mice, primary CD46+ KO neuronal cultures showed significantly lower viral RNA levels as compared to permissive BST2-expressing neurons. Similar results were obtained when analyzing the amount of detectable MV protein with time post infection (Supplementary Figure S2B). RT-qPCR and western blot analysis revealed that NSE-CD46+/BST2 KO neurons support lower viral loads over time.
Figure S2: Loss of BST2 leads to decreased detection of MV ex vivo.

Primary neurons of the indicated genotype (WT: NSE-CD46⁺; KO: NSE-CD46⁺/BST2 KO) were infected with MV at an MOI=1. A) RNA was collected at the indicated time points and analyzed by RT-qPCR for MV nucleoprotein RNA. Results of at least 6 independent experiments are represented using the ΔΔCT method. B) Western blot analysis of protein collected at the indicated times post infection. Blots were probed with a polyclonal MV fusion protein antibody and an antibody to GAPDH as a loading control. A representative image is shown. * p <0.05 Wilcoxon matched pairs
BST2 is not enriched at the synapse during neuronal MV infection

To further understand what role BST2 may be playing during trans-synaptic MV spread, we purified synaptic and cytosolic neuronal fractions from infected NSE-CD46+ neurons at varying times post infection. Synaptic termini are purified using synaptosome preparations, obtained following homogenization of neuronal tissue or cultures in isotonic buffer followed by serial centrifugations. Such preparations are enriched in synaptosome associated protein (SNAP25) and represent sealed neuronal terminals (Supplementary Figure S3B) (Sokolow et al. 2011). Using this established purification technique from the neurobiology literature, we could define the cellular localization of BST2. Our studies show that the degree of BST2 RNA expression was much greater in the non-synaptic rather than synaptic portion of the neurons (Supplementary Figure S3A). We further corroborated this observation to BST2 protein expression, which is again much greater in the non-synaptic fraction of infected neurons (Supplementary Figure S3B). These data suggest that BST2 may exert its pro-viral function upstream of the synapse (site of MV inter-neuronal spread), of an infected neuron. Further studies will be required to fully elucidate the mechanism by which BST2 promotes MV spread.
Primary NSE-CD46+ neurons were infected with MV at an MOI=1. Infected cells were collected at the indicated time post infection followed by synaptosome purification. A) RNA was collected at the indicated time points and analyzed by RT-qPCR for murine BST2 (mBST2) RNA and normalized to cyclophilin B. Data represent the results of one experiment performed in triplicate and analyzed using the ΔΔCT method. B) Western blot analysis of protein collected at the indicated times post infection from either synaptic or remaining (non-synaptic) fractions. Blots were probed with a polyclonal BST2 antibody or SNAP25 (to indicate synaptic fraction purity) and an antibody to GAPDH (loading control). Data represent the results of one experiment performed in triplicate. * p <0.05 Unpaired T test with equal standard deviations. Error bars represent SEM.
IV. Discussion

These early data contribute to a growing literature that defines the paradoxical roles of BST2/tetherin, perhaps explained by cell type specific differences. While the majority of studies have focused on how this anti-viral mediator restricts release of free virus particles, only a few studies have examined BST2 and its role in cell-cell spread of viruses. Moreover, the potential cell-specific variation in BST2 function has not been fully explored. It is well established that BST2 can block cellular egress of a multitude of enveloped viral particles (Ooi et al. 2015; Holmgren, Miller, Cavanaugh & Rall 2015b; W. Wang et al. 2015; Hammonds et al. 2010; Mahauad-Fernandez et al. 2014; Fitzpatrick et al. 2010; Y. Liu et al. 2015; Neil et al. 2008; Sakuma et al. 2009); here, we show that BST2 in primary hippocampal neurons contributes to trans-synaptic inter-neuronal MV spread both in and ex vivo.

Our data contribute to a small but growing number of studies that document the seemingly “pro-viral” functions of BST2 under some conditions. For example, in the case of cytomegalovirus (CMV) replication, in vitro studies using primary human monocytes and fibroblasts reveal that BST2 expression increases CMV cellular entry via a reverse-tethering mechanism in which BST2 expressed at the cell surface can enhance virion binding and subsequent viral entry (Viswanathan et al. 2011). Using BST2 KO mice infected with vesicular stomatitis virus and influenza virus similar results have been shown (loss of BST2 results in decreased viral loads in vivo presumably due to disruption of vesicular intracellular trafficking) (Londrigan et al. 2015; Swiecki et al. 2012).

The seemingly opposite roles of BST2 may have to do with the manner by which the virus spreads. For example, in the case of HIV infection, BST2’s contribution in
inhibiting spread of HIV particles has mainly focused on the release of infectious progeny (Fitzpatrick et al. 2010; Perez-Caballero et al. 2009; Hammonds et al. 2010). However, when investigators studied direct cell-to-cell spread of HIV-1, BST2 was found to promote spread at the virological synapse, as knockdown of BST2 by siRNA inhibited transmission of particles into uninfected cells (Jolly et al. 2010). Similar data were observed with feline immunodeficiency virus (FIV): BST2 expression could efficiently block FIV egress, but cell-to-cell transmission was enhanced, as visualized by increased syncytia formation (Dietrich et al. 2011).

This may be of particular relevance to viral transmission in neurons: many viruses, including MV and rabies, when infecting neurons, adopt a cell-to-cell spreading strategy, as opposed to budding of infectious virus particles (Lawrence et al. 2000; Makhortova et al. 2007; Gomme et al. 2012). What neuronal factors alter the viral life cycle in this manner are not yet known. In the case of MV infection of primary hippocampal neurons it is possible that BST2 aids in inter-neuronal MV trafficking, as BST2 expression has been shown in the trans-Golgi network and recycling endosomes (Masuyama et al. 2009). BST2 expression in the cytosolic neuronal trans-Golgi network could aid in directing virus or viral protein-containing vesicles from the Golgi to the synapse, facilitating MV spread across the synapse to an adjacent neuron. Loss of BST2 may consequently disrupt directed trafficking, leading to limited MV spread among neurons. Alternatively, the to-date identified cellular roles of BST2, which include clathrin-mediated endocytosis, induction of NF-κB, and impeding cellular protein synthesis, could lead to the induction or activation of other cellular pathways necessary to facilitate MV cell-to-cell spread (W. Wang et al. 2015; Narkpuk et al. 2014; Rollason et al. 2009; Masuyama et al. 2009). While these are preliminary they clearly highlight the
possible pro-viral role of BST2 after neuronal infection and underscore the need to consider cell type-specific roles of certain ISGs.
V. Conclusion

BST2 plays an important anti-viral role, given its ability to restrict viral budding from infected cells. Nevertheless, its normal cellular functions have yet to be clearly defined, and knowing the full contributions of BST2 in cell biology may inform alternative ways by which this protein can influence viral infections. The identification that BST2 can promote measles virus spread in neurons may help to elucidate the normal neuronal function of BST2 in the absence of viral infection, hinting at a possible function of BST2 in directing transport of neuronal vesicles from the trans-Golgi network. It appears that BST2 may present a possible target in preventing cell-to-cell spread of viruses; further studies will aid in understanding how BST2 enhances cell-to-cell spread of a variety of viruses in varying cell types.
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