NEURONAL DISSEMINATION PATTERNS OF THREE DISTINCT VIRUSES AND MECHANISMS REGULATING VIRAL RETROGRADE AXONAL TRANSPORT

APPROVED BY SUPERVISORY COMMITTEE

_____
Julie Pfeiffer, Ph.D.

_____
Ivan D'Orso, Ph.D.

_____
John Schoggins, Ph.D.

_____
Weichun Lin, Ph.D.

DEDICATION

To my husband Paul.

For his relentless encouragment during the tough times

and for sharing the joy and excitement of the good times.

ACKNOWLEDGEMENTS

I could not have completed this dissertation without the support of many people through research struggles and personal challenges.

To my mentor Julie Pfeiffer, who has been a huge source of support throughout my entire graduate career. Her passion and enthusiasm for the research projects in the laboratory (past, present, and future) is inspiring for developing scientists like myself. Despite some unusual setbacks through my education, Julie remained positive and supportive. Without her support, it is very unlikely that I would have remained in graduate school, and for that I am especially grateful. I look forward to watching her career and the research goals of her laboratory grow and develop, for as many years as she hopes to keep them running.

To the lab, past and present members with which I had the fortune of working closely—Karen, Sharon, Gavin, Andrea, Chris, Yao, Palmy, and Elizabeth—for excellent assistance during troubling research challenges and for the many random and enjoyable conversations, science-related or otherwise. Without their support, shared struggles, and good humor, my time spent in lab would not have been nearly as enjoyable.

To my thesis committee, for their support and guidance throughout the course of my dissertation research, especially through research areas that were new and interesting to our laboratory.

To all of my friends, whether they were in Dallas or working on other fantastic career goals across the country. Thank you for keeping my head on straight and reminding me that the world is bigger than it looks sometime, and that I always have someone to turn to.

Finally, to my family. My parents, Doug and Tammy, my siblings and siblings-in-law, Michael, Danielle, Daniel, Tyler, and Bethany, to the pets, Lexi and Matilda, and to my husband Paul, for their never-ending support throughout my education and now in my first steps into my career. I would not have made it this far without their support (and jokes!), and I hope I can be just as helpful in the future.

NEURONAL DISSEMINATION PATTERNS OF THREE DISTINCT VIRUSES AND MECHANISMS REGULATING VIRAL RETROGRADE AXONAL TRANSPORT

by

LAUREN NICOLE LUETHY

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center

Dallas, Texas

September, 2015

NEURONAL DISSEMINATION PATTERNS OF THREE DISTINCT VIRUSES AND MECHANISMS REGULATING VIRAL RETROGRADE AXONAL TRANSPORT

LAUREN NICOLE LUETHY, Ph.D.

University of Texas Southwestern Medical Center, 2015

Julie K. Pfeiffer, Ph.D.

Viruses from several distinct families can infect the central nervous system (CNS), but mechanisms and host factors that influence dissemination are not completely understood. I previously identified barriers that limit poliovirus and yellow fever virus 17D (YFV-17D) dissemination following peripheral injection of mice. To investigate how different viruses disseminate from peripheral tissue to the CNS, I intramuscularly injected mice with genetically marked pools of viruses and monitored dissemination along the sciatic nerve to the spinal cord and brain. Transport efficiency of each virus was compared in immune competent and immune deficient mice in the presence or absence of muscle damage, which was previously shown to enhance retrograde axonal transport of poliovirus in the sciatic nerve. I found that immune deficiency enhanced poliovirus and YFV-17D transport to the CNS. While

muscle damage dramatically enhanced poliovirus dissemination it did not enhance YFV-17D dissemination, likely because YFV-17D entered the CNS through the blood. Like poliovirus, reovirus type 3 Dearing strain is transported through peripheral nerves to the CNS. Using genetically marked reoviruses, I found that young age and immune deficiency, but not muscle damage, enhanced reovirus transport to the CNS from peripheral tissues. Overall, my data suggest that these three viruses access the CNS through different routes and with different efficiencies.

Though muscle damage enhances neuronal poliovirus dissemination, the mechanisms that regulate this are unclear. I tested dissemination of the marked viruses following intramuscular injection in the presence or absence of potential regulatory factors. Several growth factors, including brain-derived neurotrophic factor, were previously shown to enhance retrograde axonal transport. In conjunction with poliovirus injection, brain-derived neurotrophic factor or other growth factors were not observed to enhance viral dissemination. Microarray analysis of muscle samples was performed to compare host gene expression in damaged and non-damaged tissue. Several host transcripts had elevated transcript levels in damaged muscles, including tissue inhibitor of metalloproteinase-1 (TIMP-1) and monocyte chemoattractant protein-1 (MCP-1). The targets of TIMP regulation, matrix metalloproteinases (MMPs), were previously shown to stimulate retrograde axonal transport following damage to peripheral tissues. MCP-1 has also been suggested to enhance viral dissemination. Altering MCP-1 or MMP levels during poliovirus infection revealed no direct impact on poliovirus dissemination. Though mechanisms regulating viral dissemination following muscle damage remain unclear, the path is open for exploration.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Luethy, L.N., Erickson, A.K., Jesudhasan, P.R., Ikizler, M.R., Dermody, T.S., Pfeiffer, J.K. *Comparison of three neurotropic viruses reveals differences in viral dissemination to the central nervous system*. Manuscript under review.

Luethy, L.N. and Pfeiffer, J.K. *Preview: Viral Infection Brings Mitochondrial Traffic to a Standstill.* Cell Host Microbe 2012. 11(5):420-421.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BBB — blood-brain barrier

BDNF — brain-derived neurotrophic factor

CCL2 — C-C motif ligand 2 (a.k.a. MCP-1)

CD155 — cluster of differentiation 155 (a.k.a. PVR)

CNS — central nervous system

CNTF — ciliary neurotrophic factor

DENV — Dengue virus

DMEM — Dulbecco's modified Eagle's medium

E protein — envelope protein (of flaviviruses)

GDNF — glial cell line-derived neurotrophic factor

GSK — glycogen synthase kinase

HBSS-CMF — Hanks' Balanced Salt solution-Ca^{2+}- and Mg^{2+}-free

hpi — hours post-inoculation

IFN — interferon

IFNAR — interferon alpha/beta receptor

IM — intramuscular inoculation

IP — intraperitoneal inoculation

JAMA — junctional adhesion molecule A

JEV — Japenese encepthalitis virus

M protein — membrane protein (of flaviviruses)

MCP-1 — monocyte chemoattractant protein-1 (a.k.a. CCL2)

MMP — matrix metalloproteinase

MOI — multiplicity of infection

MVB — multivesicular body

NGF — nerve growth factor

Ngr1 — Nogo receptor 1

PFU — plaque-forming units

prM protein — precursor membrane protein (of flaviviruses)

PV — poliovirus

PVR — poliovirus receptor (a.k.a. CD155)

RdRp — RNA-dependent RNA polymerase

Reovirus T1L — Reovirus Type 1 Lang

Reovirus T3D — Reovirus Type 3 Dearing

RV — reovirus

SCG — superior cervical ganglia

TIMP — tissue inhibitor of metalloproteinase

μL — microliter

VAPP — vaccine-associated paralytic poliomyelitis

WNV — West Nile virus

YFV — yellow fever virus

CHAPTER 1: Literature Review

Nervous System and Viral Transport

The nervous system is divided into two subsections: the central nervous system (CNS) includes the spinal cord and brain, while the peripheral nervous system includes the nerves which stem from the CNS to communicate with other tissues in the body. Viruses capable of entering the CNS are called neurotropic viruses, and viruses capable of infecting cells of the CNS are considered neurovirulent [113].

The nervous system is a complex network of individual cells that stretch over long distances from peripheral tissue sites to the spinal cord or brain. Both exogenous cargoes like viruses and endogenous cargoes can reach the CNS through two different pathways [113]. The first is by crossing the blood-brain barrier directly. The blood-brain barrier is a layer of endothelial cells lining the brain vasculature system, which are permeable only to specific small molecules. Some cell types are able to cross the barrier under specific conditions, such as T-cells following viral infection. Viruses such as HIV-1 also seem to be capable of crossing the blood-brain barrier, but the mechanism by which HIV-1 crosses is unclear. The second pathway is through peripheral nerves. Cargo is constantly moving in both the anterograde (away from the CNS) and retrograde (toward the CNS) directions. The kinesin motor protein is responsible for anterograde transport and the dynein motor facilitates retrograde transport [15,56,181,188,198]. Typically, these transport systems are used to signal changes to the nerve and its surrounding tissue, as well as to provide new proteins or other molecules [3,15,50,60,135,181,188]. Exogenous cargoes, such as wheat germ agglutinin, can be injected at peripheral sites and tracked as they move retrograde toward the CNS [117]. Viruses appear to use host peripheral nerve transport systems as well, as intramuscularly-injected poliovirus can be detected in peripheral nerves prior to completion of the first round of viral replication [116,117].

Viral invasion of the CNS can yield a variety of symptoms, from fever and encephalitis to acute paralysis and death. Once in the CNS, most viruses are able to replicate within the spinal cord, brain, or

both [113,188]. In most human cases, CNS infection is a dead end for the virus, as the host will not transmit the virus. Fortunately for humans, the probability of neurological symptoms resulting from viral infection of the CNS is low for most viruses. For poliovirus, only about 1% of known infections result in paralytic disease. It is not known whether viral entry always guarantees neurological symptoms or infection of neurons, because I only measure cases where symptoms were observed.

Endogenous cargo is moved through peripheral nerves within different clusters of molecules associated with the molecular motor. In some cases, both motors are associated with an endosome or multivesicular body (MVB), but one motor dominates transport [130,188,198,231]. Neuronal transport is regulated by a number of factors, including neuron-produced factors and tissue-produced factors [22,55,60,91,135,153,181,186,197]. Though much is known regarding the regulation of host transport pathways, less is understood about how neurotropic viruses associate with the host transport system to be transported through the peripheral nerves and into the CNS.

To observe how viruses are transported through the nervous system, animal models are used. To target the nervous system directly, viruses can be inoculated through different routes to study (or bypass) different barriers to the virus [113]. Intracranial inoculation bypasses host barriers including the blood-brain barrier, intravenous inoculation can be used to study how a virus surmounts the blood-brain barrier to reach the CNS, and intramuscular inoculation helps study how the virus enters and is transported through peripheral nerves to the CNS. Since peripheral nerves extend to enervate tissues throughout the body, they are often very fine and difficult to excise, so it is important to choose an appropriate target. The sciatic nerve runs from the foot to the gastrocnemius muscle to the spinal cord, as a bundle of continuous neurons that do not synapse until reaching the spinal cord.

Poliovirus

Poliovirus is a human virus, transmitted by the fecal-oral route. It mostly likely infects and replicates initially in microfold cells in the gut as the first step of infection. Most poliovirus infections in humans are thought to be asymptomatic or cause mild flu-like symptoms. The best-known symptom of

infection, paralytic poliomyelitis, only presents in about 1% of naturally infected patients. The hallmark lower limb paralysis is the result of infection of and subsequent damage to the motor neurons in the ventral horn of the spinal cord [112,185,237]. It is unclear why poliovirus, following primary infection in the intestines, typically infects and damages these motor neurons, rather than other possible sites. Though many patients were able to recover at least partial function in the affected limb(s), the paralysis carries a lifelong impact and symptoms such as paralysis can re-emerge later in life as post-polio syndrome [51,73].

While poliovirus has a small chance of causing paralysis on its own, there are cases of enhanced paralytic poliomyelitis as a by-product of other causes. Independent studies in India, Czechoslovakia, Cameroon, and England revealed that damage to muscle tissue by injection of antibiotics or other treatments within 30 days of a poliovirus infection increased the incidence of paralysis in the injected limb [4,23,24,88,92,208,238]. In order to understand this effect, Gromeier and Wimmer tested the effect of intentional muscle damage in a mouse model. They found that mice that received muscle damage by needle sticks at the site of inoculation showed decreased survival and increased tissue titers relative to control infected mice [86]. This suggested that a component of the damage or inflammatory response altered the ability of poliovirus to access the nervous system, impeded the host's ability to respond to infection, or both. It is unclear whether this enhancement extends to other viruses.

The first poliovirus vaccine was an injectable inactivated virus developed by Jonas Salk in the 1950s, and the second vaccine, an oral vaccine developed by Albert Sabin using attenuated virus, was released two years later [142,174,189-192]. These two vaccines were responsible for the almost complete eradication of the virus that I see today, where poliovirus remains endemic in only one country [6]. There are three serologically-distinct strains of poliovirus, termed types 1, 2, and 3. Though global eradication of type 2 was reported in the past, the strain re-emerged as a vaccine-derived variant due to poor vaccination coverage [98]. Type 1 is the predominantly circulating strain, while type 3 circulates at a much lower level and is on a faster path to eradication. The Sabin attenuated strain for poliovirus 1 differs

from the wild type strain by 57 nucleotide mutations, with the predominant attenuation from reduced translation of viral mRNA in host cells [142]. The Sabin strain is also extremely inefficient at infecting nervous system tissues, but cases of vaccine-associated paralytic poliomyelitis (VAPP) have occurred. In rare cases, the virus reverts from attenuation and is able to infect the nervous system, causing symptoms indistinguishable from those of the wild virus [30,71,168,169,173]. Though the exact cause of VAPP is unclear, it is likely due to reversion of specific mutations that restore replicative fitness to the virus.

Poliovirus is a non-enveloped virus with a positive-sense, single-stranded RNA genome of 7.5 kb. It is member of the *Picornaviridae* family. At the start of infection, poliovirus enters via receptor-mediated endocytosis through CD155, the poliovirus receptor (PVR) [140]. After entry into the endosome the genetic material is released into the cytoplasm. Initially, the genome is translated by host ribosomes to produce a viral polyprotein [101,162]. The polyprotein undergoes autolytic cleavage to generate the structural and nonstructural proteins. The virally-encoded RNA-dependent RNA polymerase (RdRp) copies the positive sense segments into negative sense intermediates, which are then copied into nascent positive-sense genomes. Self-assembly of the structural capsid porteins occur on the nascent genomes and depends on genome synthesis [143,144].

Entry into peripheral neurons is PVR-dependent [116,117,159,161,178], though some work suggests that there may be a PVR-independent route to the brain [160]. It is unknown exactly what complex poliovirus is associated with after entry, but it is known that PVR is required for poliovirus retrograde axonal transport through the neuron. The cytoplasmic portion of PVR facilitates transport by associating with part of the dynein motor protein light chain called Tctex1 [150]. Tctex1 is responsible for binding cargo to dynein [41]. Without this association, retrograde neuronal transport pathways do not appear to carry poliovirus from peripheral muscle into the spinal cord [150]. It is expected that poliovirus is associated with the extracellular domain of PVR, but what membrane PVR is in and what other components might be associated with the poliovirus/PVR/dynein complex are unclear.

Since poliovirus is specific to humans (e.g. only humans are naturally susceptible and permissive to the virus), transgenic mice expressing human PVR must be used to study the virus *in vivo*. These mice show normal survival and reproduction [48,108,179]. Transgenic mice also show similar hPVR expression patterns to humans. They are susceptible to poliovirus infection by the IM and IC routes, but not by poliovirus's natural oral route of infection [48,108,179]. In addition to expressing hPVR, oral susceptibility requires ablation of the type I IFN response. For this, type I IFN α/β receptor knockout mice (IFNAR-/-) have been generated [94,158]. Mice injected IM display classical signs of paralytic poliomyelitis, with paralysis starting at the infected limb before spreading bilaterally. In contrast, orally infected mice develop encephalitis.

While viruses may encounter barriers en route to the CNS, the barriers may be difficult to detect. If a virus is capable of efficient replication both before and after a barrier, then the barrier will be masked when quantifying titer alone. To overcome this, the lab developed the viral diversity assay in which mice are infected with a pool of viruses with silent mutations and the number of viruses present at a given tissue is measured with specific, complementary probes. These viruses have similar fitness both *in vitro* and *in vivo*, such that no single pool member is found more often following *in vitro* serial passage or in a particular mouse tissue than the others. If the pool of viruses encounters a barrier but still replicates efficiently afterwards, I will still observe the barrier by low diversity (low number of pool member viruses present) [116,117].

Using the poliovirus diversity assay, the lab uncovered bottlenecks to viral dissemination in the host based on inoculation route. Initially, I observed a major barrier following oral inoculation between the gut and the nervous system. This barrier was partially reduced in IFNAR-/- mice, suggesting that the type I IFN response was part of the challenge [116]. Further study revealed that, following IM injection, the major barrier to poliovirus dissemination was between the muscle inoculation site and spinal cord, suggesting that viral transport along the peripheral nerves was inefficient, Again, IFNAR-/- mice showed somewhat enhanced viral load and pathogenesis, but the loss of the innate immune response was not the

only factor [117]. Retrograde axonal transport is inefficient, but can be enhanced by stimulating transport with muscle damage at the inoculation site [86,117]. Enhanced poliovirus titer and diversity was observed in response to muscle damage in both IFNAR+/+ and IFNAR-/- mice, suggesting that the enhancement is independent of the type I IFN response [116,117].

YFV-17D

Yellow fever virus (YFV) is disseminated to humans through the bite of an arthropod host, most commonly the mosquito *Aedes egypti* [85,102]. YFV is maintained in nature in an enzootic cycle, passing back and forth between mosquitoes and non-human primates [14,76]. In fact, viral adaptation to passage through different hosts is important to perpetuation of the virus, as YFV clones selected for increased virulence in mosquitoes will show decreased virulence in rodents [43,69,96]. Human epidemics arise in urbanized and domesticated areas that have seen rapid population growth without a concurrent increase in mosquito control [76]. Even in epidemics, YFV is transmitted between humans by the bite of *A. egypti*, not through direct human-to-human spread [76,85,102].

Though infection of arthropods fails to manifest signs of disease, human infections range from asymptomatic to flu-like symptoms and peak viremia within 3-6 days post infection. Approximately 20% of humans infected will suffer worsening symptoms, including inflammation of the liver, kidneys, and heart, as well as the hallmark jaundice [54,76,103,104,125,147]. YFV infection progresses to hemorrhagic fever in about 15% of infections, killing up to half of those patients [146]. Those that survive more aggressive symptoms face months of recovery after clearance of the virus. YFV is also neuroinvasive and neurovirulent, and in rare cases causes encephalitis [147]. YFV accesses the central nervous system predominantly through the hematogenous route, where increased viremia corresponds to earlier viral entry into the brain and enhanced viral loads in the brain [58,141,196]. Though the specific neuronal cell target of YFV remains unclear, its neuroinvasive ability maps to the viral envelope (E) protein. Enhanced neuroinvasion of YFV is likely due to enhanced binding to and/or penetration of the host cell [19,20,34,137,155,156].

An effective vaccine was developed in the 1930s and successfully triggers long-term protective immunity[76,170,172,215]. The vaccine is a live-attenuated strain called YFV-17D and encodes a series of mutations that yield a substantial reduction in both neurotopic and viscerotropic disease, such that the most common reported side effects are mild fever and pain [145,207,214]. Rare incidences of vaccine-associated neurological disease and viscerotropic disease do occur, with age (very young or old) being a risk factor [99,124,207]. In both cases, the symptoms are similar to those of a natural infection; no particular mutations in YFV-17D that correlate with these adverse events have been identified [13].

YFV is a member of the *Flaviviridae* family; the enveloped virus contains a positive-sense RNA genome of 10.9 kilobases with a 5' cap but no 3' polyadenylated tail [75,202,236]. Though detailed information about YFV replication has not been determined, investigation of related viruses such as West Nile virus (WNV), Dengue virus (DENV), and Japenese Encephalitis virus (JEV) provide information about the YFV replication cycle. Prior to replication, YFV binding to the host cell is mediated by the viral E protein [2,40,180,241] and aided by the presence of host cell sulfated glycosaminoglycans [32,38,79,115]. The virus is internalized by clathrin-mediated endocytosis [1,40,46,114,220]. Endocytosis is likely receptor-dependent but no receptors have yet been identified. In fact, YFV is capable of infecting and replicating in a broad variety of cell lines *in vitro*, as well as a variety of cell types *in vivo* [9,194]. Following entry, fusion of the viral envelope to the endosomal membrane [220] is triggered either by acidification of the endosome [1,39,81,114,220] or availability of anionic lipids in the late endosome [46,82,240]. The nucleocapsid then uncoats, releasing the viral genome into the cytoplasm [111]. The viral genome is immediately available for translation by the host machinery, encoding a single open reading frame that is cleaved into individual proteins by both host peptidases and viral proteases [35,134,154]. Nascent genomes are transcribed by the virally-encoded RNA-dependent RNA polymerase, NS5 [53,87,211], and are thought to serve as a scaffold for capsid protein assembly [235]. This complex is transported to the endoplasmic reticulum, where E and precursor membrane (prM) proteins assemble the viral envelope [95,127,129,227,235]. Virions begin maturation as they are transported along the

secretory pathway for release at the cell surface [129]. Proteolytic cleavage of prM to M by the host protease furin occurs immediately before release and is required to convey infectivity to the new virus [63,90,126,129].

In vivo study of YFV and YFV-17D is limited by rodent susceptibility to infection and the resulting difficulty with modeling both viscerotropic and encephalitic disease states [146]. In fact, adult immune-competent mice are completely resistant to infection through intraperitoneal, intramuscular, or subcutaneous inoculation with either virus strain [64,65,139]. Clinical signs similar to human encephalitis disease can be studied using several different methods: by intraperitonial, intracranial, or intravenous infections of immune-competent infant mice [67,242], by infecting immune-competent mice with mouse-adapted YFV strains [12,66,67,147,196], by inoculation of immune-competent mice intracranially or by the olfactory bulb [67,70,83,199,200,213], or by intramuscular or intraperitonial inoculation of immune-deficient mice [64,65].

Viscerotropic disease with non-adapted YFV has been more difficult to model. It was recently reported that subcutaneous inoculation of immune-deficient mice induced a non-fatal viscerotropic disease [139], but neurotropic disease was not observed in these mice. Interestingly, young immune-deficient mice inoculated either IP or IM display one of three distinct phenotypes: no clinical signs of disease, fatal viscerotropic disease, or fatal neurotropic disease [65]. The range of phenotypes more closely imitates disease outcomes observed following human infections. Though viscerotropic disease was more prominent following IP inoculation than IM, viral loads in peripheral and nervous system tissues were not significantly different based on inoculation route or viscerotropic vs. neurotropic disease outcome [65]. Mice in which no clinical signs were observed lacked also viral dissemination from the inoculation side in most tissues. The exception is the brain, where viral load was detected to similar levels in both IP and IM inoculated mice, but still at significantly lower levels than mice displaying clinical signs of disease [65]. This suggests that immune deficiency may be sufficient to permit YFV-17D replication in the brain, but that the level of replication alone is insufficient to cause disease.

In additional to developing suitable *in vivo* models for YFV infection, the molecular biology and dissemination patterns of several other related flaviruses have been studied in depth in rodents. West Nile virus (WNV), Japanese Encephalitis virus (JEV), and Dengue virus (DENV) are closely related to YFV. Several different routes are available to these viruses to reach the brain. Peripheral tissue infection induces inflammation and damage response signals, which can cause a weakening of the blood-brain barrier through cytokine-dependent increases in permeability of endothelial cells [128,229] or the production of other molecules such as MMPs, which are responsible for degrading cells in preparation for replacement [37,106,222,228]. Damage to the blood-brain barrier permits the virus to cross into the brain. Similar to poliovirus, WNV is capable of entering peripheral nerves and undergoing retrograde transport to access the spinal cord and brain [28,93,148,152,157,175,195,226]. However, YFV CNS invasion is comparatively understudied.

Reovirus

Mammalian orthoreoviruses (*reo* stands for respiratory enteric orphan viruses, hereafter called reoviruses) are transmitted through the fecal-oral route. They are nonenveloped viruses with a segmented double-stranded RNA genome of 23.5 kbp from the *Reoviridae* family and the *spinareovirinae* subfamily. Disease occurs primarily in children, where infection of the respiratory and intestinal tracts typically yields minimal associated symptoms. By ten years of age, about 50% of children in urban areas are seropositive for reovirus [123,210] suggesting that infection is widespread. In rare instances, reovirus is capable of entering the CNS in humans.

Individual reovirus strains differ in their dissemination patterns and receptor specificities [18,232,233]. In mice, strain type 1 Lang (T1L) disseminates to the CNS primarily through a hematogenous route, while strain type 3 Dearing (T3D) disseminates to the CNS through neural or hematogenous routes [7,25,149,217]. Reovirus uses the viral protein σ1 to attach to cell-surface glycans and enter via receptor-mediated endocytosis through junctional adhesion molecule-A (JAMA) [16,31,36,68,121,131,177]. Glycan attachment promotes reovirus entry, but is not necessary for infection

[17]. T3D infects neurons using the Nogo receptor NgR1 [72,110], although glycan interactions are also important for viral attachment [77,78,163]. Endocytosis is likely clathrin-dependent [26,27,176,184,209], but instances of caveolin-dependent endocytosis have been observed [119,171]. Following entry, the outer capsid proteins are cleaved by host proteases [8,57,209] and the genomic dsRNA segments are transcribed within the viral core [203]. The 10 dsRNA segments yield 10 full-length mRNA segments, which are translated into 12 viral proteins. Single-stranded positive-sense RNA segments are sorted to developing viral cores, where minus-strand synthesis occurs to produce nascent dsRNA genomes [203]. Though the mechanism of reovirus release from host cells is not clear, many reovirus strains can induce apoptosis *in vitro* in immortalized cells and primary neuron culture [42,45,182,193,218], but apoptotic release does not seem to correlate with enhanced viral yield [183].

When reovirus T3D is inoculated into the hind limb muscle of newborn mice, the virus spreads to the CNS by trafficking through the sciatic nerve to the spinal cord. Sciatic nerve transection inhibits T3D dissemination from the hind limb muscle to the spinal cord [25,217]. However, sciatic nerve transection delays, but does not prevent, T3D spread to the brain following intramuscular inoculation, highlighting the importance of hematogenous spread following this inoculation route [25]. NgR1 is a GPI-anchored protein that is unlikely to interact directly with dynein [187], but dynein is required for reovirus entry and endocytic transport in non-neuronal cells [132]. An inhibitor of fast retrograde axonal transport inhibits T3D dissemination to the CNS in mice [217], suggesting that dynein-mediated fast retrograde axonal transport is involved in reovirus transport in neurons. The age-dependent barrier to reovirus CNS entry is also recapitulated in mice, as reovirus disseminates to the brain of young but not adult mice following intramuscular inoculation [133,212]. This barrier could be the result of receptor availability in young mice. Further study of the mechanisms regulating neuronal dissemination of reovirus T3D is required to clarify this complex system.

CHAPTER 2: Materials and Methods

Plasmid construction

The plasmids used to prepare the nine genetically marked reovirus strains were engineered using site-directed mutagenesis of the M1 gene segment of the reovirus T3D M1 cDNA plasmid beginning with nucleotide 426 and ending at nucleotide 444 (see Chap. 3, Fig. 5A). PCR products were subcloned using Bgl II and Mfe I restriction sites. Fidelity of mutagenesis for each PCR-generated region was confirmed by sequencing (Sequencing Core, UT Southwestern Medical Center, Dallas, TX). The 10 poliovirus plasmids and six YFV-17D plasmids have been described [64,116,165].

Viruses and cells

L929 cells (reovirus) and BHK cells (YFV-17D) were propagated in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum, and HeLa cells (poliovirus) were propagated in DMEM with 10% calf serum. Reovirus plaque assays were performed as described using 6 x 10^5 L929 cells seeded into wells of 6-well plates. Monolayers were stained with neutral red at 6 d post-infection, and plaques were counted at 7 d post-infection [224]. Poliovirus plaque assays were performed as described using 10^6 HeLa cells seeded into wells of 6-well plates. Monolayers were stained with crystal violet at 2 d post-infection [165]. YFV-17D plaque assays were performed as described using 10^6 BHK cells seeded into wells of 6-well plates. Monolayers were stained with crystal violet at 5 d post-infection [64]. For co-infection experiments with poliovirus and reovirus, I could discriminate reovirus plaques from poliovirus plaques because L929 cells do not support poliovirus replication due to the absence of PVR.

Reoviruses harboring the nine different mutated M1 genome segments were recovered by plasmid-based rescue [105]. Monolayers of BHK-T7 cells at 90% confluency ($\sim 3 \times 10^6$ cells) seeded in 60-mm dishes (Costar; Corning Inc., Corning, NY) were co-transfected with 10 plasmids representing the cloned reovirus T3D genome using 3 μl of TransIT-LT1 transfection reagent (Mirus Bio LLC; Madison,

WI) per μg of plasmid DNA. Following 72 h of incubation, recombinant viruses were isolated from transfected cells by plaque purification using monolayers of L929 cells. [224]. High-titer reovirus stocks were prepared by large-scale infections and purification by cesium chloride gradient centrifugation [74].

The relative fitness of the nine genetically marked reoviruses was evaluated by a serial passage competition experiment [64,116]. Each of the nine viruses at a dose of 10^6 PFU was mixed together and adsorbed to 10^6 L929 cells. After 24 h of incubation, infected cells were collected, and 25% of the harvested cells were plated on a fresh monolayer of 10^6 L929 cells to initiate further replication cycles. This process was repeated for a total of 7 passages, and the relative ratios of each virus in each passage were quantified by hybridization assay as described below. Pool member fitness was assessed *in vivo* by calculating the number of times each pool member was detected relative to the total possible number of times that the pool member could have been detected. Pool member totals were from all reovirus mouse experiments, with the exception of any experiments where all nine pool members were detected in all possible tissues.

Poliovirus growth curves were performed on HeLa cells to determine whether brain derived neurotrophic factor (BDNF) altered viral growth rate *in vitro*. Briefly, HeLa cells were infected with poliovirus as described at an MOI of 0.1 or 10. Inoculation solution contained either PBS alone or 25 ng BDNF. Cells were harvested in PBS at 2, 4, 6, or 8 hours post infection. Cells were freeze-thawed three times and centrifuged to separate virus-containing supernatant. Viral titers were quantified by plaque assay.

Mouse experiments

All animals were handled in strict accordance with good animal practice as defined by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mouse studies were performed at the University of Texas Southwestern Medical Center (Animal Welfare Assurance no. A3472-01) using protocols approved by the UT Southwestern Institutional Animal Care and Use Committee (IACUC). All studies were performed in a manner designed to minimize pain and suffering,

and any animals that exhibited severe disease signs were euthanized immediately in accordance with IACUC-approved endpoints.

C57BL/6 hPVR-Tg21 mice (called IFNAR+/+ throughout) and C57BL/6 hPVR-Tg21 mice lacking the IFN-α/β receptor (called IFNAR-/- throughout) were provided by S. Koike (Tokyo, Japan) [94,107]. Although PVR expression is required only for poliovirus infection, I used the PVR-transgenic mice for all viruses in this study to allow direct comparisons to be made using isogenic strains.

For studies comparing the neuronal dissemination patterns of poliovirus, YFV-17D, and reovirus, I mixed equal PFU of each pool member for a total of 10^7 PFU/mouse in 30 µL for adult 6-8 week-old mice mice or 10 µL for 3-day-old mice and inoculated the mixture into the lower left gastrocnemius muscle [116,117]. For YFV-17D infection of 3-day-old mice, the inoculum was 10^4 PFU due to low virus concentration and the small 10 µL inoculum. Unless otherwise stated, all muscle damage is needle sticks damage, which was induced in adult mice by inserting a 29-gauge needle 5 times once daily into the muscle around the inoculation site (Chap 3, Fig. 1). Muscle damage was not induced in 3-day old mice due to their small size. For one set of experiments, sterile crush damage was tested. This is separately denoted, and was performed by crushing the lower quadriceps with clean forceps four times at 15 seconds per crush, with 30-45 seconds of rest between each crush. Mice were euthanized at a predetermined endpoint or at the onset of clinical signs, whichever occurred earliest.

For studies investigating the potential effect of exogenous growth factors upon neuronal poliovirus dissemination, 6-10 week old immune-competent mice were intramuscularly inoculated with equal PFU of each pool member for a total of $3x10^4$ or $1x10^5$ PFU poliovirus for survival studies, or $1x10^6$ or $2x10^7$ PFU poliovirus for studies in which mice were euthanized prior to clinical signs of disease to monitor tissue titer and viral diversity. Control mice received poliovirus alone or poliovirus mixed with 50 µg BSA to control for the presence of exogenous protein, while experimental mice received 50 µg of either BDNF (Invitrogen), glial cell line derived neurotophic factor (GDNF, Invitrogen), glycogen synthase kinase (GSK, Santa Cruz), nerve growth factor (NGF, Invitrogen), or ciliary neurotrophic factor

(CNTF, Invitrogen) intramuscularly concurrently with the poliovirus inoculation. As a control, growth factors mixed with with 5 μg wheat germ agglutinin (Sigma-Aldrich) were injected intramuscularly to measure trafficking in neurons independent of viral infection. Muscle damage was performed as described above, using a 29-gauge needle inserted five times at the inoculation site. Mice were euthanized according to IACUC-approved methods at pre-determined endpoints or at the onset of clinical signs, whichever occurred earliest.

RNA for microarray was isolated following the same method of muscle damage as described above, by needle sticks five times per mouse with a 29-gauge needle once per day over three days. Muscle damage and control mice were given a mock injection of 30 μL PBS on the second day. Muscle tissue was collected on the fourth day, emulating a 48 hour post infection time point. Following dissection, each tissue was immediately flash-frozen and stored at -80ºC until processing.

To understand whether TIMP1 and MMPs may be regulating enhanced neuronal poliovirus dissemination in response to muscle damage, 6-8 week-old immune-competent mice were injected intraperitoneally once per day for four days with pan-MMP inhibitor GM6001 (Sigma-Aldrich) in DMSO at 0.5 mg/mouse/day and concurrently given muscle damage by needle sticks as described above. On the second day, mice were intramuscularly inoculated with equal PFU of each poliovirus pool member, for a total of 10^7 PFU per mouse. Control mice were treated with vehicle with or without muscle damage and the same poliovirus inoculation. Tissues were harvested at three days post-infection.

Macrophage chemoattractant protein-1 (MCP-1) was also tested for a possible role in stimulating retrograde axonal transport to enhance neuronal poliovirus dissemination. 6-8 week-old immune competent mice were injected intramuscularly once per day for two days (one day before and the day of viral inoculation) or for four days with PBS alone or 0.5 μg MCP-1 (BioLegend) in PBS. Mice were inoculated on the second day of MCP-1 injections with 10^5 or 10^7 PFU diversity poliovirus. Tissues were harvested at three days post-infection.

Tissue harvest, processing and viral diversity assays

Several tissues (muscle, sciatic nerve, spinal cord, brain, blood) were collected as described [64,117]. Adult mice infected with YFV-17D were perfused with PBS prior to tissue collection due to moderate viral titers in blood. Perfusion was not performed in poliovirus- or reovirus-infected mice due to negligible viral titers in blood. Tissues were isolated and processed as follows, including those from mice injected with virus in conjunction with exogenous growth factors, GM6001, or MCP-1. In experiments using adult mice, the sciatic nerve was sectioned into lower, middle, and upper segments [117]. The upper sciatic nerve segment included both the dorsal root ganglia and the motor nerves. The spinal cord was collected as the whole spine from the lumbar vertebrae (L5-L6) to the middle of the cervical vertebrae (C4-C5). Tissues were weighed and suspended in 1-3 volumes of PBS and homogenized with a Bullet Blender (Next Advance, Inc, Averill Park, NY) according to the manufacturer's instructions. Tissues from YFV-17D or reovirus-infected mice were frozen and thawed once prior to homogenization, whereas poliovirus-infected tissues were frozen and thawed three times after homogenization. All samples were centrifuged at 13,000 rpm for 1 min to isolate virus-containing supernatant.

Virus titers in supernatants were quantified by plaque assay and amplified prior to the viral population diversity assay to aid in detection of pool members. Poliovirus was amplified in HeLa cells until cytopathic effects were apparent (6-48 h, depending on the tissue). YFV-17D was amplified for 48 h in BHK cells [64]. Reovirus was amplified following a 1:10 dilution of supernatant and plated on L929 cells. Cells were incubated at 37°C for 12 h to 5 d, depending upon tissue titer, and monitored regularly for cytopathic effects. In all cases, cells were collected in 1 mL TRI Reagent, and RNA was extracted as described [116,117]. RT-PCR of poliovirus and YFV-17D samples was performed as described [64,116]. RT-PCR of reovirus samples was performed as described [116,166] with a few modifications to facilitate amplification of reovirus dsRNA. Total cellular RNA, the REO M1 614 antisense primer 5' TAGAGTGAGGAACACGACC 3', and all reaction components except for SSII reverse transcriptase were incubated at 100°C for 20 min to separate dsRNA, allowed to cool slowly to 42°C, and then

incubated with SSII reverse transcriptase at 42°C for 1 h. PCR reactions were performed as described [116], but with the M1 297 sense primer 5' CGGACAACGTTGATCGTCG 3' and the M1 542 antisense primer 5' CGCATAGTACTTTCTAGGAGC 3'. Blotting and hybridization were performed as described [116] but with reovirus-specific probes. The reovirus probe sequences are the reverse complement of sequences shown in Fig. 5A, but with an extra T on the 5' end of each. Reovirus probes were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P] ATP, and excess nucleotides were removed with the Qiagen Nucleotide Removal Kit (Qiagen, Valencia, CA). Hybridization was performed at 56°C overnight, followed by washing. Membranes were exposed to a Phosphor Screen for 2 d and scanned with a STORM Scanner. Signals were normalized to a mismatched control sample on each membrane as described [116].

For microarray RNA samples, each tissue sample was processed in 700 µL Tri-Reagent with a Bullet Blender at 4ºC, 15 seconds homogenization and 1 minute rest on ice, for 12 cycles. Following processing, an additional 300 µL Tri-Reagent was added to each sample, and samples allowed to incubate at room temperature for 5 minutes prior to isolating RNA with RNeasy Mini Kit (Qiagen). RNA samples were stored at -80ºC until processing for microarray on the Mouse WG-6 v2.0 BeadChip (Illumina) by the UT Southwestern Genomics and Microarray Core Facility (Dallas, TX).

In vitro cultures of neurons from embryonic superior cervical ganglia

Neuronal transport systems can be modeled *in vitro* using the compartmented chamber system. A segmented camber is placed on a plate and sealed with grease. Neurons isolated from embryonic mice are placed in the segment at one end of the chamber, and when properly stimulated will extend processes underneath the partitions between each segment. This allows separation of the growth medias and supernatant along the length of the neuron. Neurons can be treated or infected at either end, and the supernatant of the opposite segment can be monitored for any changes. Neurons of the superior cervical ganglia (SCG) were isolated and cultured as follows.

The SCG were isolated from embryonic day 14 C57Bl/6-hPVR (immune-competent) mice and neurons cultured as previously described for rats [33,49,97]. A brief summary follows. Chambers were prepared in advance: each plate was incubated with poly-D-ornithine for at least six hours, washed with sterile water, incubated with laminin overnight, washed with HBSS CMF, then scored with a pin rake. Methocellulose solution was applied to the fresh grooves. Teflon chamber was greased then placed gently onto the plate over the methocellulose-containing scored section. Each chamber section was filled with medium and plates were incubated until neuron isolation. Embryos were dissected from mother at embryonic day 14 and SCG isolated as follows. Embryonic sac was gently removed and embryos were placed in 2X HBSS. Cuts were made with microdissecting knives on a glass dish containing HBSS, the first cut along the chin and the second parallel to the mouth. From the resulting slice, the spinal cord segments were removed and the esophageal tissue was pulled to expose the SCGs, one on each side of the esophagus. The SCGs have a "dolphin-like" appearance and were carefully removed with the knives. SCGs were pooled and the cells were dissociated by incubation with trypsin, followed by a wash with trypsin inhibitor, then vigorous repeated passage through a blood-blocked flame-narrowed Pasteur pipet. Dissociated cells were added to one end of the chamber, over the grooves. Cells were incubated at 37°C for two days to permit attachment, then treated overnight with anti-mitotic AraC to eliminate any non-neuronal cells. Media was replaced every two to three days and neurons showed fully-extended processes by 14 days after plating, at which time infection experiments began.

CHAPTER 3: Comparison of three neurotropic viruses reveals differences in viral dissemination to the central nervous system

Introduction

Neurotropic viruses can invade and infect the central nervous system (CNS), which includes the spinal cord and brain. These viruses come from a variety of different families and include several that infect humans [113]. Neurotropic viruses enter the CNS through peripheral nerves or by crossing the blood-brain barrier following hematogenous dissemination. Different viruses target different cell types within the nervous system, causing symptoms ranging from seizures to paralysis or death. Here, I investigated the dissemination patterns of three neurotropic viruses, each from a different family, to discern whether common mechanisms are employed to invade the CNS. Viral dissemination barriers can be masked by titer-based assays since post-barrier viral replication can produce high viral titers [64,116,117,167]. Therefore, I used genetically marked virus pools to examine dissemination of poliovirus, yellow fever virus 17D (YFV-17D), and reovirus.

Poliovirus is a nonenveloped virus with a positive-sense, single-stranded RNA genome from the *Picornaviridae* family. It is most commonly transmitted in humans by the fecal-oral route and causes paralysis in less than 1% of infected individuals due to damage to motor neurons. Poliovirus accesses the CNS by either crossing the blood-brain barrier [239] or via retrograde axonal transport in peripheral nerves [161,164,178]. Young mice and immune-deficient mice have enhanced susceptibly to poliovirus infection [48,94,158]. When poliovirus is inoculated into the hind limb muscle of mice, the virus spreads to the CNS by transport through the sciatic nerve to the spinal cord and brain. Concordantly, sciatic nerve transection limits poliovirus spread to the CNS following intramuscular inoculation [86,161,178]. Poliovirus enters neurons and other cell types by binding the poliovirus receptor (PVR/CD155), followed by endocytosis and uncoating [109,158,178]. The cytoplasmic domain of PVR interacts with Tctex-1, a light chain of cytoplasmic dynein, which facilitates transport of virus-containing endosomes through the

fast retrograde transport system [150,159]. It is unclear whether other molecules are associated with retrograde axonal transport of poliovirus in neurons.

YFV is an enveloped virus with a positive-sense, single-stranded RNA genome from the *Flaviviridae* family. YFV usually infects humans through the bite of a mosquito, with outcomes ranging from asymptomatic infection to severe hemorrhagic fever. YFV rarely enters the CNS, but the virus is capable of causing encephalitis [147]. The receptor for YFV and its mechanism of entry into the CNS are not known. YFV strain 17D (YFV-17D) is a live-attenuated vaccine strain derived from the virulent Asibi strain. It is one of the safest live-attenuated vaccines available, but in rare cases, YFV-17D can enter the CNS and induce encephalitis following vaccination [147]. Laboratory rodents have varying degrees of susceptibility to YFV-17D, with young and immune-deficient animals having the greatest susceptibility [67,120,139,213,216]. Early studies suggested that YFV disseminates to the CNS hematogenously [141,196]; indeed, many flaviviruses access the CNS through blood. It is unclear whether YFV can undergo retrograde transport in neurons.

Mammalian orthoreoviruses (hereafter called reoviruses) are nonenveloped viruses with a segmented double-stranded RNA genome from the *Reoviridae* family. Reovirus infects humans by the fecal-oral route, but individual strains differ in their dissemination patterns and receptor specificities [18,232,233]. Reovirus infects most humans during childhood and, in rare instances, reovirus is capable of entering the CNS in humans. The age-dependent barrier to reovirus CNS entry is recapitulated in mice, as reovirus disseminates to the brain of young but not adult mice following intramuscular inoculation [133,212]. In mice, strain type 1 Lang (T1L) disseminates to the CNS primarily through a hematogenous route, while strain type 3 Dearing (T3D) disseminates to the CNS through neural or hematogenous routes [7,25,149,217]. When reovirus T3D is inoculated into the hind limb muscle of newborn mice, the virus spreads to the CNS by trafficking through the sciatic nerve to the spinal cord. Sciatic nerve transection inhibits T3D dissemination from the hind limb muscle to the spinal cord [25,217]. However, sciatic nerve transection delays, but does not prevent, T3D spread to the brain following intramuscular inoculation,

highlighting the importance of hematogenous spread following this inoculation route [25]. T1L and T3D reoviruses attach to host cells using cell-surface glycans and junctional adhesion molecule A, followed by receptor-mediated endocytosis [16,17,31,68,131,177]. T3D infects neurons using the Nogo receptor NgR1, although glycan interactions are also important for viral attachment to neurons [72,110]. NgR1 is a GPI-anchored protein that is unlikely to interact directly with dynein [187]. However, dynein is required for reovirus entry and endocytic transport in non-neuronal cells [132]. An inhibitor of fast retrograde axonal transport inhibits T3D dissemination to the CNS in mice [217], suggesting that dynein-mediated fast retrograde axonal transport is involved in reovirus transport in neurons.

The mouse sciatic nerve is a tractable model system to study how viruses move from the periphery to the CNS because of its length, width, and ease of dissection. Following intramuscular inoculation of the hind limb, viruses can enter the sciatic nerve and transit by retrograde fast axonal transport using dynein motors [161,178,217]. This transport system is essential, considering that viruses travel long distances within an axon. For example, viruses inoculated into the gastrocnemius muscle of the adult mouse travel over 2 cm within a single cell prior to reaching the site of viral replication in the cell body. The sciatic nerve model has been used to examine the routes by which neurotropic viruses gain access to the CNS and to study the effects of the type I interferon (IFN) response and stimulation of retrograde axonal transport [25,86,94,113,117,133,217]. For example, poliovirus transport in the sciatic nerve of mice is inefficient, but efficiency is enhanced by ablating the type I IFN response or increasing retrograde axonal transport via muscle damage. Importantly, virulence correlates with viral diversity in the CNS: mice with high viral diversity in the CNS have higher mortality than mice with low viral diversity in the CNS [116,117,166,223].

Although the mechanism is unclear, damage to muscle increases transport of both poliovirus and non-viral cargo protein in the sciatic nerve [86,117]. Muscle damage also decreases the time to disease onset in poliovirus-infected mice [86,117]. This damage response and subsequent enhanced poliovirus transport in neurons also may be operative in humans. In children, injury to muscle from trauma or

intramuscular injections increases the incidence of poliovirus-induced paralysis, particularly in the damaged limb [4,88,92,136,208,238]. The effect of muscle damage on transport of other viruses is unclear.

To investigate differences in dissemination patterns of distinct neurotropic viruses, I intramuscularly inoculated adult or newborn immune-competent or immune-deficient mice lacking the type I IFN-α/β receptor (IFNAR-/-) with genetically marked pools of poliovirus, YFV-17D, or reovirus T3D. I quantified viral dissemination at 72 hours post-inoculation (hpi) and found that dissemination of reovirus T3D was more restricted than poliovirus and YFV-17D in adult immune-competent mice. All three viruses had enhanced dissemination in IFNAR-/- mice. Stimulating retrograde axonal transport with muscle damage enhanced poliovirus dissemination but did not enhance dissemination of either YFV-17D or reovirus T3D. Dissemination of poliovirus and reovirus was substantially enhanced in newborn mice, with reovirus showing the largest differences as a function of host age. Overall, my results suggest that poliovirus, YFV-17D, and reovirus T3D disseminate to the CNS using different pathways with different efficiencies.

Immune deficiency, muscle damage, and young age enhance poliovirus transport to the CNS

Analysis of viral titer is not always sufficient to define viral dissemination barriers; in fact, robust viral replication following traverse of a barrier can mask barriers completely [116,117,167]. To overcome this obstacle, I inoculated mice with genetically marked viruses and used a hybridization-based assay to quantify the viral population diversity in a given tissue [116,117]. A decrease in viral population diversity from one tissue to another indicates a viral transport barrier. Using this approach, I previously found that poliovirus transport to the CNS is inefficient, but ablating the type I IFN response enhanced viral transport to the CNS. Additionally, I found that muscle damage increased poliovirus transport to the CNS by enhancing retrograde axonal transport in the sciatic nerve [117]. However, the previous study analyzed viral titer and population diversity in tissues at disease onset, days 3-8 post-inoculation. Here, I examined

viral transport to the CNS at an early time point, 72 hpi, to determine whether the type I IFN response, muscle damage, or host age influences viral dissemination early in infection.

To examine viral transport early in infection, IFNAR+/+ or IFNAR-/- mice were inoculated intramuscularly with 10 genetically marked polioviruses (Fig. 1). Tissues were harvested at 72 hpi, and viral titer and population diversity were quantified. Similar to previous studies at late time points [116,117], I found increased viral titers in all tissues of IFNAR-/- mice relative to IFNAR+/+ mice (Fig. 2A, white vs. gray bars). Similarly, viral population diversity was greater in IFNAR-/- mice relative to IFNAR+/+ mice, and viral population diversity was less in brain compared with muscle (Fig. 2B, white vs. gray bars). In contrast to previous studies at later time points (disease onset, 5-8 days post-infection) where viral brain titers were high [117], viral titers in the brain were very low at 72 hpi in immune-competent mice. However, mean poliovirus titers in the brain were >8,000-fold higher in IFNAR-/- mice compared to IFNAR+/+ mice. Overall, these data suggest that poliovirus transport to the CNS is inefficient, particularly in adult immune-competent mice, at 72 hpi.

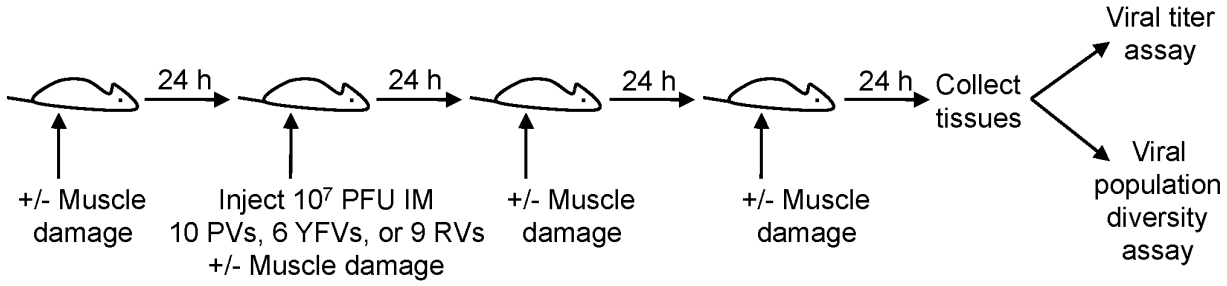

Figure 1. Experimental design. Mice were inoculated intramuscularly (IM) with 10^7 PFU of 10 poliovirus (PV), 6 YFV-17D, or 9 reovirus (RV) pool members, and tissues were collected at 72 hpi unless otherwise indicated. Muscle damage was induced in a subset of mice before, during, and after viral inoculation to stimulate retrograde axonal transport in the sciatic nerve.

I next examined the effect of muscle damage on poliovirus transport to the CNS at 72 hpi. Muscle damage was induced daily starting 1 d before viral inoculation and concluding 1 d prior to tissue collection (Fig. 1). Titers of poliovirus in tissues were generally higher in IFNAR+/+ mice with damage compared with those in IFNAR+/+ mice without damage (Fig. 2A, white vs. white hatched bars). In fact, mean poliovirus titers in brain were >270,000-fold higher in mice with muscle damage compared with mice lacking muscle damage. The muscle and brain tissues of IFNAR-/- mice with muscle damage also show increased titer compared with IFNAR-/- mice without damage (Fig. 2A, gray vs. gray hatched bars). Not surprisingly, viral population diversity was also higher for mice with muscle damage (Fig. 2B). Strikingly, the effect of muscle damage on viral titer and viral population diversity in the brain was greater than ablation of the type I IFN response. Although poliovirus replicated more efficiently in IFNAR-/- mice, trafficking to the CNS was still restricted by a barrier that was overcome by muscle damage. These results suggest that early in infection inefficient retrograde axonal transport and type I IFN restrict poliovirus trafficking to the CNS by different mechanisms. Furthermore, inefficient retrograde axonal transport is a stronger barrier for poliovirus dissemination to the CNS than the type I IFN response.

To determine whether barriers to poliovirus CNS dissemination exist in very young mice, I inoculated 3-day-old IFNAR+/+ or IFNAR-/- mice with the pool of genetically marked viruses and assessed viral titers and population diversity. Infected mice were moribund at 48 hpi; therefore, tissues were collected at this earlier time point. Viral titers were very high in muscle, spinal cord, and brain in both IFNAR+/+ and IFNAR-/- mice, and all pool members were present in all tissues (Fig. 2C-D). In agreement with previous studies, these data suggest that a major barrier limiting viral dissemination develops as mice age [48]. Overall, poliovirus dissemination is limited most by age-specific factors, followed by inefficient retrograde axonal transport, and the type I IFN response.

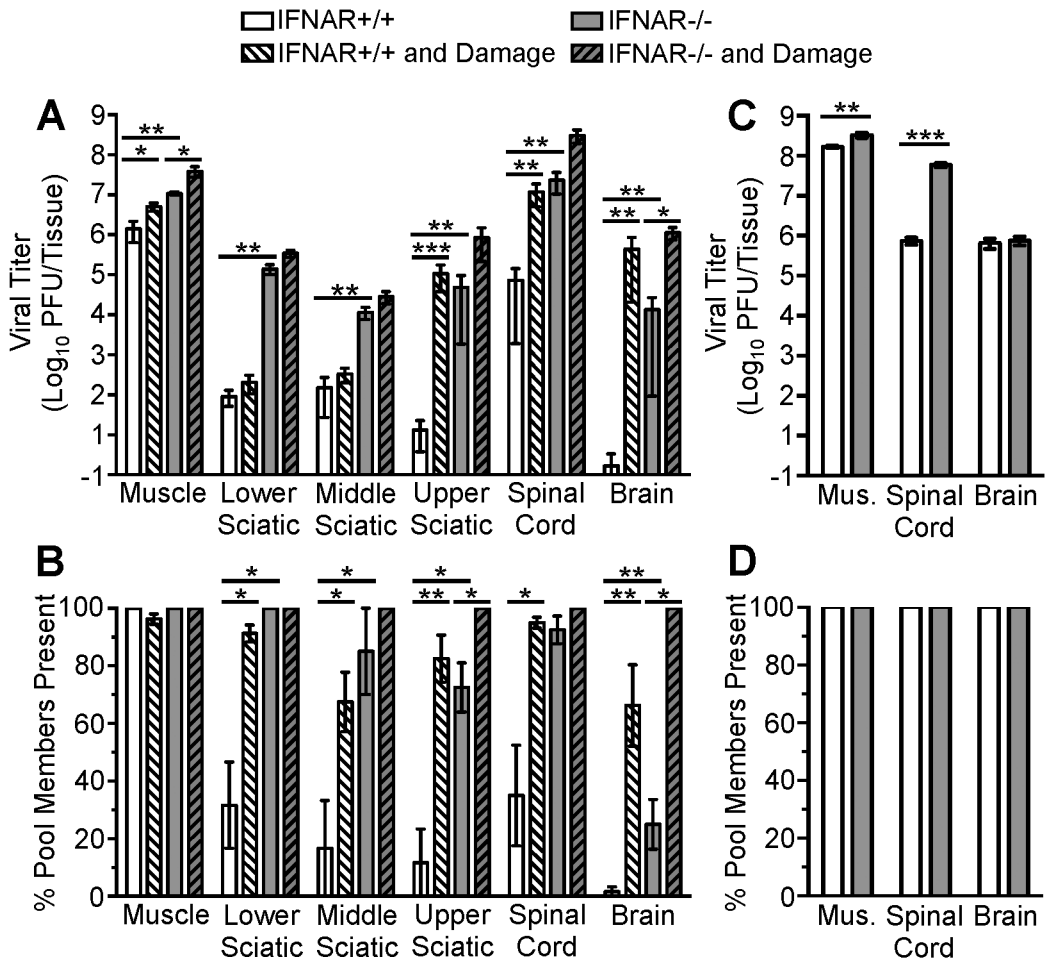

Figure 2. Dissemination of poliovirus to the CNS is limited by inefficient retrograde axonal transport, type I IFN responses, and older age. IFNAR+/+ and IFNAR-/- mice were inoculated intramuscularly with 10^7 PFU total of 10 genetically marked polioviruses, with or without additional muscle damage. Tissues were collected, viral titers were determined by plaque assay, and viral population diversity was determined using a hybridization-based assay [116]. Poliovirus titer (A) and viral population diversity (B) in tissues harvested from adult IFNAR+/+ or IFNAR-/- mice with or without muscle damage. Tissues were collected at 72 hpi, prior to disease onset. Poliovirus titer (C) and viral population diversity (D) in tissues harvested from 3-day-old IFNAR+/+ or IFNAR-/- mice. Tissues were collected at 48 hpi in C and D due to the onset of disease. Results are presented as mean +/- standard error of the mean from 4-8 mice per condition. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$. Mus., muscle.

Immune deficiency, but not muscle damage, enhances YFV-17D transport to the CNS

Using a pool of six genetically marked viruses, I previously found that YFV-17D inoculated intramuscularly disseminated to the brain relatively efficiently in IFNAR-/- mice at 7-8 dpi [64]. Here, I examined YFV-17D dissemination at 72 hpi to better understand the kinetics of viral transport by evaluating the effect of immune deficiency, muscle damage, and host age on viral dissemination. I found that immune-competent adult IFNAR+/+ mice had moderate titers of YFV-17D in all tissues tested (Fig. 3A, white bars). While immune-deficient IFNAR-/- mice had higher YFV-17D titers in several tissues, I was surprised to find that viral titers in brain were only 1.3-fold higher than those in IFNAR+/+ mice (Fig. 3A, white vs. gray bars). For most tissues, viral population diversity was greater in IFNAR-/- mice compared with that in IFNAR+/+ mice (Fig. 3B, white vs. gray bars). Although viral titers in the brain were similar in IFNAR-/- and IFNAR+/+ mice, viral population diversity was greater in IFNAR-/- mice. These results suggest that YFV-17D may encounter a barrier in IFNAR+/+ mice en route to the brain that was overcome in IFNAR-/- mice or that clearance of the virus differs in the two mouse strains.

Because YFV and YFV-17D are thought to enter the CNS predominantly via the hematogenous route [141,196], and muscle damage stimulates transport in neurons, I hypothesized that muscle damage would not alter YFV-17D dissemination to the brain. Indeed, viral titers were not higher in IFNAR+/+ mouse tissues at 72 hpi following muscle damage (Fig. 3A, white vs. hatched bars). In fact, viral titers were lower in mice with muscle damage, suggesting that muscle injury reduced viral replication and dissemination. Muscle damage was associated with increased YFV-17D population diversity in muscle tissue but not in any of the peripheral or central nervous system tissues (Fig. 3B, white vs. hatched bars). It is possible that inflammation associated with muscle damage reduced YFV-17D replication.

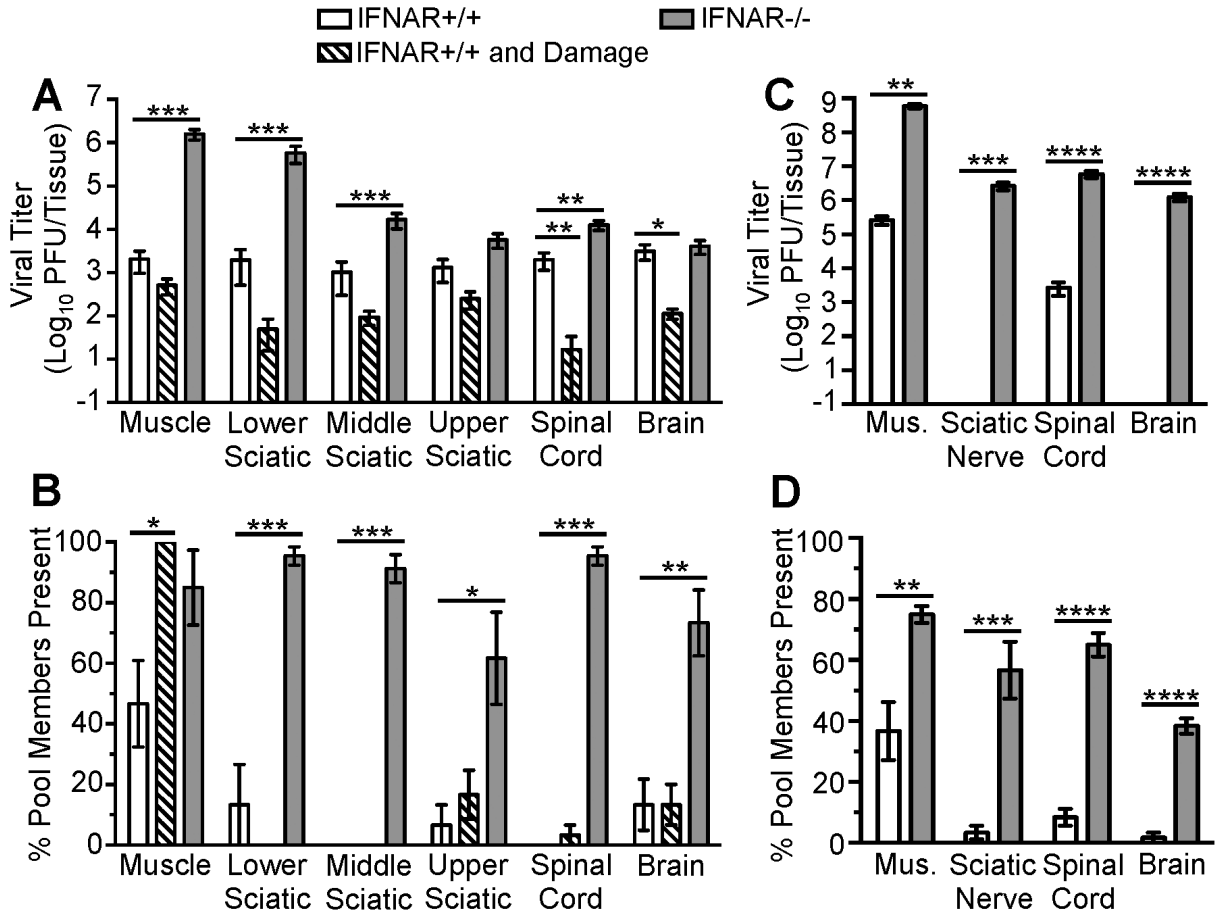

Figure 3. Dissemination of YFV-17D to the CNS is limited by type I IFN responses but not inefficient retrograde axonal transport. IFNAR+/+ and IFNAR-/- mice were inoculated intramuscularly with 10^7 PFU total of six genetically marked YFV-17D viruses, with or without additional muscle damage for IFNAR+/+ mice. Tissues were collected at 72 hpi, prior to disease onset. Viral titers were determined by plaque assay, and viral population diversity was determined using a hybridization-based assay. YFV-17D titer (A) and viral population diversity (B) in tissues harvested from adult IFNAR+/+ or IFNAR-/- mice with or without muscle damage. YFV-17D titer (C) and viral population diversity (D) in tissues harvested from 3-day-old IFNAR+/+ or IFNAR-/- mice infected with 10^4 PFU. Results are presented as mean +/- standard error of the mean from 6-8 mice per condition. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$. Mus., Muscle.

To determine whether YFV-17D disseminates more efficiently in very young mice, I inoculated 3-day-old IFNAR+/+ or IFNAR-/- mice with the pool of genetically marked viruses and assessed viral titers and population diversity. Due to the small inoculation volume required for infant mice and low YFV-17D concentration, mice were inoculated with 10^4 PFU in these experiments rather than the 10^7 PFU inoculum used in experiments with adult animals. Therefore, direct comparisons between YFV-17D dissemination in adult vs. young mice could not be made. However, I were able to evaluate the relative viral dissemination efficiencies in IFNAR+/+ and IFNAR-/- infant mice. Not surprisingly, YFV-17D titers were higher and population diversity was greater in IFNAR-/- mice compared with those parameters in IFNAR+/+ mice (Fig. 3C-D). Strikingly, YFV-17D titers were undetectable in the brain of immune-competent young mice, while immune-deficient mice contained $>10^4$ PFU in the brain. Therefore, the type I IFN response is a major barrier in young mice to dissemination of YFV-17D.

Tracing individual viral pool members reveals different transport routes of poliovirus and YFV-17D to the CNS

A major strength of using genetically marked viruses for viral dissemination studies is that they can be used to determine viral transport routes from the periphery to the CNS. To determine whether intramuscularly inoculated YFV-17D disseminated to the brain through the blood rather than through peripheral nerves, we compared specific pool members found in tissues of adult mice. YFV-17D pool members detected in the brain were scored relative to the pool members detected in the sciatic nerve, spinal cord, or blood of each mouse to determine the degree of overlap. For this analysis, we used only mice in which less than or equal to half of the total pool members were detected in the brain, since animals with most or all of the pool members present in the brain would be uninformative for determining trafficking routes. We found that, on average, a YFV-17D pool member detected in the brain was detected in the sciatic nerve in 70% of mice, in the spinal cord in 45% of mice, and in the blood in 75% of mice (Fig. 4). Therefore, viruses found in the brain matched viruses found in the blood to a greater extent

than viruses found in the sciatic nerve or spinal cord. In contrast, poliovirus pool members detected in the brain matched the pool members detected in the sciatic nerve and spinal cord in 98-100% of cases, and poliovirus was undetectable in the blood (Fig. 4). These data are consistent with previous studies showing that poliovirus disseminates through nerves to reach the mouse brain [159,161,178] and support the idea that YFV-17D disseminates primarily through the blood to infect the CNS [141,196].

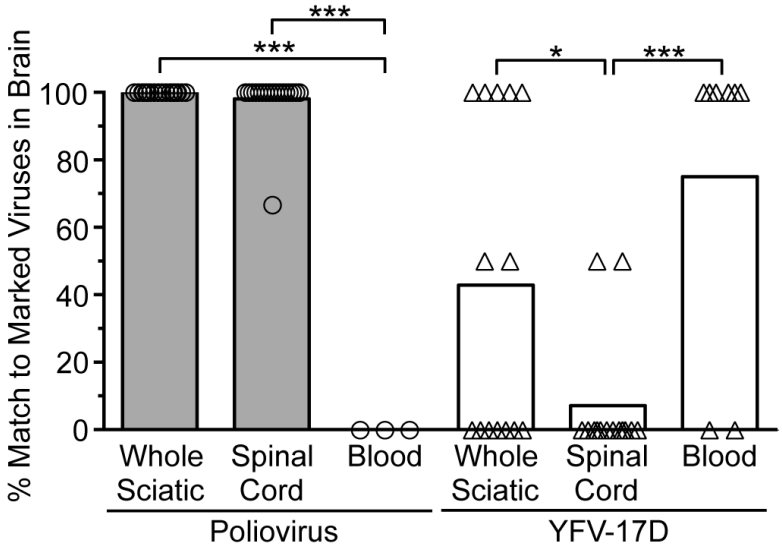

Figure 4. Poliovirus and YFV-17D likely reach the brain through different routes. Viral pool members detected in the brain were scored relative to the pool members detected in the sciatic nerve, spinal cord, or blood of each IFNAR+/+ or IFNAR-/- mouse to determine the degree of overlap. We used only mice in which less than or equal to half of the total pool members were detected in the brain, since animals with most or all of the pool members present in the brain would be uninformative for determining trafficking routes. Each symbol represents a single mouse. Bars represent means of 3-18 mice. Data are shown as percent match of viral pool members present in the brain to viral pool members present in each tissue. As an example, if the brain contained poliovirus pool members 2 and 4 (of 10 total) and the spinal cord contained poliovirus pool members 4, 6, 8, and 10, then the percent match of brain viruses to viral pool members present in the spinal cord is 50% ([1 matching virus/2 total viruses present in brain] x 100). Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$.

Development of a reovirus T3D population diversity assay

To examine reovirus dissemination in mice, I engineered a panel of nine genetically marked reovirus strains that can be distinguished using a hybridization-based population diversity assay. The strains each contain a combination of unique silent mutations in the M1 gene segment such that RT-PCR products of each pool member are recognized by specific oligonucleotide probes (Fig. 5A). The specificity of each probe was confirmed by blotting RT-PCR products from individual strains on membranes, followed by hybridization with each ^{32}P-labeled oligonucleotide probe (Fig. 5B). To determine whether any of the strains have altered replication efficiency compared with the others, I performed an *in vitro* viral serial passage experiment. This type of analysis is more sensitive than single-cycle replication assays for detection of subtle replication differences [116]. After an initial infection with a mixture containing equal aliquots of each pool member, I used the infected cells to initiate viral replication in naïve cells, and relative levels of each pool member were quantified over seven serial passages. In this experiment, all pool members were present at similar levels in the initial and final passages (Fig. 5C), suggesting that the pool members have similar fitness *in vitro*. To determine whether the genetically marked reovirus strains differ in fitness *in vivo*, I inoculated mice intramuscularly with a mixture of all nine pool members and examined viral population diversity at 72 hpi. A blot of the reovirus pool members in peripheral and CNS tissues of a representative IFNAR-/- mouse is shown in Fig. 5D. To confirm that reovirus pool members have equivalent fitness *in vivo*, viral population diversity data for all reovirus mouse experiments were compiled to determine the relative number of times each pool member was detected. All nine reovirus pool members were detected with similar frequency (Fig. 5E), providing strong evidence that the pool members have similar fitness *in vivo*.

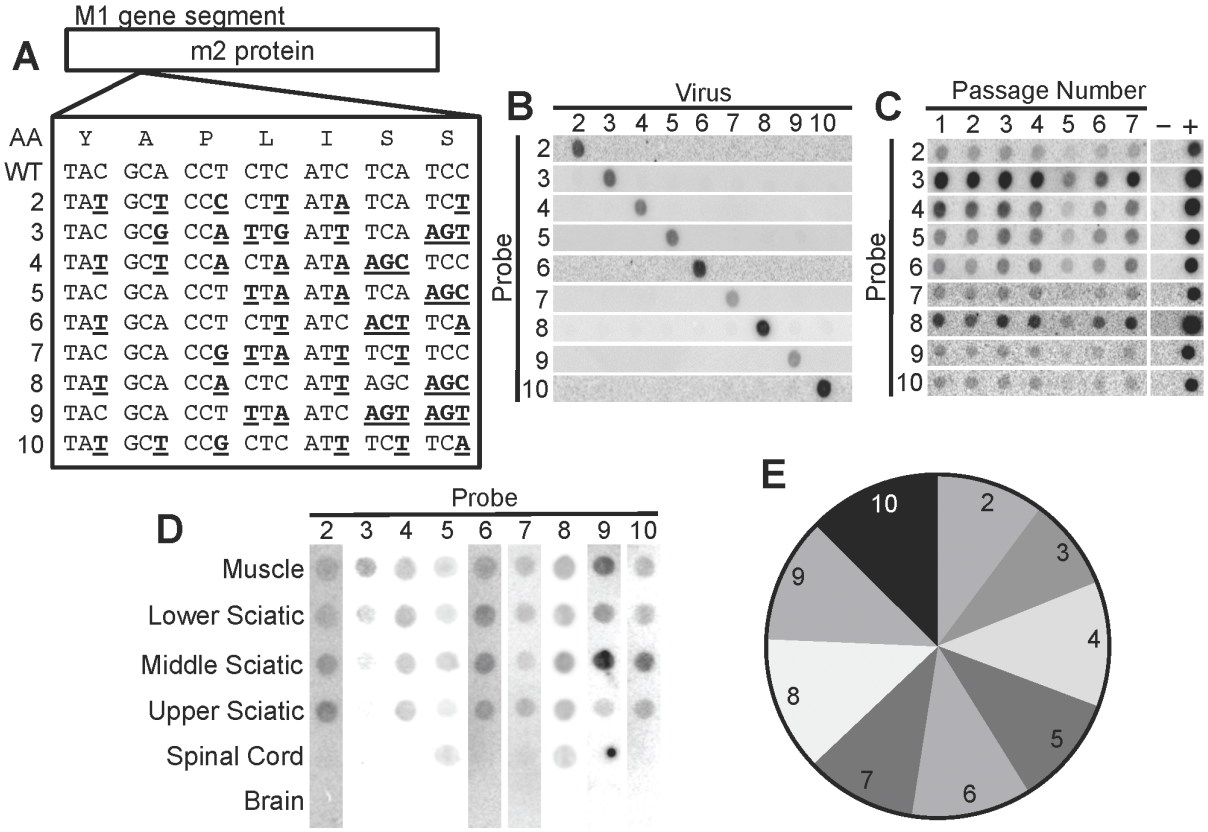

Figure 5. Development of a hybridization-based reovirus T3D viral population diversity assay. (A) Sequence alignment showing groups of silent point mutations introduced into the reovirus T3D M1 gene segment. The amino acid sequence is shown at the top. (B) Blots demonstrating specificity of each probe for its cognate viral RT-PCR product. (C) Serial passage competition experiment showing maintenance of all pool members. L929 cells were adsorbed with equivalent PFU of each reovirus pool member. Infected cells were collected at 24 hpi and used to initiate another cycle of replication in naïve cells. Following seven passages, the ratios of viruses were compared by hybridization assay. + indicates positive match control, - indicates mismatch control as previously described [116]. (D) Representative blot of RT-PCR products from a reovirus-infected IFNAR-/- mouse following intramuscular inoculation. Mice were inoculated intramuscularly with 10^7 PFU total of the nine genetically marked reoviruses. Tissues were collected at 72 hpi, and viral population diversity was assessed using the hybridization assay. (E) Relative prevalence of each reovirus pool member in all mouse tissues collected during this study. Data were derived from tissues of 63 mice. The relatively equal prevalence of each pool member indicates that pool members do not have significant fitness differences *in vivo*.

Young age and immune deficiency, but not muscle damage, enhances reovirus T3D transport to the CNS

To define factors that influence reovirus dissemination from the periphery to the CNS in mice, I examined trafficking of the nine genetically marked reoviruses following intramuscular inoculation. In accordance with previous work [59], viral titers in muscle, sciatic nerve, and spinal cord were significantly higher in adult IFNAR-/- mice than those in adult IFNAR+/+ mice (Fig. 6A, white vs. gray bars). Remarkably, reovirus dissemination to the upper sciatic nerve and spinal cord was observed only in IFNAR-/- mice (Fig. 6A). Viral population diversity was modest in IFNAR+/+ tissues and generally greater in IFNAR-/- tissues (Fig. 6B, white vs. gray bars). Reovirus was not detected in the brain of adult mice of either strain at 72 hpi, which was not surprising given the well-established restriction of reovirus dissemination to the CNS in adult mice [133,212].

Because reovirus T3D and poliovirus both infect neurons and are transported by fast retrograde axonal transport, I hypothesized that muscle damage would enhance reovirus transport as observed in the case of poliovirus transport. Although damage induced small increases in reovirus titers in muscle of both IFNAR+/+ and IFNAR-/- mice, muscle injury was not sufficient to enhance reovirus dissemination to the sciatic nerve, spinal cord, or brain in IFNAR+/+ mice (Fig. 6A, white vs. white hatched bars). Muscle damage was associated with increased viral population diversity in muscle of IFNAR+/+ mice but generally not in other tissues (Fig. 6B). In IFNAR-/- mice, increases in viral population diversity following muscle damage were only significant in the spinal cord (Fig. 6B). Overall, these experiments show that dissemination of intramuscularly inoculated reovirus is severely restricted in immune-competent adult mice and that muscle damage does not enhance viral dissemination from the inoculation site.

I next determined whether a longer infectious time course would allow increased reovirus dissemination to the CNS. Reovirus replicates more slowly than poliovirus and may require a longer interval to reach the brain. Therefore, I compared viral titers in adult IFNAR+/+ and IFNAR-/- mice with

and without muscle damage at 7 d post-inoculation. In most tissues tested, viral titers were lower at 7 days post-inoculation than at 72 hpi (Fig. 6C vs. Fig. 6A). Reovirus was not detected in the brain of adult mice under any of the conditions tested (Fig. 6C). Therefore, the lack of reovirus dissemination to the brain of adult mice at 72 hpi is not simply due to the relatively early time point of tissue collection.

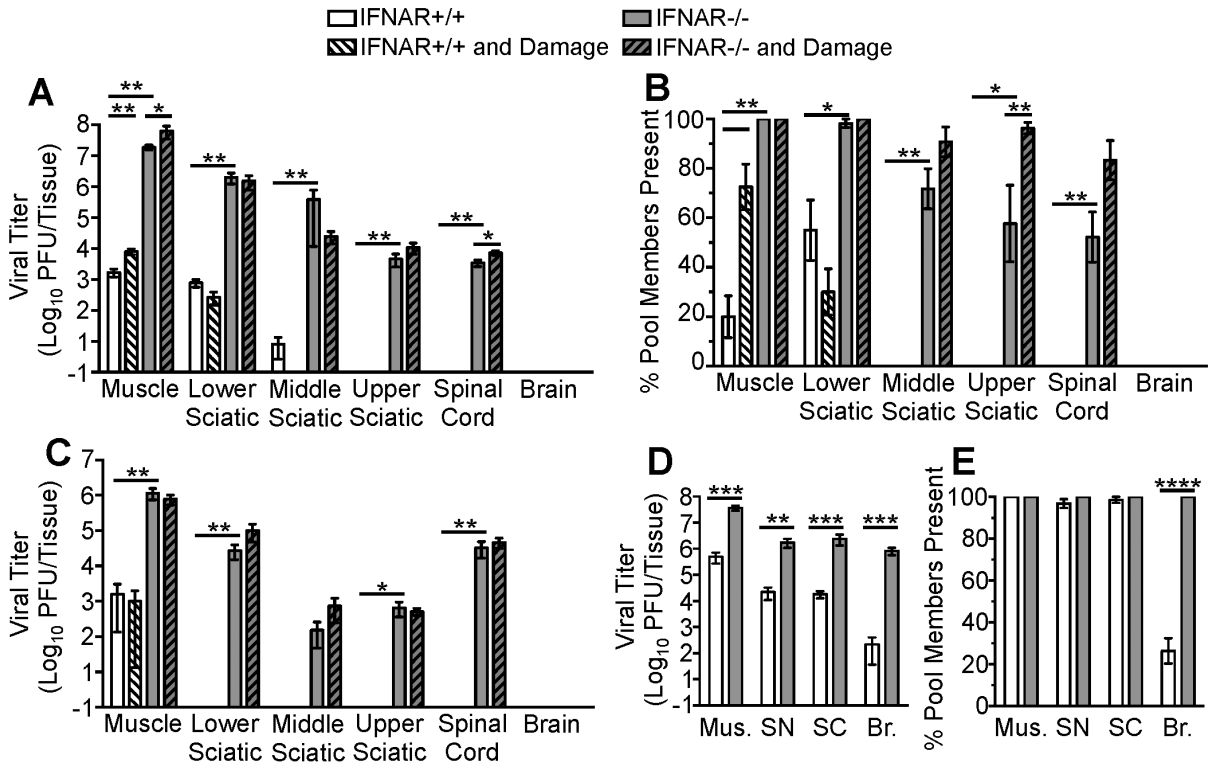

Figure 6. Dissemination of reovirus T3D to the CNS is limited by type I IFN responses and older age but not inefficient retrograde axonal transport. IFNAR+/+ and IFNAR-/- mice were inoculated intramuscularly with 10^7 PFU total of nine genetically marked reoviruses, with or without additional muscle damage. Tissues were collected at 72 hpi, prior to disease onset. Viral titers were determined by plaque assay, and viral population diversity was determined using a hybridization-based assay. Reovirus T3D titer (A) and viral population diversity (B) in tissues harvested from adult IFNAR+/+ or IFNAR-/- mice with or without muscle damage. (C) Reovirus T3D titer in tissues from adult mice collected at 7 d post-inoculation. Reovirus T3D titer (D) and viral population diversity (E) in tissues from 3-day-old IFNAR+/+ or IFNAR-/- mice. Results are presented as mean +/- standard error of the mean from 5-9 mice per condition. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$. Mus., Muscle, SN, Sciatic Nerve, SC, Spinal Cord, Br., Brain.

To determine whether reovirus T3D disseminates more efficiently in very young mice, I inoculated 3-day-old IFNAR+/+ or IFNAR-/- mice with the pool of genetically marked viruses and assessed viral titers and population diversity. Reovirus titers were detectable in all tissues of young mice, including brain, with significantly higher titers in IFNAR-/- mice compared with those in INFAR+/+ mice (Fig. 6D). Notably, all reovirus pool members were detected in the brains of INFAR-/- mice (Fig. 6E). However, only about 25% of pool members were detected in the brains of IFNAR+/+ mice, revealing an IFN-mediated barrier limiting reovirus infection of the brain in young mice. Collectively, these experiments are consistent with previous findings on age-dependent and IFN-dependent barriers to reovirus dissemination [59,133,201,212].

Co-infection with poliovirus does not enhance reovirus dissemination to the CNS

Since poliovirus is more efficiently transported than reovirus in neurons of IFNAR+/+ and IFNAR-/- mice, I were curious about whether co-infection with poliovirus could enhance reovirus transport. I thought it possible that poliovirus infection might stimulate host pathways that mediate viral transport in neurons, which in turn would enhance transport of other types of cargoes. To test this hypothesis, adult mice were inoculated intramuscularly with 10^7 PFU of the 10 genetically marked polioviruses and 10^7 PFU of the nine genetically marked reoviruses for a total inoculum of 2×10^7 PFU. Tissues were resected at 72 hpi and processed for virus titer determination and population diversity assay using L929 cells, which do not support poliovirus replication. When titer and diversity data from co-infected mice ('co') are compared with data from individually infected mice ('ind', from Fig. 6A-B), I found no detectable change (enhancement or diminishment) in reovirus transport following co-infection with poliovirus (Fig. 7A-B). This finding suggests that poliovirus and reovirus disseminate to the CNS using distinct pathways.

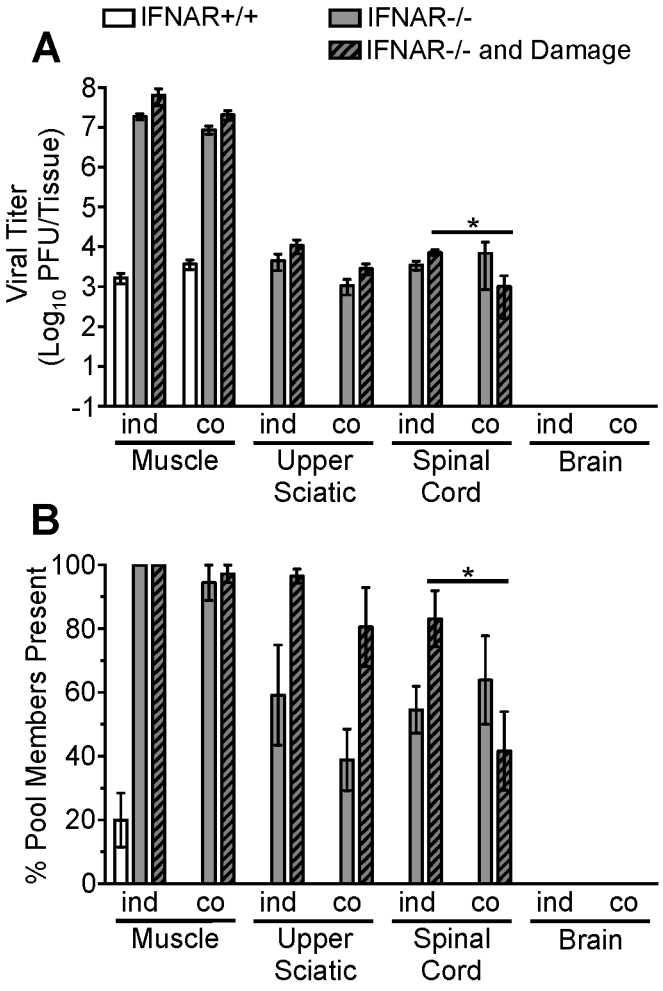

Figure 7. Co-infection with poliovirus does not enhance reovirus dissemination to the CNS. IFNAR+/+ and IFNAR-/- mice were inoculated intramuscularly with 10^7 PFU total of nine genetically marked reoviruses and 10^7 PFU total of 10 genetically marked polioviruses. Tissues were collected at 72 hpi. Reovirus titers and population diversity were determined by plaque assay and hybridization-based assay, respectively, using L929 cells, which do not support poliovirus replication. Reovirus T3D titer (A) and viral population diversity (B) in tissues harvested from adult IFNAR+/+ or IFNAR-/- mice with or without muscle damage. 'ind' indicates data from infections with reovirus only, and 'co' indicates data from the reovirus-poliovirus co-infection. Results are presented as mean +/- standard error of the mean from 5-7 mice per condition. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$.

Discussion

Knowledge about how different viruses disseminate to the CNS is essential for a comprehensive understanding of the pathogenesis of neurotropic viral infections. In this study, I compared the transport of three different neurotropic viruses following intramuscular inoculation of mice using viral titer and population diversity assays. I found that poliovirus, YFV-17D, and reovirus T3D disseminate to the CNS with varying efficiencies and use distinct mechanisms that are affected differently by host damage responses (Table 1).

Although injury to the muscle at the inoculation site enhanced neuronal transport of poliovirus in both IFNAR+/+ and IFNAR-/- mice, muscle damage did not enhance transport of either YFV-17D or reovirus T3D. In fact, muscle damage reduced YFV-17D dissemination. YFV-17D likely enters the brain by crossing the blood-brain barrier following hematogenous dissemination rather than by transport through peripheral nerves into the spinal cord. Therefore, enhancement of retrograde axonal transport in neurons induced by muscle damage would be less likely to increase YFV-17D dissemination to the CNS. Interestingly, blood-brain barrier disruption by needle puncture enhances dissemination of peripherally inoculated YFV to the brain, but peripheral tissue damage does not [141,196]. Poliovirus and reovirus share many biological properties such as fecal-oral transmission, dissemination in peripheral nerves, dependence on dynein for transport, and spread in neurons by fast retrograde axonal transport. Therefore, it was somewhat surprising that muscle damage enhanced neuronal transport of poliovirus but not reovirus. Damage-dependent enhancement of retrograde axonal transport occurs for wheat germ agglutinin [117], indicating that accelerated transport as a consequence of muscle injury can occur with cargoes other than viruses. Collectively, these observations provide additional evidence that poliovirus and reovirus traffic in the nervous system using different mechanisms.

Injury to muscle at the inoculation site enhanced YFV-17D and reovirus population diversity in the muscle of IFNAR+/+ mice as well as reovirus titer in the muscle of IFNAR+/+ and IFNAR-/- animals. However, increased viral titer and population diversity in muscle as a consequence of damage

did not enhance transport through peripheral nerves for either virus, suggesting that the effect may be due to muscle-specific factors that enhance viral replication or decrease viral clearance.

Co-infection with poliovirus did not accelerate neuronal transport of reovirus, likely due to differences in the host transport mechanisms used by these viruses. I think it possible that each virus associates with different endogenous cargoes during transport, and that poliovirus is associated with a more efficiently transported cargo than reovirus. Differences in receptor utilization also may control transport efficiency. The PVR cytoplasmic domain associates with dynein light chain Tctex-1 to transport poliovirus using dynein motors [150,159]. Reovirus T3D requires NgR1 to infect neurons, but NgR1 is GPI-anchored and therefore not likely to associate directly with any dynein components outside of reovirus-containing endosomes [110,187]. NgR1 is expressed at high levels in the adult brain but, because of myelination in the adult, the receptor may not be available for reovirus T3D binding and entry [110]. This aspect of NgR1 physiology could explain the age-specific barrier to reovirus infection. In contrast, PVR is widely expressed and available for poliovirus binding and entry into the brain.

Findings made in this study show that poliovirus, YFV-17D, and reovirus T3D disseminate to the CNS by distinct mechanisms. By using assays of viral load and viral population diversity, I uncovered dissemination routes to the CNS and identified barriers to dissemination for each of these viruses. Enhancement of fast retrograde axonal transport by muscle damage increased dissemination of poliovirus but not YFV-17D or reovirus T3D. These findings highlight the complexity of neurotropic virus access to the CNS and provide a framework to define virus-specific neural transportation routes.

CHAPTER 4: Attempting to clarify the mechanism of muscle damage-induced enhancement to neuronal poliovirus dissemination

Introduction

Medical evidence suggests that a damaged muscle correlates with development of paralytic poliomyelitis following infection [4,23,24,88,92,208,238], and previous research in mice reveals that muscle damage enhances retrograde axonal transport of the virus and decreases mouse survival [86,118]. Despite this, the mechanisms which regulate damage-dependent enhancement of poliovirus dissemination are still unclear. In fact, it is not even clear whether poliovirus is spreading through a single particular pathway and whether it has cofactors during transport. Multiple molecules have been implicated in stimulating retrograde axonal transport and I were interested to investigate them for their potential to stimulate viral transport.

Neuron-derived growth factors were previously studied for their effects in preventing damage to neurons and axonal transport. Ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) were both demonstrated to enhance retrograde axonal transport in the sciatic nerve following intramuscular co-injection with a tracer [22,186]. Other molecules such as glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) were demonstrated to protect neurons following damage by signaling through the retrograde axonal transport pathway, but are incapable of enhancing transport [135,186]. Similar to poliovirus, CNTF, BDNF, GDNF, and NGF enter motor neurons by receptor-dependent endocytosis and are transported through peripheral nerves in conjunction with their receptor [22,91,181,186]. It is unclear whether poliovirus may use one or multiple of these same pathways to spread through peripheral nerves to the CNS.

In addition to testing co-injection of growth factors and poliovirus, an unbiased approach was used to compare changes to gene expression between damaged and non-damaged muscle. Microarray analysis of uninfected tissue allows me to investigate the effects of damage independent of changes in transcription due to viral infection. Elevated levels of two particular transcripts stood out in damaged

muscle compared to non-damanged muscle. The first was tissue inhibitor of metalloproteinase-1 (TIMP-1). TIMP-1 regulates matrix metalloproteinases (MMPs), which are shown to stimulate retrograde axonal transport, the same mechanism by which poliovirus is disseminated through the nervous system [86,117,178,197,228]. MMPs are produced immediately following damage and are responsible for degradation of damaged tissue [100,225,234]. As MMPs are extremely effective at degrading damaged tissue for clearance, their activity is closely regulated by TIMPs, which are expressed roughly 24 hours after damage [100,230].

The second transcript with elevated levels in damaged muscle was monocyte chemoattractant protein-1 (MCP-1), expression of which is triggered in neurons, astrocytes, and microglia by inflammatory responses [11,21,44,80,89,122,138] and peripheral nerve damage [221]. Though constitutive expression of MCP1 in the nervous system is detected in resident immune cells [5], discrete brain regions [10,47,84,206,219] and in dorsal horn of the spinal cord [52,221], it has inflammatory-stimulated expression which yields enhanced permeability across the endothelial cells of the blood brain barrier [62,151,204,206]. Enhanced blood brain barrier permeability may in turn reduce barriers to poliovirus dissemination into the CNS. The above molecules were individually tested to determine whether their functions in altering host retrograde axonal transport or blood brain barrier permeability facilitates poliovirus dissemination through peripheral nerves and into the CNS.

Exogenous BDNF does not alter poliovirus replication in HeLa cells

Neuron-derived growth factors were previously shown to enhance host retrograde axonal transport, including the dynein motor used by poliovirus [15,22,50,60,91,135,181,186]. I hypothesized that stimulating retrograde axonal transport with growth factors would enhance poliovirus dissemination similar to the effect of muscle damage.

Prior to testing the effect of growth factors on poliovirus dissemination *in vivo*, I wanted to examine whether BDNF altered poliovirus entry *in vitro*. I infected HeLa cells with poliovirus in the presence or absence of BDNF, then quantified viral titer by plaque assay at predetermined times

following infection. Infections at either an MOI of 0.1 or 10 revealed that BDNF does not alter poliovirus entry *in vitro* (Fig. 8). Regardless of treatment with BDNF, viral output was the same, suggesting that BDNF neither enhances nor impedes poliovirus replication in HeLa cells.

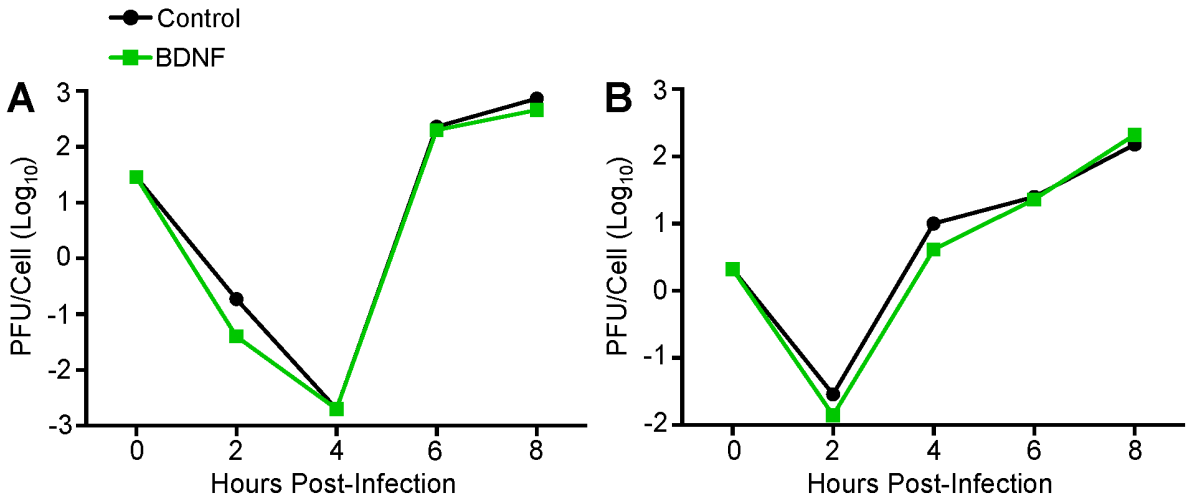

Figure 8. Exogenous BDNF does not alter poliovirus replication in HeLa cells. HeLa cells were infected at an MOI of 10 (A) or 0.1 (B) with poliovirus alone (black) or poliovirus with 25 ng of BDNF (green). Cells were harvested at 0, 2, 4, 6, or 8 hours post-infection and titers were determined by plaque assay on HeLa cells.

Exogenous neuron-derived growth factors do not alter survival of infected mice, enhance poliovirus load per tissue, or neuronal poliovirus dissemination

As previous work has shown that muscle damage decreases mouse survival following intramuscular poliovirus inoculation [86,117], and neuron-derived growth factors increased host retrograde axonal transport [22,186], I hypothesized that treatment with BDNF may also decrease survival. Mice were inoculated intramuscularly with either $1x10^5$ or $3x10^4$ PFU poliovirus alone, or mixed with 50 ng BSA or BDNF, then monitored for clinical signs. There were no statistically significant differences in the onset of clinical signs of disease between the different conditions (Fig. 9A,C). In fact, all mice were euthanized by 8 days post infection due to complete hind limb paralysis. This suggests that injection of an exogenous protein alone, such as BSA, does not alter the course of poliovirus infection following intramuscular inoculation. It also reveals that a single injection of BDNF at the inoculation site does not alter mouse survival. Quantification of viral load in the muscle, spinal cord, and brain collected at the onset of clinical signs show that there is no statistically significant difference in any of the tissues (Fig. 9B).

Though a single injection of BDNF insufficient to enhance poliovirus dissemination significantly enough to alter survival, perhaps a different growth factor enhances poliovirus transport. The effect of a single BDNF injection may occur early during the infection, but wane as BDNF is degraded [153]. To overcome this, tissues were collected at 24 hours post inoculation, rather than later timepoints. Immune-competent 6-8 week old mice were injected intramuscularly with poliovirus with or without 50 ng of an exogenous growth factor added. Tissues were processed for quantification of poliovirus titer by plaque assay. Separately, poliovirus-containing cleared tissue lysates were amplified on HeLa cells for isolation of RNA for the viral diversity assay. Plaque assay data reveal that growth factors do not alter viral load close to the inoculation site in the muscle and lower sciatic nerve Fig. 10A). Some growth factors like BDNF and GSK show trends toward enhanced viral load in the spinal cord (Fig. 10D), but further investigation into the data reveals a very broad spread of PFU/tissue from mouse to mouse (Fig. 10E).

Past research shows that stimulation of neuronal poliovirus dissemination by muscle damage yields enhanced viral load in the brain [117], but plaque assay data lack a clear enhancement of viral load in the brain (Fig. 10A,F), suggesting that neuronal poliovirus transport through the spinal cord was not enhanced by the addition of exogenous growth factors. These data suggest that BDNF and other growth factors are unlikely to play a direct role in enhancing viral load of poliovirus per tissue following muscle damage.

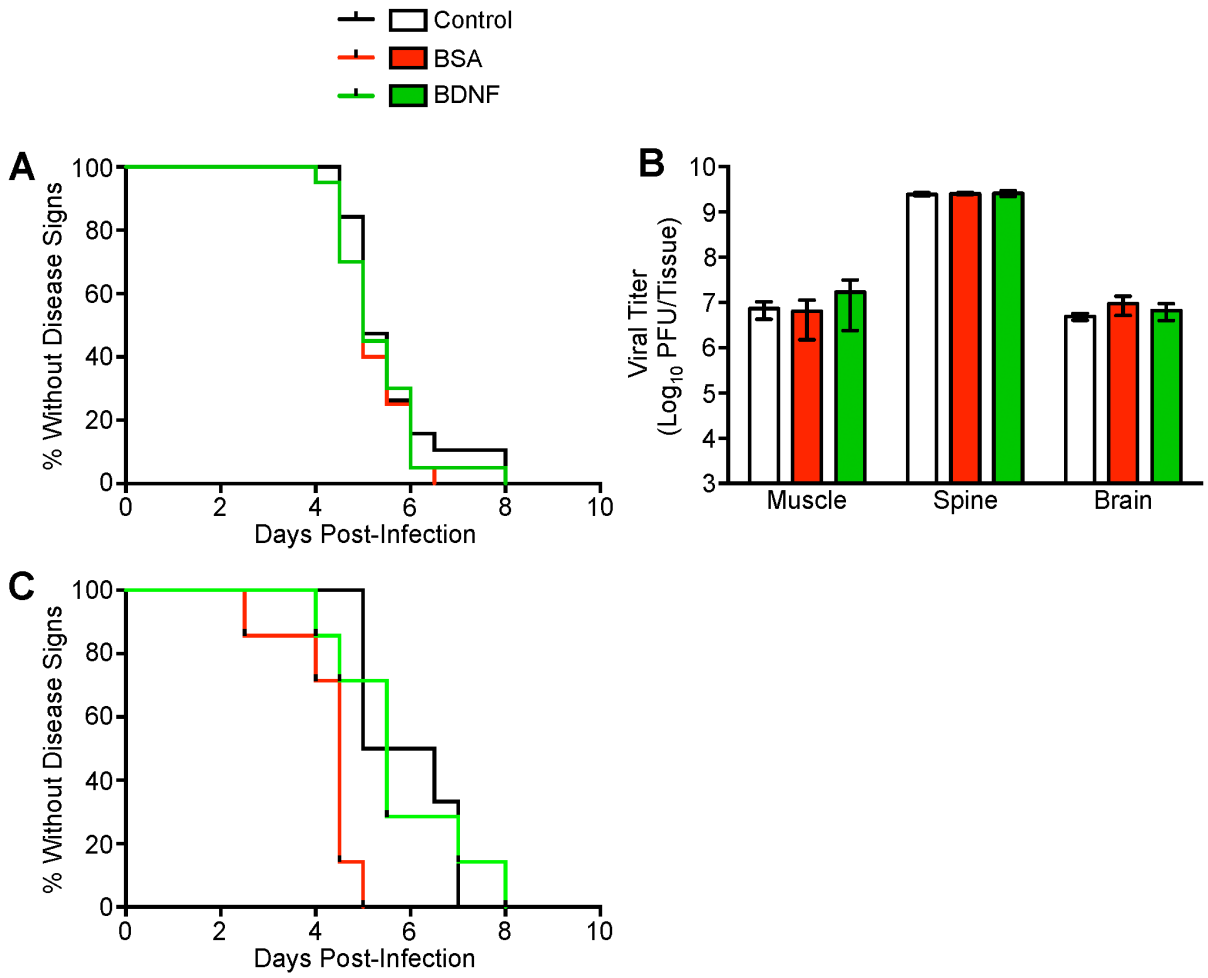

Figure 9. Exogenous BDNF does not decrease the time of onset of clinical signs of disease or enhance viral load per tissue in immune-competent mice. IFNAR+/+ mice were inoculated intramuscularly with poliovirus alone (black line/white bar), with 50 µg BSA (red), or with 50 µg BDNF (green). Disease signs were the earliest point at which at least one hind limb was fully paralyzed. (A) Mice were inoculated with 10^5 PFU poliovirus alone, with BSA, or with BDNF as indicated above. (B) Poliovirus titer was determined by plaque assay from tissues collected at disease onset in A. Results are presented as mean +/- standard error. (C) Mice were inoculated with $3x10^4$ PFU poliovirus alone, with BSA, or with BDNF as indicated above. Data represent 5-7 mice per condition. For both A and C, differences to time of onset of disease signs were not significantly different by the Mantel-Cox test.

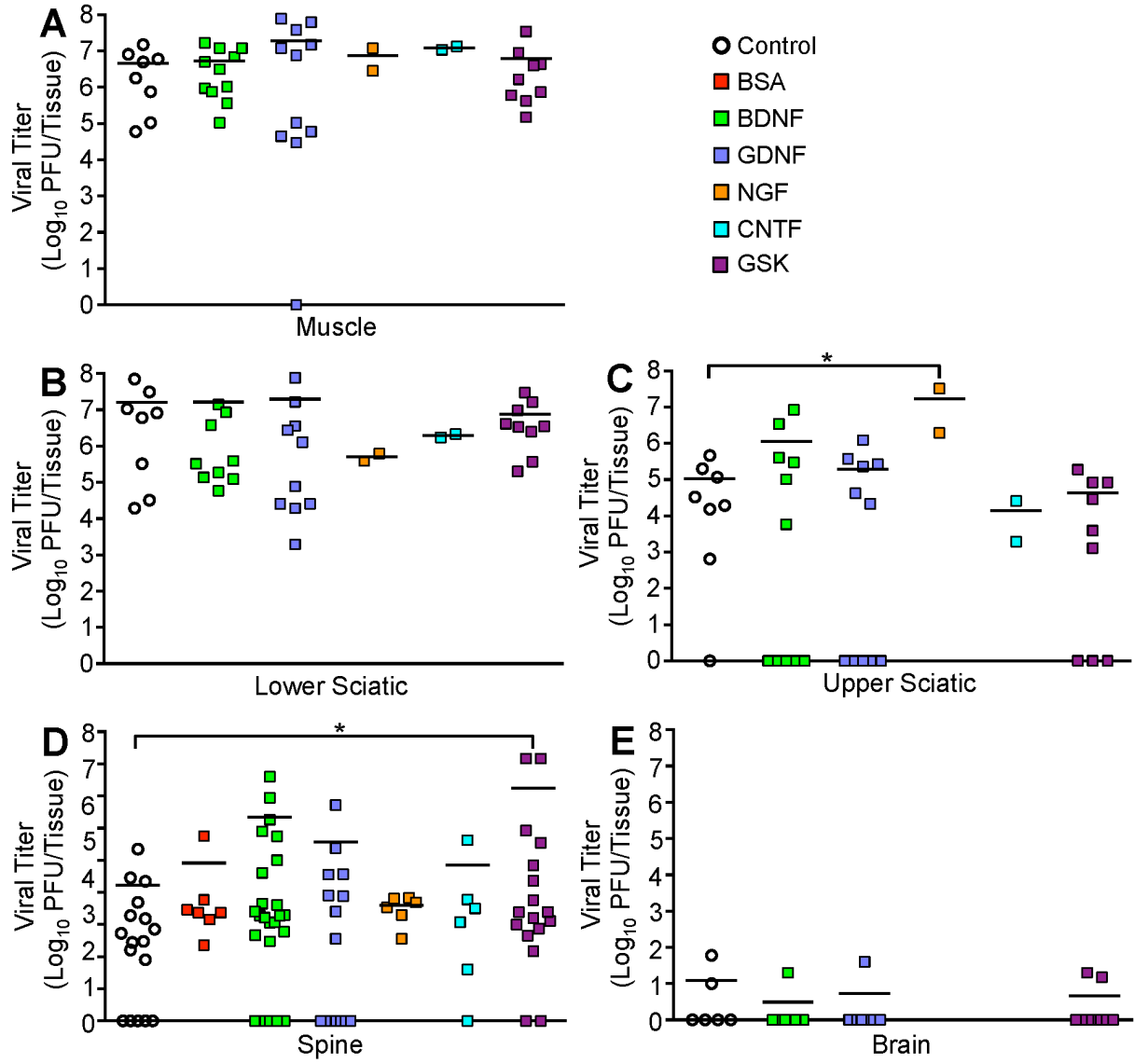

Figure 10. Single dose of individual exogenous growth factors does not play a significant role in enhancing poliovirus titers. IFNAR+/+ mice were inoculated intramuscularly with $2x10^7$ PFU total of 10 genetically marked polioviruses with or without 50 µg of an individual exogenous protein: BSA (red), BDNF (green), GDNF (dark blue), NGF (orange), CNTF (light blue), or GSK (purple). Tissues were collected at 24 hours post-inoculation and viral titers were determined by plaque assay. Each dot represents poliovirus titer of a single mouse for the muscle (A), lower sciatic nerve (B), upper sciatic nerve (C), spine (D), or brain (E). Black line indicates mean of 2-11 mice per condition (A-C), 10-24 mice per condition (D), or 6-9 mice per condition (E). Values that are significantly different, as determined by the Mann-Whitney test, were calculated between the control values and each condition, and are indicated by asterisks as follows: *, $P < 0.05$.

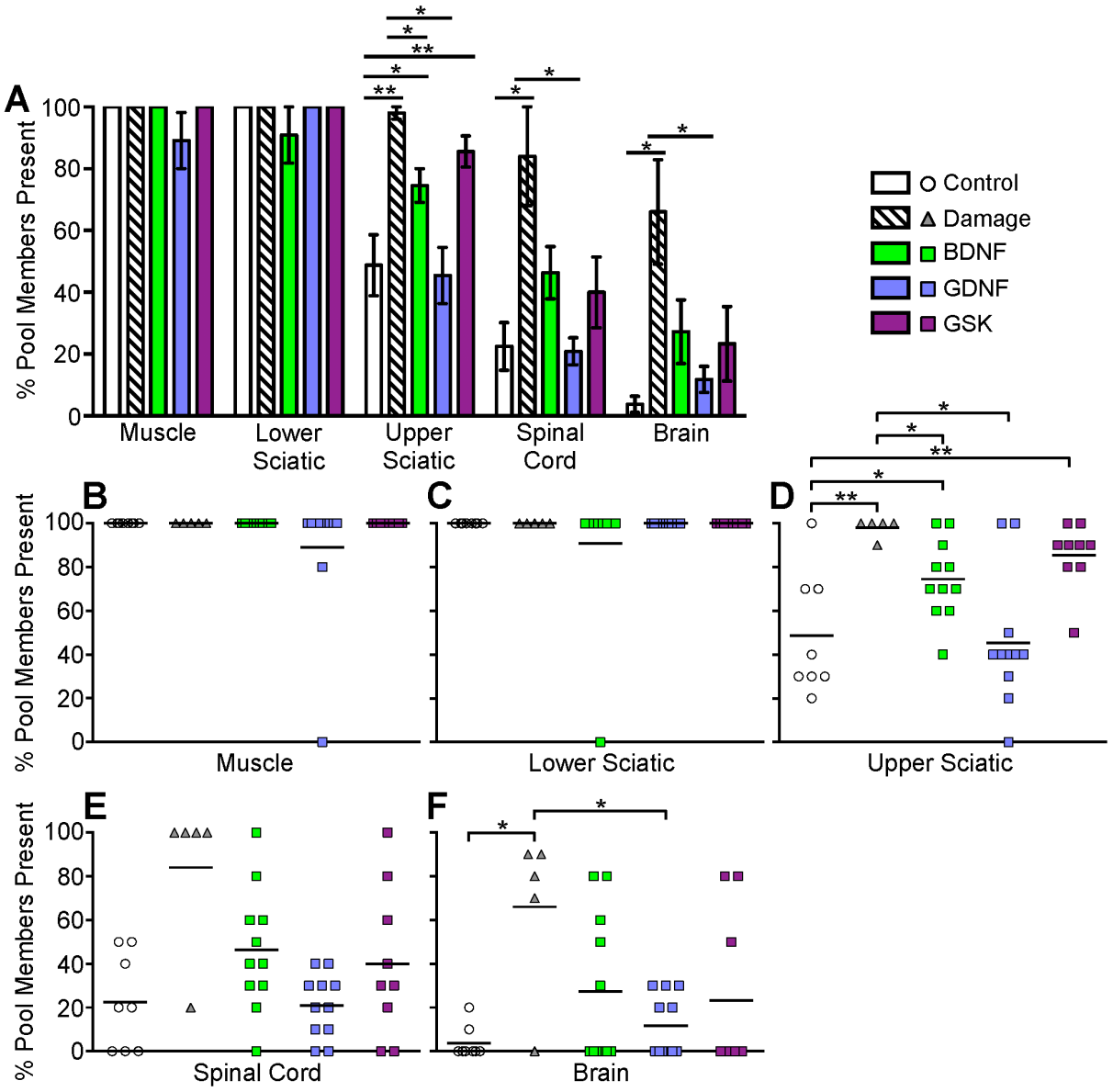

Figure 11. Single dose of individual exogenous growth factors does not play a significant role in enhancing neuronal poliovirus dissemination. IFNAR+/+ mice were inoculated intramuscularly with $2x10^7$ PFU total of 10 genetically marked polioviruses alone (white), with muscle damage (diagonal stripes/gray), or with 50 µg of an individual exogenous protein: BDNF (green), GDNF (dark blue), or GSK (purple). Tissues were collected at 24 hours post-inoculation and viral population diversity was determined used a hybridization-based assay [116]. (A) Results are presented as mean +/- standard error of the mean from 5-11 mice per condition. (B-F) % pool members present per individual tissue from A. Each dot represents the poliovirus population diversity of a single mouse for the muscle (B), lower sciatic nerve (C), upper sciatic nerve (D), spine (E), or brain (F). Black line indicates mean of 5-11 mice per condition. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$.

Quantifying titer alone could cause poliovirus replication to mask enhancements in dissemination [116,117,167], so changes in poliovirus diversity along the neuronal dissemination route were also quantified. Similar to the plaque assay data, treatment with damage or growth factors revealed no detectable changes at tissues closest to the inoclulation site (Fig. 11A). In agreement with previous work, poliovirus diversity is enhanced by muscle damage relative to controls (Fig. 11A,D-F and [117]). Mean poliovirus diversity in the spine was elevated with the addition of BDNF or GSK relative to controls, but viral diversity was not enhanced to the same magnitude as following muscle damage, and review of individual data points again reveal wide variability from mouse to mouse for each condition (Fig. 11B-F). Interestingly, higher levels of viral diversity were detected in the brains of some growth factor-treated mice, but the majority of mice had no detectable virus in the brain (Fig. 11F). As with the plaque assay data, these data suggest that neuronal poliovirus dissemination is not enhanced by concurrent treatment with exogenous growth factors.

Microarray of muscle following needle sticks damage compared to non-damaged muscle

To gain more insight into host reponses to muscle damage using a non-biased approach, microarrays comparing needle stick-damaged muscle to non-damaged muscle were performed. As I expect that the damage-stimulated enhancement to neuronal poliovirus dissemination is the result of a host response to damage, mice were not infected with poliovirus to eliminate changes in expression due to viral infection. Muscle damage was performed as described above and damage was given daily for three days. Mock IM inoculations with PBS alone were given on the second day of damage. Muscle tissue was collected one the second day after the mock inoculation, as most damage-dependent changes in expression occur within the first 24-48 hours following damage [100,230]. Data were compared as the fold change in transcript levels between damaged muscle and non-damaged muscle (Table 1).

Table 1. Transcripts with greater than 5-fold change in damage vs. non-damaged muscle tissue.

Gene	Fold Change (Damage/No Damage)	*Mus musculus* Definition	Synonyms
TIMP1	20.43	tissue inhibitor of metalloproteinase 1 (Timp1), transcript variant 2, mRNA.	MGC7143; Clgi; TIMP-1; Timp
HMOX1	13.49	heme oxygenase (decycling) 1 (Hmox1), mRNA.	Hsp32; HO1; Hemox; Hmox; HO-1; D8Wsu38e
LOC100034251	12.96	predicted gene, OTTMUSG00000000971 (OTTMUSG00000000971), mRNA.	RP23-430I21.1
LYZS	11.30		
LGALS3	10.85	lectin, galactose binding, soluble 3 (Lgals3), mRNA.	L-34; Mac-2; GBP; gal3
MS4A6D	10.81		
FCER1G	10.05		
ALOX5AP	9.72	arachidonate 5-lipoxygenase activating protein (Alox5ap), mRNA.	Flap
CD44	9.03		
CCL9	9.00		
HIST1H2AI	8.91	histone cluster 1, H2ai (Hist1h2ai), mRNA.	H2a-291A
GPNMB	8.86	glycoprotein (transmembrane) nmb (Gpnmb), mRNA.	ipd; Dchil
HIST1H2AF	8.78	histone cluster 1, H2af (Hist1h2af), mRNA.	H2a-221
THBS2	8.73	thrombospondin 2 (Thbs2), mRNA.	Thbs-2; TSP2
CCL8	8.69	chemokine (C-C motif) ligand 8 (Ccl8), mRNA.	Mcp2; HC14; 1810063B20Rik; AB023418; Scya8; MCP-2
HIST1H2AO	8.63	histone cluster 1, H2ao (Hist1h2ao), mRNA.	
TUBB2B	8.50	tubulin, beta 2b (Tubb2b), mRNA.	2410129E14Rik
LYZS	8.48	lysozyme (Lyzs), mRNA.	Lys; Lzm; Lzp; Lzm-s1; AI326280
HIST1H2AG	8.32		
HIST1H2AH	8.07	histone cluster 1, H2ah (Hist1h2ah), mRNA.	RP23-9O16.9
FCRL3	8.06	Fc receptor, IgG, low affinity IV (Fcgr4), mRNA.	4833442P21Rik; CD16-2; FcgRIV; FcgammaRIV; Fcrl3; Fcgr3a
ALOX5AP	7.99		
PTPNS1	7.84		
SLC11A1	7.83		
LAPTM5	7.80	lysosomal-associated protein transmembrane 5 (Laptm5), mRNA.	E3
HIST1H2AN	7.79	histone cluster 1, H2an (Hist1h2an), mRNA.	
HIST1H2AD	7.61	histone cluster 1, H2ad (Hist1h2ad), mRNA.	
SLC11A1	7.58	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (Slc11a1), mRNA.	MGC129157; Lsh; Nramp; Ity; Bcg; MGC129156; Nramp1
HIST1H2AK	7.53	histone cluster 1, H2ak (Hist1h2ak), mRNA.	
C1QB	7.49	complement component 1, q subcomponent, beta polypeptide (C1qb), mRNA.	
HIST1H2AH	7.26	histone cluster 1, H2ah (Hist1h2ah), mRNA.	RP23-9O16.9
LIP1	7.22		
COTL1	7.02	coactosin-like 1 (Dictyostelium) (Cotl1), mRNA.	2010004C08Rik; 1810074P22Rik; Clp
LOX	6.97	lysyl oxidase (Lox), mRNA.	TSC-160; AI893619
PRC1	6.95	protein regulator of cytokinesis 1 (Prc1), mRNA.	D7Ertd348e; MGC6745
SDC3	6.72		
GLIPR2	6.61		
B230343A10RIK	6.48		

Table 1 (continued)

Gene	Fold Change (Damage/No Damage)	*Mus musculus* Definition	Synonyms
CD44	6.46	CD44 antigen (Cd44), transcript variant 2, mRNA.	AU023126; Ly-24; AW146109; HERMES; AW121933; Pgp-1
MS4A7	6.43		
CYBA	6.40	cytochrome b-245, alpha polypeptide (Cyba), mRNA.	b558
CXCL4	6.40	chemokine (C-X-C motif) ligand 4 (Cxcl4), mRNA.	Pf4; Scyb4
STAB1	6.36	stabilin 1 (Stab1), mRNA.	KIAA0246; STAB-1; MS-1; mKIAA0246; FEEL-1
HP	6.32	haptoglobin (Hp), mRNA.	HP-1
SERPINA3N	6.27	serine (or cysteine) peptidase inhibitor, clade A, member 3N (Serpina3n), mRNA.	Spi2/eb.4; Spi2-2; Spi2.2
PSCD4	6.25		
LGMN	6.25		
MRC1	6.21	mannose receptor, C type 1 (Mrc1), mRNA.	CD206; AW259686
F13A1	6.17	coagulation factor XIII, A1 subunit (F13a1), mRNA.	1200014I03Rik; AI462306; F13a
CD68	6.17		
P2RY6	6.17		
HP	6.13	haptoglobin (Hp), mRNA.	HP-1
SAA3	6.13		
PRG4	6.09		
GLIPR2	6.02	GLI pathogenesis-related 2 (Glipr2), mRNA.	5730414A08Rik; GAPR-1; C77180
EMP3	5.91	epithelial membrane protein 3 (Emp3), mRNA.	HNMP-1; Ymp; MI-35; H4; H-4
CORO1A	5.83	coronin, actin binding protein 1A (Coro1a), mRNA.	TACO; p57; Clabp
EDNRB	5.80	endothelin receptor type B (Ednrb), mRNA.	ETb; s; ET-B; Sox10m1; ET>B<; AU022549
HCPH	5.77		
PFC	5.76		
WBSCR5	5.73		
MPEG1	5.72		
CD52	5.66	CD52 antigen (Cd52), mRNA.	CLS1; B7-Ag; B7; AI463198; MB7; CAMPATH-1
MMP14	5.61	matrix metallopeptidase 14 (membrane-inserted) (Mmp14), mRNA.	AI325305; MT-MMP-1; MT1-MMP
GRN	5.59	granulin (Grn), mRNA.	epithelin
GAS7	5.57	growth arrest specific 7 (Gas7), mRNA.	AW124766; Gas7-cb
FXYD5	5.46	FXYD domain-containing ion transport regulator 5 (Fxyd5), mRNA.	RIC; Oit2; EF-8
C1QG	5.44		
KCNAB2	5.44	potassium voltage-gated channel, shaker-related subfamily, beta member 2 (Kcnab2), mRNA.	I2rf5; F5; Kcnb3
LY86	5.37	lymphocyte antigen 86 (Ly86), mRNA.	MD-1; MD1
FER1L3	5.27		
EMR1	5.21	EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (Emr1), mRNA.	Ly71; EGF-TM7; F4/80; TM7LN3; Gpf480; DD7A5-7
LYZ	5.19	lysozyme (Lyz), mRNA.	Lzp-s
FKBP11	5.08		
EMP3	5.07	epithelial membrane protein 3 (Emp3), mRNA.	HNMP-1; Ymp; MI-35; H4; H-4
HP	5.05	haptoglobin (Hp), mRNA.	HP-1
FN1	5.05		

The top microarray result was tissue inhibitor of metalloproteinase-1 (TIMP-1); transcript levels from damaged muscle were 20-fold higher than transcript expression in control muscle (Table 1). TIMP-1 regulates matrix metalloproteinases (MMPs), which stimulate retrograde axonal transport immediately following tissue damage [100,225,234]. I hypothesized that MMPs are responsible for enhanced retrograde axonal transport following muscle damage, and that this enhancement is also responsible for enhanced neuronal dissemination of poliovirus.

To examine whether MMPs influence poliovirus transport, three conditions were tested for differences in viral load and diversity per tissue: vehicle only, vehicle and damage, or pan-MMP inhibitor GM6001 and damage. Mice were treated with vehicle or GM6001 IP once per day for four days, inoculated IM with 10^7 PFU poliovirus on the second day, and tissues were collected three days after infection. If stimulation of retrograde axonal transport by MMPs is involved in enhanced poliovirus dissemination following muscle damage, I expect that inhibiting MMPs with GM6001 would counteract the effect of muscle damage and return poliovirus titer and diversity to similar levels as in vehicle alone. The titer data, summarized in Figure 12A-B, shows no significant difference between the muscle damage condition and treatment with GM6001 plus muscle damage. I also examined poliovirus diversity and found no significant differences (Fig. 12C-D). These data suggest that GM6001 treatment does not have a detectable effect upon poliovirus replication orwa dissemination. It is unusual that the viral load and diversity per tissue was almost as high in the control mice as the muscle damage mice in these experiments (Fig. 12); in fact, the viral load in the sciatic nerve segments, spinal cord, and brain for control mice was higher than observed previously for poliovirus (Fig. 2 and [117]). This difference, in conjunction with titer differences discussed in other experiments below, suggests that repeated IM injections of vehicle is sufficient to cause similar tissue damage to needle sticks at the muscle.

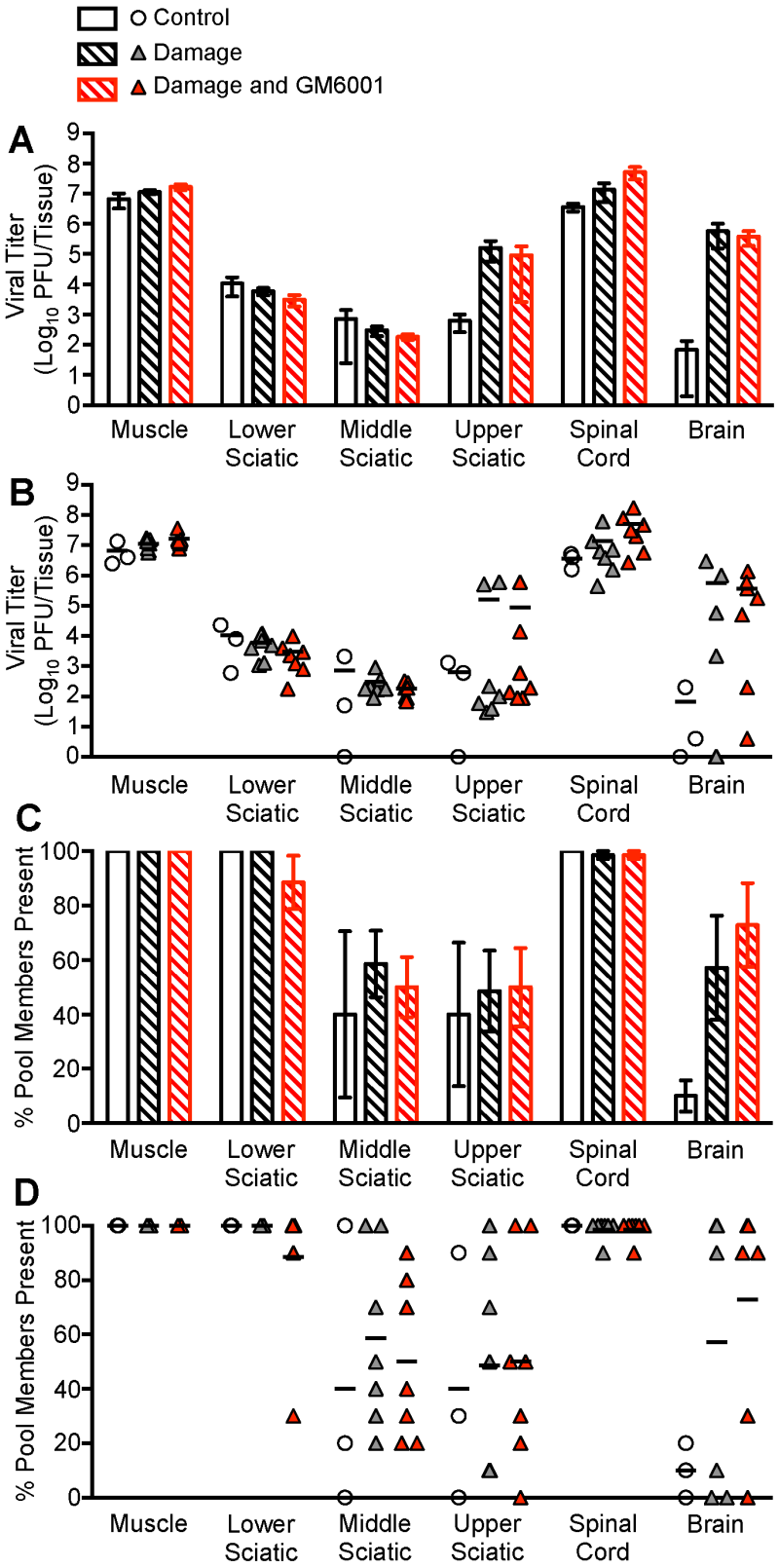

Figure 12. *(legend on following page)*

Figure 12. Pan-MMP inhibitor GM6001 does not block damage-induced enhancement in neuronal poliovirus dissemination. IFNAR+/+ mice were inoculated intramuscularly with 10^7 PFU total of 10 genetically marked polioviruses. Damage (black slashes/grey triangles) or intraperitoneal injection of 0.5 mg GM6001 (red slashes/red triangles) was given once daily for four days. Mice were inoculated with poliovirus on the second day of treatment. Tissues were collected three days post-inoculation, viral titers were determined by plaque assay, and viral population diversity was determined by a hybridization-based assay [116]. Poliovirus titer (A) and viral population diversity (C) are presented as mean +/- standard error of the mean. (B) Individual tissue titers from A. Each dot represents poliovirus titer of an individual tissue from a single mouse. (D) Viral population diversity per individual tissue from C. Each dot represents diversity in an individual tissue from a single mouse. All means and standard errors were calculated from 3-7 mice per condition.

Macrophage chemoattractant protein-1 alone is insufficient to stimulate neuronal poliovirus dissemination

In conjunction with the microarray, unpublished cytokine ELISA data from Karen Lancaster (previously a graduate student in the laboratory) suggested that macrophage chemoattractant protein-1 (MCP-1) expression at the transcript and protein level was also increased following muscle damage, independent of poliovirus infection. Constitutive expression of MCP-1 in the nervous system is detected in resident immune cells [5], discrete brain regions [10,47,84,206,219] and in dorsal horn of the spinal cord [52,221]. Increased expression of both MCP-1 and its cognate receptor CCR2 from neurons, astrocytes, and microglia is triggered by inflammatory responses [11,21,44,80,89,122,138] and as a result of peripheral nerve damage [221]. This yields enhanced permeability across the endothelial cells of the blood brain barrier [62,151,204,206]. I hypothesized that MCP-1 could enhance neuronal poliovirus dissemination by relieving barriers to poliovirus access to the CNS.

To test whether MCP-1 could enhance neuronal poliovirus dissemination in a similar manner as muscle damage, MCP-1 or vehicle was injected IM once per day for two or four days. As in previous experiments, mice were inoculated IM with 10^7 (or 10^5, see below) PFU diversity poliovirus on the second day of treatment. Tissues were collected at three days post-inoculation. Tissues were prepared for titer analysis by plaque assay alone, as previous data suggested that viral load and diversity data show similar trends at three days post-inoculation (Fig. 2).

Initially, MCP-1 or vehicle injections were given once per day over four days to simulate the needle sticks damage procedure and mice were inoculated with 10^7 PFU poliovirus to follow most previous experiments. I observed that the viral load per tissue was similar between the needle sticks, PBS control, and MCP-1 conditions (Fig. 13A-B). This suggests that a series of vehicle injections could simulate muscle damage sufficiently to enhance neuronal poliovirus dissemination. If MCP-1 were stimulating poliovirus dissemination, the result would be obfuscated by the enhancement in dissemination due to the injection volume alone.

To attempt to counteract this enhancement, MCP-1 or vehicle was injected once per day for only two days and in a smaller injection volume (30 μL here vs. 50 μL above). This means that the mice were receiving only one more injection than a typical infection, as the second MCP-1 injection was given in conjunction with the poliovirus inoculation. These mice were still inoculated with 10^7 PFU poliovirus. Even with fewer injections, injections with vehicle alone still showed viral loads per tissue similar to loads measured following needle sticks damage (Fig. 13C). Viral loads following MCP-1 injections were similar to those after needle sticks damage as well, suggesting no MCP-1-stimulated enhancement of neuronal poliovirus dissemination over that caused by repeated IM injections (Fig. 13C). An additional experiment was performed with the same conditions, except mice were inoculated with 10^5 PFU poliovirus to determine whether a high input titer was overwhelming existing barriers and masking any potential changes in poliovirus dissemination. This method was also unsuccessful in distinguishing between vehicle and MCP-1-treated conditions (Fig. 13D). In fact, other than a single vehicle-treated mouse, poliovirus was not detected in the upper sciatic, spinal cord, or brain of any mice. If MCP-1 were playing a major and direct role in enhancing neuronal poliovirus dissemination, I would have expected to detect some small amount of poliovirus in at least the upper sciatic and spinal cord of MCP-1-treated mice.

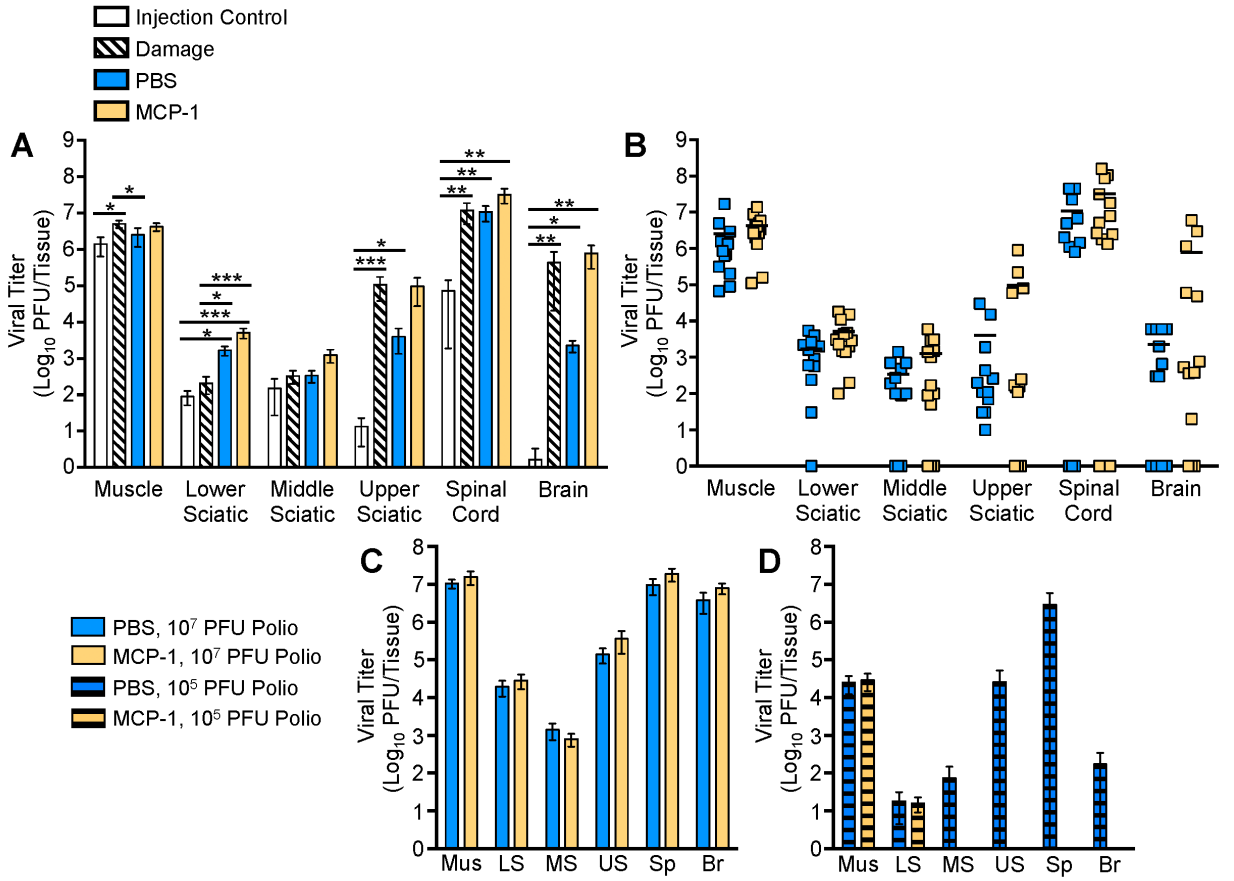

Figure 13. MCP-1 does not directly stimulate neuronal poliovirus dissemination. (A) Four conditions were tested: Injection Control (single poliovirus inoculation, no other injections; white), Damage (damage once daily for four days; black slashes), PBS (PBS injected intramuscularly once daily for four days; blue), or MCP-1 (0.5 µg MCP-1 injected intramuscularly once daily for four days; yellow). IFNAR+/+ mice were inoculated intramuscularly with 10^7 PFU poliovirus on the second day of treatment based on conditions above. Tissues were collected at three days post-inoculation and viral titer quantified by plaque assay for 6-12 mice per condition. (B) Individual tissue titers from A, where each dot represents an individual tissue from a single mouse. Black lines represent mean. (C-D) IFNAR+/+ mice were intramuscularly injected with PBS (blue) or 0.5 µg MCP-1 (yellow) twice, once 24 hours prior to poliovirus inoculation and once concurrently with poliovirus inoculation. Mice were intramuscularly inoculated with 10^7 PFU (C) or 10^5 PFU (D) poliovirus and tissues were collected three days post-inoculation. Viral titers were quantified by plaque assay for 5 mice per condition. For A, C, and D, results are presented as mean +/- standard error of the mean. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows:*, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$. Mus, muscle, LS, lower sciatic, MS, middle sciatic, US, upper sciatic, Sp, spine, Br, brain.

Damage-induced enhancement of neuronal poliovirus dissemination occurs independently of secondary infections

Damaging the mouse hindlimb muscle with needle sticks could allow for secondary bacterial infections to take hold. In fact, microarray analysis indicated increased transcript expression for genes related to host response to bacterial infection. It is important to clarify that enhanced poliovirus dissemination is the result of muscle injury itself, and not a bacterial infection. To test this, needle stick (open tissue) damage was compared to muscle crush damage, which maintains tissue sterility. Sterile damage is frequently used to test response to and recovery from muscle, peripheral nerve, and spinal cord trauma [29,61,205]. Similar to previous experiments, 6-8 week old immune-competent mice were inoculated IM with 10^7 PFU diversity poliovirus. Sterile crush damage was given once daily for four days—the same timing as needle sticks damage—by repeated crushes with clean forceps. Tissues were collected at three days post-inoculation and analyzed for viral load and diversity. Crush damage mice were separated into two groups based on the vascular damage observed at the inoculation site; the muscle of mice with "less damage observed" had no obvious bruising or damage, whereas mice with "more damage observed" had apparent bruising.

Plaque assay data show that there is no statistical difference between the damage conditions at the muscle, lower sciatic, middle sciatic, or spinal cord (Fig. 14A-B). In the upper sciatic and brain, there are not statistically significant differences between tissues from mice with more crush damage and needle sticks damage, suggesting that the crush damage stimulated retrograde axonal transport and therefore neuronal poliovirus dissemination to a similar degree (Fig. 14A-B). There are statistically significant differences between tissues from less damage and more damage. This suggests that the factor enhancing neuronal poliovirus replication or dissemination scales with the amount of vascular damage; it may scale up and enhance replication or dissemination, or scale down and relieve inhibition on the system.

Compared to viral load trends, the diversity data show a similar pattern in the tissues nearest to the inoculation site: muscle, lower sciatic, and middle sciatic. While there are detectable differences

between control and damage conditions, there are no detectable differences between the different damage conditions. (Fig. 14C-D) Interestingly, diversity at the upper sciatic, spinal cord, and brain show different trends than the viral load data. In the upper sciatic, there are no significant differences between damage conditions, similar to the other tissues closer to the inoculation site (Fig. 14C-D). In the spinal cord and brain it appears that crush damage is either inhibiting dissemination or not stimulating it as strongly. Diversity for the crush damage conditions is lower than needle sticks damage, though still higher than controls. Though the brains from mice with more crush damage contained higher viral loads than tissues with less damage, the mean percent diversity for both conditions is similar (Fig. 14C). This suggests that replication may be independent from dissemination; while a similar percent of viral pool members are able to reach the brain regardless of the amount of crush damage, greater crush damage allows enhanced replication in the brain.

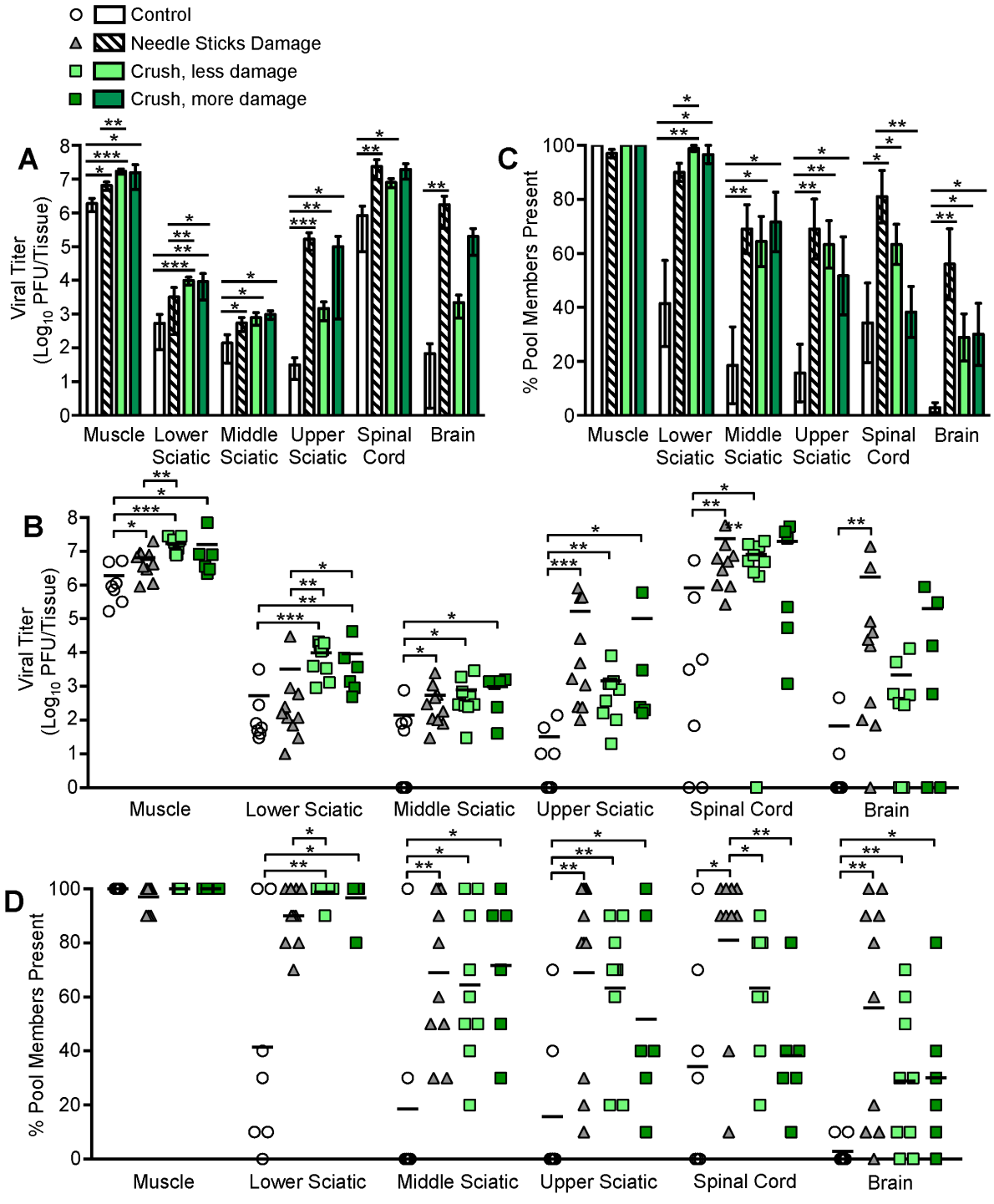

Figure 14. *(legend on following page)*

Figure 14. Damage-induced enhancement of neuronal poliovirus dissemination does not depend on secondary infections. IFNAR+/+ mice were inoculated intramuscularly with 10^7 PFU total of 10 genetically marked polioviruses without damage, with needle sticks damage given once daily for four days, or with crush damage given once daily for four days. Mice were inoculated the second day of damage treatment and tissues were collected three days post-inoculation. (A) Viral titers were quantified by plaque assay and results are presented as mean +/- standard error of the mean. (B) Individual tissue titers from A, where each dot represents poliovirus titer of an individual tissue for a single mouse. Black lines represent mean. (C) Viral population diversity was quantified using a hybridization-based assay [Kuss 2008] and results are presented as mean +/- standard error of the mean. (D) Individual viral diversity per tissue from C, where each dot represents population diversity in an individual tissue for a single mouse. Black lines represent mean. Values that are significantly different, as determined by the Mann-Whitney test, are indicated by asterisks as follows: *, $P < 0.05$, **, $P < 0.005$, ***, $P < 0.0005$. Results determined from 6-10 mice per condition.

Discussion

Clarifying the mechanisms which regulate poliovirus dissemination through the nervous system provides a better understanding of neurotropic infections and possible targets for treatment. I investigated the effects of molecules such as neutrotrophic growth factors, TIMP-1, and MMPs on neuronal poliovirus dissemination in mice use viral titer and population diversity assays. Neuronal poliovirus dissemination was enhanced by stimulating retrograde axonal transport with muscle damage, but injection of growth factors such as BDNF and CNTF did not enhance poliovirus dissemination or alter mouse survival after infection. It is possible that a single growth factor alone is insufficient to stimulate a detectable change in poliovirus dissemination, or that the growth factors stimulate retrograde axonal transport in an alternative pathway not used by poliovirus.

Microarray analysis was performed to detect differences between transcript expression in damaged and non-damaged tissue. Top results with previously identified functions in reducing barriers to peripheral nerve and CNS transport were tested for effects upon poliovirus dissemination. MMPs, targeted for regulation by top hit TIMP-1, can stimulate retrograde axonal transport following tissue damage. Inhibiting MMPs following damage of poliovirus-infected mice did not yield detectable differences in viral dissemination. MCP-1 was another transcript elevated in damaged muscle; MCP-1 induces increased blood-brain barrier permeability. I expected that increased permeability would yield

enhanced poliovirus dissemination into the CNS, but this was not the case in mice. These results again suggest that damage-dependent increases in neuronal poliovirus dissemination require an alternative pathway from those tested above.

Sterile crush damage also showed similar patterns of neuronal poliovirus dissemination as needle sticks damage, suggesting that secondary infections are not playing a detectable role in enhancing poliovirus dissemination after damage. This was important to verify, as bacterial factors can stimulate retrograde axonal transport in some cases.

Though a single molecule responsible for enhancing neuronal poliovirus dissemination in mice was not identified, several molecules known for enhancing retrograde axonal transport were tested. It is possible the retrograde axonal transport system used by poliovirus is more complex and that a single molecule is insufficient to trigger detectable differences in viral dissemination. It may also be that poliovirus is exploiting a separate pathway that was not tested. This work serves as a starting point for future studies of the mechanisms regulating enhanced neuronal poliovirus dissemination following muscle damage, and how these mechanisms may play a role in neuronal dissemination of other neurotropic viruses.

CHAPTER 5: Discussion and Future Directions

All three viruses disseminate by different mechanisms. Though this was somewhat expected as YFV-17D accesses the CNS by the hematogenous route, it was surprising that poliovirus and reovirus T3D appeared to disseminate differently from one another. Both viruses access the CNS following retrograde axonal transport through peripheral nerves, but only poliovirus dissemination is enhanced following muscle damage. This suggests that reovirus T3D depends on a different pathway than poliovirus for transport through the peripheral nerves and that this pathway is not stimulated by tissue damage. Determining the difference between these two retrograde axonal transport pathways, if there is one, would provide more breadth of knowledge on dissemination of neurotropic viruses, as previous research has focused predominantly on a small selection of viruses.

In additional to clarifying differences in retrograde axonal transport requirements for distinct viruses, it would be interesting to pursue differences in CNS access ability between distinct viruses. Poliovirus can access the CNS of adult immune-competent mice, whereas reovirus can not. One possible explanation is that retrograde axonal transport of reovirus is less efficient than poliovirus, such that fewer viruses even reach the spinal cord following intramuscular inoculation. Reovirus may also be more efficiently cleared from peripheral nerves than poliovirus, whether this is a result of using a different transport pathway or more prompt identification of reovirus as foreign material by host cells. Finally, it is likely that receptor avaiability is not equal for both viruses. In fact, it was recently suggested that the age-dependent difference in reovirus access to the CNS may be the result of decreased receptor availability throughout the mouse life-span. Determining whether altered receptor expression in adult-hood could relieve the block to reovirus dissemination into the CNS is an interesting next step in understanding the differences among neurotropic viruses.

Though there is still no clear mechanism by which damage-dependent enhancement of retrograde axonal transport increases poliovirus dissemination, multiple pathways of interest shown to play roles in stimulating retrograde axonal transport and host responses to tissue damage were tested. The mechanisms

regulating host response to damage are complex, making it difficult to identify a single molecule capable of detactably altering polivirus dissemination. Neurotrophic growth factors such as BDNF were previously demonstrated to enhance retrograde axonal transport, but the data above suggest that poliovirus does not associate with the growth factor-containing complexes during transport. In fact, even though these growth factors are capable of protecting host cells during viral infection, there were no detectable differences in the survival or viral dissemination in mice co-injected with individual growth factors and polivirus. This suggests that the growth factors may not be playing the same role healing role during viral infection. Some research suggests that viruses similar to poliovirus may be moved along the retrograde axonal transport system by inclusion in multivesicular bodies. Identifying the other molecules or cofactors with which poliovirus, PVR, and the dynein motor are associated prior to and during poliovirus dissemination through the sciatic nerve would help clarify the mechanism of enhancement for polivirus and. It would also serve as a point of comparison with other viruses, to understand why muscle damage does or does not enhance viral dissemination.

It was unexpected, yet interesting, to observe that even single mock injections can cause sufficient damage to stimulate enahanced poliovirus dissemination. I had struggled with stimulating a sufficient amount of damage with needlesticks (data not shown). Congruently, sterile crush damage at the inoculation site was also sufficient to enhanced poliovirus titer in the brain. In contrast to previous experiments investigating changes in poliovirus titer and diversity, I observed that there was not a strong correlation between altered viral titer and population diversity. In the brain, increased poliovirus titer but not viral diversity suggests that polivoirus may be replicating more efficiently. Due to this and failure of other individual molecules to stimulate transport, it is possible that enhanced poliovirus dissemination following damage is a result of changes to vascular factors rather than neurotrophic factors. Therefore, it is important to test for differences in vascular factor expression and function following muscle damage, with and without polioirus infection. Understanding the mechanisms behind poliovirus dissemination will

help to clarify the details not only of poliovirus transport, but help develop targets for future pharmacological treatments.

BIBLIOGRAPHY

1. Acosta, E.G., Castilla, V., Damonte, E.B.: **Functional entry of dengue virus into Aedes albopictus mosquito cells is dependent on clathrin-mediated endocytosis**. *J Gen Virol* 2008, **89**:474-84.

2. Allison, S.L., Stiasny, K., Stadler, K., Mandl, C.W., Heinz, F.X.: **Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E**. *J Virol* 1999, **73**:5605-12.

3. Ambron, R.T., Walters, E.T.: **Priming events and retrograde injury signals. A new perspective on the cellular and molecular biology of nerve regeneration**. *Molecular neurobiology* 1996, **13**:61-79.

4. Anderson, G.W., Skaar, A.E.: **Poliomyelitis occurring after antigen injections**. *Pediatrics* 1951, **7**:741-59.

5. Andjelkovic, A.V., Pachter, J.S.: **Characterization of binding sites for chemokines MCP-1 and MIP-1alpha on human brain microvessels**. *J Neurochem* 2000, **75**:1898-906.

6. Anonymous: **Polio this week as of 9 September 2015**. Edited by. http://www.polioeradication.org/Dataandmonitoring/Poliothisweek.aspx: The Global Polio Eradication Initiative; 2015. vol 2015.]

7. Antar, A.A., Konopka, J.L., Campbell, J.A., Henry, R.A., Perdigoto, A.L., Carter, B.D., Pozzi, A., Abel, T.W., Dermody, T.S.: **Junctional adhesion molecule-A is required for hematogenous dissemination of reovirus**. *Cell host & microbe* 2009, **5**:59-71.

8. Baer, G.S., Dermody, T.S.: **Mutations in reovirus outer-capsid protein sigma3 selected during persistent infections of L cells confer resistance to protease inhibitor E64**. *J Virol* 1997, **71**:4921-8.

9. Balsitis, S.J., Coloma, J., Castro, G., Alava, A., Flores, D., McKerrow, J.H., Beatty, P.R., Harris, E.: **Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining**. *Am J Trop Med Hyg* 2009, **80**:416-24.

10. Banisadr, G., Fontanges, P., Haour, F., Kitabgi, P., Rostene, W., Melik Parsadaniantz, S.: **Neuroanatomical distribution of CXCR4 in adult rat brain and its localization in cholinergic and dopaminergic neurons**. *Eur J Neurosci* 2002, **16**:1661-71.

11. Barna, B.P., Pettay, J., Barnett, G.H., Zhou, P., Iwasaki, K., Estes, M.L.: **Regulation of monocyte chemoattractant protein-1 expression in adult human non-neoplastic astrocytes is sensitive to tumor necrosis factor (TNF) or antibody to the 55-kDa TNF receptor**. *J Neuroimmunol* 1994, **50**:101-7.

12. Barrett, A.D., Gould, E.A.: **Comparison of neurovirulence of different strains of yellow fever virus in mice**. *J Gen Virol* 1986, **67 (Pt 4)**:631-7.

13. Barrett, A.D., Teuwen, D.E.: **Yellow fever vaccine - how does it work and why do rare cases of serious adverse events take place?** *Curr Opin Immunol* 2009, **21**:308-13.

14. Barros, M.L., Boecken, G.: **Jungle yellow fever in the central Amazon**. *Lancet* 1996, **348**:969-70.

15. Bartlett, S.E., Reynolds, A.J., Hendry, I.A.: **Retrograde axonal transport of neurotrophins: differences between neuronal populations and implications for motor neuron disease**. *Immunol Cell Biol* 1998, **76**:419-23.

16. Barton, E.S., Connolly, J.L., Forrest, J.C., Chappell, J.D., Dermody, T.S.: **Utilization of sialic acid as a coreceptor enhances reovirus attachment by multistep adhesion strengthening**. *J Biol Chem* 2001, **276**:2200-11.

17. Barton, E.S., Forrest, J.C., Connolly, J.L., Chappell, J.D., Liu, Y., Schnell, F.J., Nusrat, A., Parkos, C.A., Dermody, T.S.: **Junction adhesion molecule is a receptor for reovirus**. *Cell* 2001, **104**:441-51.

18. Barton, E.S., Youree, B.E., Ebert, D.H., Forrest, J.C., Connolly, J.L., Valyi-Nagy, T., Washington, K., Wetzel, J.D., Dermody, T.S.: **Utilization of sialic acid as a coreceptor is required for reovirus-induced biliary disease**. *The Journal of clinical investigation* 2003, **111**:1823-33.

19. Beasley, D.W., Li, L., Suderman, M.T., Barrett, A.D.: **Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype**. *Virology* 2002, **296**:17-23.

20. Beasley, D.W., Whiteman, M.C., Zhang, S., Huang, C.Y., Schneider, B.S., Smith, D.R., Gromowski, G.D., Higgs, S., Kinney, R.M., Barrett, A.D.: **Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains**. *J Virol* 2005, **79**:8339-47.

21. Berman, J.W., Guida, M.P., Warren, J., Amat, J., Brosnan, C.F.: **Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat**. *J Immunol* 1996, **156**:3017-23.

22. Bhattacharyya, A., Watson, F.L., Bradlee, T.A., Pomeroy, S.L., Stiles, C.D., Segal, R.A.: **Trk receptors function as rapid retrograde signal carriers in the adult nervous system**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1997, **17**:7007-16.

23. Bodian, D.: **Viremia in experimental poliomyelitis. II. Viremia and the mechanism of the provoking effect of injections or trauma**. *American journal of hygiene* 1954, **60**:358-70.

24. Bodian, D.: **Viremia, invasiveness, and the influence of injections**. *Ann N Y Acad Sci* 1955, **61**:877-82.

25. Boehme, K.W., Frierson, J.M., Konopka, J.L., Kobayashi, T., Dermody, T.S.: **The reovirus sigma1s protein is a determinant of hematogenous but not neural virus dissemination in mice**. *J Virol* 2011, **85**:11781-90.

26. Borsa, J., Morash, B.D., Sargent, M.D., Copps, T.P., Lievaart, P.A., Szekely, J.G.: **Two modes of entry of reovirus particles into L cells**. *J Gen Virol* 1979, **45**:161-70.

27. Borsa, J., Sargent, M.D., Lievaart, P.A., Copps, T.P.: **Reovirus: evidence for a second step in the intracellular uncoating and transcriptase activation process**. *Virology* 1981, **111**:191-200.

28. Brown, A.N., Kent, K.A., Bennett, C.J., Bernard, K.A.: **Tissue tropism and neuroinvasion of West Nile virus do not differ for two mouse strains with different survival rates**. *Virology* 2007, **368**:422-30.

29. Bunn, J.R., Canning, J., Burke, G., Mushipe, M., Marsh, D.R., Li, G.: **Production of consistent crush lesions in murine quadriceps muscle--a biomechanical, histomorphological and immunohistochemical study**. *J Orthop Res* 2004, **22**:1336-44.

30. Burns, C.C., Diop, O.M., Sutter, R.W., Kew, O.M.: **Vaccine-derived polioviruses**. *J Infect Dis* 2014, **210 Suppl 1**:S283-93.

31. Campbell, J.A., Schelling, P., Wetzel, J.D., Johnson, E.M., Forrest, J.C., Wilson, G.A., Aurrand-Lions, M., Imhof, B.A., Stehle, T., Dermody, T.S.: **Junctional adhesion molecule a serves as a receptor for prototype and field-isolate strains of mammalian reovirus**. *J Virol* 2005, **79**:7967-78.

32. Castle, E., Wengler, G.: **Nucleotide sequence of the 5'-terminal untranslated part of the genome of the flavivirus West Nile virus**. *Arch Virol* 1987, **92**:309-13.

33. Ch'ng, T.H., Flood, E.A., Enquist, L.W.: **Culturing primary and transformed neuronal cells for studying pseudorabies virus infection**. *Methods Mol Biol* 2005, **292**:299-316.

34. Chambers, T.J., Droll, D.A., Walton, A.H., Schwartz, J., Wold, W.S., Nickells, J.: **West Nile 25A virus infection of B-cell-deficient ((micro)MT) mice: characterization of neuroinvasiveness and pseudoreversion of the viral envelope protein**. *J Gen Virol* 2008, **89**:627-35.

35. Chambers, T.J., Nestorowicz, A., Amberg, S.M., Rice, C.M.: **Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication**. *J Virol* 1993, **67**:6797-807.

36. Chappell, J.D., Duong, J.L., Wright, B.W., Dermody, T.S.: **Identification of carbohydrate-binding domains in the attachment proteins of type 1 and type 3 reoviruses**. *J Virol* 2000, **74**:8472-9.

37. Chaturvedi, U.C., Dhawan, R., Khanna, M., Mathur, A.: **Breakdown of the blood-brain barrier during dengue virus infection of mice**. *J Gen Virol* 1991, **72 (Pt 4)**:859-66.

38. Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., Marks, R.M.: **Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate**. *Nat Med* 1997, **3**:866-71.

39. Chu, J.J., Ng, M.L.: **Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway**. *J Virol* 2004, **78**:10543-55.

40. Chu, J.J., Rajamanonmani, R., Li, J., Bhuvanakantham, R., Lescar, J., Ng, M.L.: **Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein**. *J Gen Virol* 2005, **86**:405-12.

41. Chuang, J.Z., Yeh, T.Y., Bollati, F., Conde, C., Canavosio, F., Caceres, A., Sung, C.H.: **The dynein light chain Tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth**. *Dev Cell* 2005, **9**:75-86.

42. Clarke, P., Meintzer, S.M., Gibson, S., Widmann, C., Garrington, T.P., Johnson, G.L., Tyler, K.L.: **Reovirus-induced apoptosis is mediated by TRAIL**. *J Virol* 2000, **74**:8135-9.

43. Coffey, L.L., Vasilakis, N., Brault, A.C., Powers, A.M., Tripet, F., Weaver, S.C.: **Arbovirus evolution in vivo is constrained by host alternation**. *Proc Natl Acad Sci U S A* 2008, **105**:6970-5.

44. Conductier, G., Blondeau, N., Guyon, A., Nahon, J.L., Rovere, C.: **The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases**. *J Neuroimmunol* 2010, **224**:93-100.

45. Connolly, J.L., Rodgers, S.E., Clarke, P., Ballard, D.W., Kerr, L.D., Tyler, K.L., Dermody, T.S.: **Reovirus-induced apoptosis requires activation of transcription factor NF-kappaB**. *J Virol* 2000, **74**:2981-9.

46. Corver, J., Ortiz, A., Allison, S.L., Schalich, J., Heinz, F.X., Wilschut, J.: **Membrane fusion activity of tick-borne encephalitis virus and recombinant subviral particles in a liposomal model system**. *Virology* 2000, **269**:37-46.

47. Coughlan, C.M., McManus, C.M., Sharron, M., Gao, Z., Murphy, D., Jaffer, S., Choe, W., Chen, W., Hesselgesser, J., Gaylord, H., Kalyuzhny, A., Lee, V.M., Wolf, B., Doms, R.W., Kolson, D.L.:

Expression of multiple functional chemokine receptors and monocyte chemoattractant protein-1 in human neurons. *Neuroscience* 2000, **97**:591-600.

48. Crotty, S., Hix, L., Sigal, L.J., Andino, R.: **Poliovirus pathogenesis in a new poliovirus receptor transgenic mouse model: age-dependent paralysis and a mucosal route of infection**. *J Gen Virol* 2002, **83**:1707-20.

49. Curanovic, D., Ch'ng, T.H., Szpara, M., Enquist, L.: **Compartmented neuron cultures for directional infection by alpha herpesviruses**. *Curr Protoc Cell Biol* 2009, **Chapter 26**:Unit 26 4.

50. Curtis, R., Adryan, K.M., Zhu, Y., Harkness, P.J., Lindsay, R.M., DiStefano, P.S.: **Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury**. *Nature* 1993, **365**:253-5.

51. Dalakas, M., Illa, I.: **Post-polio syndrome: concepts in clinical diagnosis, pathogenesis, and etiology**. *Adv Neurol* 1991, **56**:495-511.

52. Dansereau, M.A., Gosselin, R.D., Pohl, M., Pommier, B., Mechighel, P., Mauborgne, A., Rostene, W., Kitabgi, P., Beaudet, N., Sarret, P., Melik-Parsadaniantz, S.: **Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats**. *J Neurochem* 2008, **106**:757-69.

53. Davidson, A.D.: **Chapter 2. New insights into flavivirus nonstructural protein 5**. *Adv Virus Res* 2009, **74**:41-101.

54. De Brito, T., Siqueira, S.A., Santos, R.T., Nassar, E.S., Coimbra, T.L., Alves, V.A.: **Human fatal yellow fever. Immunohistochemical detection of viral antigens in the liver, kidney and heart**. *Pathol Res Pract* 1992, **188**:177-81.

55. Deinhardt, K., Reversi, A., Berninghausen, O., Hopkins, C.R., Schiavo, G.: **Neurotrophins Redirect p75NTR from a clathrin-independent to a clathrin-dependent endocytic pathway coupled to axonal transport**. *Traffic* 2007, **8**:1736-49.

56. Deinhardt, K., Schiavo, G.: **Endocytosis and retrograde axonal traffic in motor neurons**. *Biochem Soc Symp* 2005:139-50.

57. Dermody, T.S., Nibert, M.L., Wetzel, J.D., Tong, X., Fields, B.N.: **Cells and viruses with mutations affecting viral entry are selected during persistent infections of L cells with mammalian reoviruses**. *J Virol* 1993, **67**:2055-63.

58. Diamond, M.S., Shrestha, B., Marri, A., Mahan, D., Engle, M.: **B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus**. *J Virol* 2003, **77**:2578-86.

59. Dionne, K.R., Galvin, J.M., Schittone, S.A., Clarke, P., Tyler, K.L.: **Type I interferon signaling limits reoviral tropism within the brain and prevents lethal systemic infection**. *Journal of neurovirology* 2011, **17**:314-26.

60. DiStefano, P.S., Friedman, B., Radziejewski, C., Alexander, C., Boland, P., Schick, C.M., Lindsay, R.M., Wiegand, S.J.: **The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons**. *Neuron* 1992, **8**:983-93.

61. Dobek, G.L., Fulkerson, N.D., Nicholas, J., Schneider, B.S.: **Mouse model of muscle crush injury of the legs**. *Comparative medicine* 2013, **63**:227-32.

62. Dzenko, K.A., Andjelkovic, A.V., Kuziel, W.A., Pachter, J.S.: **The chemokine receptor CCR2 mediates the binding and internalization of monocyte chemoattractant protein-1 along brain microvessels**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2001, **21**:9214-23.

63. Elshuber, S., Allison, S.L., Heinz, F.X., Mandl, C.W.: **Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus**. *J Gen Virol* 2003, **84**:183-91.

64. Erickson, A.K., Pfeiffer, J.K.: **Dynamic viral dissemination in mice infected with yellow fever virus strain 17D**. *J Virol* 2013, **87**:12392-7.

65. Erickson, A.K., Pfeiffer, J.K.: **Spectrum of disease outcomes in mice infected with YFV-17D**. *J Gen Virol* 2015, **96**:1328-39.

66. Fitzgeorge, R., Bradish, C.J.: **Differentiation of strains of yellow fever virus in gamma-irradiated mice**. *J Gen Virol* 1980, **50**:345-56.

67. Fitzgeorge, R., Bradish, C.J.: **The in vivo differentiation of strains of yellow fever virus in mice**. *J Gen Virol* 1980, **46**:1-13.

68. Forrest, J.C., Campbell, J.A., Schelling, P., Stehle, T., Dermody, T.S.: **Structure-function analysis of reovirus binding to junctional adhesion molecule 1. Implications for the mechanism of reovirus attachment**. *J Biol Chem* 2003, **278**:48434-44.

69. Forrester, N.L., Guerbois, M., Seymour, R.L., Spratt, H., Weaver, S.C.: **Vector-borne transmission imposes a severe bottleneck on an RNA virus population**. *PLoS Pathog* 2012, **8**:e1002897.

70. Fox, J.P.: **Immunity to Yellow Fever Encephalitis of Monkeys and Mice Immunized by Neural and Extraneural Routes**. *The Journal of experimental medicine* 1943, **77**:487-506.

71. Friedrich, F.: **Rare adverse events associated with oral poliovirus vaccine in Brazil**. *Braz J Med Biol Res* 1997, **30**:695-703.

72. Frierson, J.M., Pruijssers, A.J., Konopka, J.L., Reiter, D.M., Abel, T.W., Stehle, T., Dermody, T.S.: **Utilization of sialylated glycans as coreceptors enhances the neurovirulence of serotype 3 reovirus**. *J Virol* 2012, **86**:13164-73.

73. Frustace, S.J.: **Poliomyelitis: late and unusual sequelae**. *Am J Phys Med* 1987, **66**:328-37.

74. Furlong, D.B., Nibert, M.L., Fields, B.N.: **Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles**. *J Virol* 1988, **62**:246-56.

75. Furuichi, Y., Shatkin, A.J.: **Viral and cellular mRNA capping: past and prospects**. *Adv Virus Res* 2000, **55**:135-84.

76. Gardner, C.L., Ryman, K.D.: **Yellow fever: a reemerging threat**. *Clin Lab Med* 2010, **30**:237-60.

77. Gentsch, J.R., Pacitti, A.F.: **Effect of neuraminidase treatment of cells and effect of soluble glycoproteins on type 3 reovirus attachment to murine L cells**. *J Virol* 1985, **56**:356-64.

78. Gentsch, J.R., Pacitti, A.F.: **Differential interaction of reovirus type 3 with sialylated receptor components on animal cells**. *Virology* 1987, **161**:245-8.

79. Germi, R., Crance, J.M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R.W., Zarski, J.P., Drouet, E.: **Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus**. *Virology* 2002, **292**:162-8.

80. Glabinski, A.R., Balasingam, V., Tani, M., Kunkel, S.L., Strieter, R.M., Yong, V.W., Ransohoff, R.M.: **Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain**. *J Immunol* 1996, **156**:4363-8.

81. Gollins, S.W., Porterfield, J.S.: **Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry**. *J Gen Virol* 1985, **66 (Pt 9)**:1969-82.

82. Gollins, S.W., Porterfield, J.S.: **pH-dependent fusion between the flavivirus West Nile and liposomal model membranes**. *J Gen Virol* 1986, **67 (Pt 1)**:157-66.

83. Goodman, G.T., Koprowski, H.: **Macrophages as a cellular expression of inherited natural resistance**. *Proc Natl Acad Sci U S A* 1962, **48**:160-5.

84. Gourmala, N.G., Buttini, M., Limonta, S., Sauter, A., Boddeke, H.W.: **Differential and time-dependent expression of monocyte chemoattractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration**. *J Neuroimmunol* 1997, **74**:35-44.

85. Grard, G., Moureau, G., Charrel, R.N., Holmes, E.C., Gould, E.A., de Lamballerie, X.: **Genomics and evolution of Aedes-borne flaviviruses**. *J Gen Virol* 2010, **91**:87-94.

86. Gromeier, M., Wimmer, E.: **Mechanism of injury-provoked poliomyelitis**. *J Virol* 1998, **72**:5056-60.

87. Guyatt, K.J., Westaway, E.G., Khromykh, A.A.: **Expression and purification of enzymatically active recombinant RNA-dependent RNA polymerase (NS5) of the flavivirus Kunjin**. *Journal of virological methods* 2001, **92**:37-44.

88. Guyer, B., Bisong, A.A., Gould, J., Brigaud, M., Aymard, M.: **Injections and paralytic poliomyelitis in tropical Africa**. *Bulletin of the World Health Organization* 1980, **58**:285-91.

89. Hanisch, U.K.: **Microglia as a source and target of cytokines**. *Glia* 2002, **40**:140-55.

90. Heinz, F.X., Stiasny, K., Puschner-Auer, G., Holzmann, H., Allison, S.L., Mandl, C.W., Kunz, C.: **Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM**. *Virology* 1994, **198**:109-17.

91. Hibbert, A.P., Kramer, B.M., Miller, F.D., Kaplan, D.R.: **The localization, trafficking and retrograde transport of BDNF bound to p75NTR in sympathetic neurons**. *Mol Cell Neurosci* 2006, **32**:387-402.

92. Hill, A.B., Knowelden, J.: **Inoculation and poliomyelitis; a statistical investigation in England and Wales in 1949**. *British medical journal* 1950, **2**:1-6.

93. Hunsperger, E.A., Roehrig, J.T.: **Temporal analyses of the neuropathogenesis of a West Nile virus infection in mice**. *Journal of neurovirology* 2006, **12**:129-39.

94. Ida-Hosonuma, M., Iwasaki, T., Yoshikawa, T., Nagata, N., Sato, Y., Sata, T., Yoneyama, M., Fujita, T., Taya, C., Yonekawa, H., Koike, S.: **The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus**. *J Virol* 2005, **79**:4460-9.

95. Ishak, R., Tovey, D.G., Howard, C.R.: **Morphogenesis of yellow fever virus 17D in infected cell cultures**. *J Gen Virol* 1988, **69 (Pt 2)**:325-35.

96. Jerzak, G., Bernard, K.A., Kramer, L.D., Ebel, G.D.: **Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection**. *J Gen Virol* 2005, **86**:2175-83.

97. Johnson, M.I.: **Primary Cultures of Sympathetic Ganglia**. In *Protocols for Neural Cell Culture*, edn 3rd. Edited by Federoff, S., Richardson, A.: Humana Press; 2001:71-94.

98. Kew, O.M., Cochi, S.L., Jafari, H.S., Wassilak, S.G., Mast, E.E., Diop, O.M., Tangermann, R.H., Armstrong, G.L., Centers for Disease, C., Prevention: **Possible eradication of wild poliovirus type 3--worldwide, 2012**. *MMWR Morb Mortal Wkly Rep* 2014, **63**:1031-3.

99. Khromava, A.Y., Eidex, R.B., Weld, L.H., Kohl, K.S., Bradshaw, R.D., Chen, R.T., Cetron, M.S., Yellow Fever Vaccine Safety Working, G.: **Yellow fever vaccine: an updated assessment of advanced age as a risk factor for serious adverse events**. *Vaccine* 2005, **23**:3256-63.

100. Kim, Y., Remacle, A.G., Chernov, A.V., Liu, H., Shubayev, I., Lai, C., Dolkas, J., Shiryaev, S.A., Golubkov, V.S., Mizisin, A.P., Strongin, A.Y., Shubayev, V.I.: **The MMP-9/TIMP-1 axis controls the status of differentiation and function of myelin-forming Schwann cells in nerve regeneration**. *PLoS One* 2012, **7**:e33664.

101. Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., van der Werf, S., Anderson, C.W., Wimmer, E.: **Primary structure, gene organization and polypeptide expression of poliovirus RNA**. *Nature* 1981, **291**:547-53.

102. Kitchen, A., Shackelton, L.A., Holmes, E.C.: **Family level phylogenies reveal modes of macroevolution in RNA viruses**. *Proc Natl Acad Sci U S A* 2011, **108**:238-43.

103. Klotz, O., Belt, T.H.: **The Pathology of the Liver in Yellow Fiver**. *The American journal of pathology* 1930, **6**:663-88 1.

104. Klotz, O., Belt, T.H.: **The Pathology of the Spleen in Yellow Fever**. *The American journal of pathology* 1930, **6**:655-62 3.

105. Kobayashi, T., Ooms, L.S., Ikizler, M., Chappell, J.D., Dermody, T.S.: **An improved reverse genetics system for mammalian orthoreoviruses**. *Virology* 2010, **398**:194-200.

106. Kobiler, D., Lustig, S., Gozes, Y., Ben-Nathan, D., Akov, Y.: **Sodium dodecylsulphate induces a breach in the blood-brain barrier and enables a West Nile virus variant to penetrate into mouse brain**. *Brain Res* 1989, **496**:314-6.

107. Koike, S., Ise, I., Nomoto, A.: **Functional domains of the poliovirus receptor**. *Proc Natl Acad Sci U S A* 1991, **88**:4104-8.

108. Koike, S., Taya, C., Aoki, J., Matsuda, Y., Ise, I., Takeda, H., Matsuzaki, T., Amanuma, H., Yonekawa, H., Nomoto, A.: **Characterization of three different transgenic mouse lines that carry human poliovirus receptor gene--influence of the transgene expression on pathogenesis**. *Arch Virol* 1994, **139**:351-63.

109. Koike, S., Taya, C., Kurata, T., Abe, S., Ise, I., Yonekawa, H., Nomoto, A.: **Transgenic mice susceptible to poliovirus**. *Proc Natl Acad Sci U S A* 1991, **88**:951-5.

110. Konopka-Anstadt, J.L., Mainou, B.A., Sutherland, D.M., Sekine, Y., Strittmatter, S.M., Dermody, T.S.: **The Nogo receptor NgR1 mediates infection by mammalian reovirus**. *Cell host & microbe* 2014, **15**:681-91.

111. Koschinski, A., Wengler, G., Wengler, G., Repp, H.: **The membrane proteins of flaviviruses form ion-permeable pores in the target membrane after fusion: identification of the pores and analysis of their possible role in virus infection**. *J Gen Virol* 2003, **84**:1711-21.

112. Kovacs, E., Baratawidjaja, R.K., Labzoffsky, N.A.: **Visualization of Poliovirus Type Iii in Paraffin Sections of Monkey Spinal Cord by Indirect Immuno-Fluorescence**. *Nature* 1963, **200**:497-8.

113. Koyuncu, O.O., Hogue, I.B., Enquist, L.W.: **Virus infections in the nervous system**. *Cell host & microbe* 2013, **13**:379-93.

114. Krishnan, M.N., Sukumaran, B., Pal, U., Agaisse, H., Murray, J.L., Hodge, T.W., Fikrig, E.: **Rab 5 is required for the cellular entry of dengue and West Nile viruses**. *J Virol* 2007, **81**:4881-5.

115. Kroschewski, H., Allison, S.L., Heinz, F.X., Mandl, C.W.: **Role of heparan sulfate for attachment and entry of tick-borne encephalitis virus**. *Virology* 2003, **308**:92-100.

116. Kuss, S.K., Etheredge, C.A., Pfeiffer, J.K.: **Multiple host barriers restrict poliovirus trafficking in mice**. *PLoS Pathog* 2008, **4**:e1000082.

117. Lancaster, K.Z., Pfeiffer, J.K.: **Limited trafficking of a neurotropic virus through inefficient retrograde axonal transport and the type I interferon response**. *PLoS Pathog* 2010, **6**:e1000791.

118. Lancaster, K.Z., Pfeiffer, J.K.: **Mechanisms controlling virulence thresholds of mixed viral populations**. *J Virol* 2011, **85**:9778-88.

119. Laniosz, V., Holthusen, K.A., Meneses, P.I.: **Bovine papillomavirus type 1: from clathrin to caveolin**. *J Virol* 2008, **82**:6288-98.

120. Lee, E., Lobigs, M.: **E protein domain III determinants of yellow fever virus 17D vaccine strain enhance binding to glycosaminoglycans, impede virus spread, and attenuate virulence**. *J Virol* 2008, **82**:6024-33.

121. Lee, P.W., Hayes, E.C., Joklik, W.K.: **Protein sigma 1 is the reovirus cell attachment protein**. *Virology* 1981, **108**:156-63.

122. Lee, Y.B., Nagai, A., Kim, S.U.: **Cytokines, chemokines, and cytokine receptors in human microglia**. *J Neurosci Res* 2002, **69**:94-103.

123. Leers, W.D., Rozee, K.R.: **A survey of reovirus antibodies in sera of urban children**. *Can Med Assoc J* 1966, **94**:1040-2.

124. Lindsey, N.P., Schroeder, B.A., Miller, E.R., Braun, M.M., Hinckley, A.F., Marano, N., Slade, B.A., Barnett, E.D., Brunette, G.W., Horan, K., Staples, J.E., Kozarsky, P.E., Hayes, E.B.: **Adverse event reports following yellow fever vaccination**. *Vaccine* 2008, **26**:6077-82.

125. Lloyd, W.: **The Myocardium in Yellow Fever**. *Science* 1930, **72**:18.

126. Lorenz, I.C., Allison, S.L., Heinz, F.X., Helenius, A.: **Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum**. *J Virol* 2002, **76**:5480-91.

127. Lorenz, I.C., Kartenbeck, J., Mezzacasa, A., Allison, S.L., Heinz, F.X., Helenius, A.: **Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus**. *J Virol* 2003, **77**:4370-82.

128. Lustig, S., Danenberg, H.D., Kafri, Y., Kobiler, D., Ben-Nathan, D.: **Viral neuroinvasion and encephalitis induced by lipopolysaccharide and its mediators**. *The Journal of experimental medicine* 1992, **176**:707-12.

129. Mackenzie, J.M., Westaway, E.G.: **Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively**. *J Virol* 2001, **75**:10787-99.

130. Maday, S., Twelvetrees, A.E., Moughamian, A.J., Holzbaur, E.L.: **Axonal transport: cargo-specific mechanisms of motility and regulation**. *Neuron* 2014, **84**:292-309.

131. Maginnis, M.S., Forrest, J.C., Kopecky-Bromberg, S.A., Dickeson, S.K., Santoro, S.A., Zutter, M.M., Nemerow, G.R., Bergelson, J.M., Dermody, T.S.: **Beta1 integrin mediates internalization of mammalian reovirus**. *J Virol* 2006, **80**:2760-70.

132. Mainou, B.A., Zamora, P.F., Ashbrook, A.W., Dorset, D.C., Kim, K.S., Dermody, T.S.: **Reovirus cell entry requires functional microtubules**. *mBio* 2013, **4**.

133. Mann, M.A., Knipe, D.M., Fischbach, G.D., Fields, B.N.: **Type 3 reovirus neuroinvasion after intramuscular inoculation: direct invasion of nerve terminals and age-dependent pathogenesis**. *Virology* 2002, **303**:222-31.

134. Markoff, L.: **5'- and 3'-noncoding regions in flavivirus RNA**. *Adv Virus Res* 2003, **59**:177-228.

135. Matheson, C.R., Carnahan, J., Urich, J.L., Bocangel, D., Zhang, T.J., Yan, Q.: **Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: comparison with the effects of the neurotrophins**. *J Neurobiol* 1997, **32**:22-32.

136. McCloskey, B.P., Melb, M.B.: **The relation of prophylactic inoculations to the onset of poliomyelitis.** *Lancet* 1950, **255**:659-63.

137. McMinn, P.C.: **The molecular basis of virulence of the encephalitogenic flaviviruses**. *J Gen Virol* 1997, **78 (Pt 11)**:2711-22.

138. Meda, L., Bernasconi, S., Bonaiuto, C., Sozzani, S., Zhou, D., Otvos, L., Jr., Mantovani, A., Rossi, F., Cassatella, M.A.: **Beta-amyloid (25-35) peptide and IFN-gamma synergistically induce the production of the chemotactic cytokine MCP-1/JE in monocytes and microglial cells**. *J Immunol* 1996, **157**:1213-8.

139. Meier, K.C., Gardner, C.L., Khoretonenko, M.V., Klimstra, W.B., Ryman, K.D.: **A mouse model for studying viscerotropic disease caused by yellow fever virus infection**. *PLoS Pathog* 2009, **5**:e1000614.

140. Mendelsohn, C.L., Wimmer, E., Racaniello, V.R.: **Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily**. *Cell* 1989, **56**:855-65.

141. Mims, C.A.: **The invasion of the brain by yellow fever virus present in the blood of mice**. *British journal of experimental pathology* 1957, **38**:329-38.

142. Minor, P.D.: **Attenuation and reversion of the Sabin vaccine strains of poliovirus**. *Dev Biol Stand* 1993, **78**:17-26.

143. Molla, A., Paul, A.V., Wimmer, E.: **Cell-free, de novo synthesis of poliovirus**. *Science* 1991, **254**:1647-51.

144. Molla, A., Paul, A.V., Wimmer, E.: **In vitro synthesis of poliovirus**. *Dev Biol Stand* 1993, **78**:39-53.

145. Monath, T.P.: **Yellow fever vaccine**. *Expert Rev Vaccines* 2005, **4**:553-74.

146. Monath, T.P.: **Treatment of yellow fever**. *Antiviral Res* 2008, **78**:116-24.

147. Monath, T.P., Barrett, A.D.: **Pathogenesis and pathophysiology of yellow fever**. *Adv Virus Res* 2003, **60**:343-95.

148. Morrey, J.D., Siddharthan, V., Wang, H., Hall, J.O., Skirpstunas, R.T., Olsen, A.L., Nordstrom, J.L., Koenig, S., Johnson, S., Diamond, M.S.: **West Nile virus-induced acute flaccid paralysis is prevented by monoclonal antibody treatment when administered after infection of spinal cord neurons**. *Journal of neurovirology* 2008, **14**:152-63.

149. Morrison, L.A., Sidman, R.L., Fields, B.N.: **Direct spread of reovirus from the intestinal lumen to the central nervous system through vagal autonomic nerve fibers**. *Proc Natl Acad Sci U S A* 1991, **88**:3852-6.

150. Mueller, S., Cao, X., Welker, R., Wimmer, E.: **Interaction of the poliovirus receptor CD155 with the dynein light chain Tctex-1 and its implication for poliovirus pathogenesis**. *J Biol Chem* 2002, **277**:7897-904.

151. Murawski, M.R., McGinnes, L.W., Finberg, R.W., Kurt-Jones, E.A., Massare, M.J., Smith, G., Heaton, P.M., Fraire, A.E., Morrison, T.G.: **Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with no evidence of immunopathology**. *J Virol* 2010, **84**:1110-23.

152. Myint, K.S., Raengsakulrach, B., Young, G.D., Gettayacamin, M., Ferguson, L.M., Innis, B.L., Hoke, C.H., Jr., Vaughn, D.W.: **Production of lethal infection that resembles fatal human disease by intranasal inoculation of macaques with Japanese encephalitis virus**. *Am J Trop Med Hyg* 1999, **60**:338-42.

153. Nawa, H., Carnahan, J., Gall, C.: **BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels**. *Eur J Neurosci* 1995, **7**:1527-35.

154. Nestorowicz, A., Chambers, T.J., Rice, C.M.: **Mutagenesis of the yellow fever virus NS2A/2B cleavage site: effects on proteolytic processing, viral replication, and evidence for alternative processing of the NS2A protein**. *Virology* 1994, **199**:114-23.

155. Nickells, J., Cannella, M., Droll, D.A., Liang, Y., Wold, W.S., Chambers, T.J.: **Neuroadapted yellow fever virus strain 17D: a charged locus in domain III of the E protein governs heparin binding activity and neuroinvasiveness in the SCID mouse model**. *J Virol* 2008, **82**:12510-9.

156. Nickells, M., Chambers, T.J.: **Neuroadapted yellow fever virus 17D: determinants in the envelope protein govern neuroinvasiveness for SCID mice**. *J Virol* 2003, **77**:12232-42.

157. Nir, Y., Beemer, A., Goldwasser, R.A.: **West Nile Virus infection in mice following exposure to a viral aerosol**. *British journal of experimental pathology* 1965, **46**:443-9.

158. Ohka, S., Igarashi, H., Nagata, N., Sakai, M., Koike, S., Nochi, T., Kiyono, H., Nomoto, A.: **Establishment of a poliovirus oral infection system in human poliovirus receptor-expressing**

transgenic mice that are deficient in alpha/beta interferon receptor. *J Virol* 2007, **81**:7902-12.

159. Ohka, S., Matsuda, N., Tohyama, K., Oda, T., Morikawa, M., Kuge, S., Nomoto, A.: **Receptor (CD155)-dependent endocytosis of poliovirus and retrograde axonal transport of the endosome**. *J Virol* 2004, **78**:7186-98.

160. Ohka, S., Sakai, M., Bohnert, S., Igarashi, H., Deinhardt, K., Schiavo, G., Nomoto, A.: **Receptor-dependent and -independent axonal retrograde transport of poliovirus in motor neurons**. *J Virol* 2009, **83**:4995-5004.

161. Ohka, S., Yang, W.X., Terada, E., Iwasaki, K., Nomoto, A.: **Retrograde transport of intact poliovirus through the axon via the fast transport system**. *Virology* 1998, **250**:67-75.

162. Pallansch, M.A., Kew, O.M., Semler, B.L., Omilianowski, D.R., Anderson, C.W., Wimmer, E., Rueckert, R.R.: **Protein processing map of poliovirus**. *J Virol* 1984, **49**:873-80.

163. Paul, R.W., Choi, A.H., Lee, P.W.: **The alpha-anomeric form of sialic acid is the minimal receptor determinant recognized by reovirus**. *Virology* 1989, **172**:382-5.

164. Pfeiffer, J.K.: **Innate host barriers to viral trafficking and population diversity: lessons learned from poliovirus**. *Adv Virus Res* 2010, **77**:85-118.

165. Pfeiffer, J.K., Kirkegaard, K.: **A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity**. *Proc Natl Acad Sci U S A* 2003, **100**:7289-94.

166. Pfeiffer, J.K., Kirkegaard, K.: **Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice**. *PLoS Pathog* 2005, **1**:e11.

167. Pfeiffer, J.K., Kirkegaard, K.: **Bottleneck-mediated quasispecies restriction during spread of an RNA virus from inoculation site to brain**. *Proc Natl Acad Sci U S A* 2006, **103**:5520-5.

168. Piyasirisilp, S., Hemachudha, T.: **Neurological adverse events associated with vaccination**. *Curr Opin Neurol* 2002, **15**:333-8.

169. Platt, L.R., Estivariz, C.F., Sutter, R.W.: **Vaccine-associated paralytic poliomyelitis: a review of the epidemiology and estimation of the global burden**. *J Infect Dis* 2014, **210 Suppl 1**:S380-9.

170. Pulendran, B.: **Learning immunology from the yellow fever vaccine: innate immunity to systems vaccinology**. *Nat Rev Immunol* 2009, **9**:741-7.

171. Querbes, W., O'Hara, B.A., Williams, G., Atwood, W.J.: **Invasion of host cells by JC virus identifies a novel role for caveolae in endosomal sorting of noncaveolar ligands**. *J Virol* 2006, **80**:9402-13.

172. Querec, T.D., Akondy, R.S., Lee, E.K., Cao, W., Nakaya, H.I., Teuwen, D., Pirani, A., Gernert, K., Deng, J., Marzolf, B., Kennedy, K., Wu, H., Bennouna, S., Oluoch, H., Miller, J., Vencio, R.Z., Mulligan, M., Aderem, A., Ahmed, R., Pulendran, B.: **Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans**. *Nat Immunol* 2009, **10**:116-25.

173. Querfurth, H., Swanson, P.D.: **Vaccine-associated paralytic poliomyelitis. Regional case series and review**. *Arch Neurol* 1990, **47**:541-4.

174. Racaniello, V.R.: **One hundred years of poliovirus pathogenesis**. *Virology* 2006, **344**:9-16.

175. Raengsakulrach, B., Nisalak, A., Gettayacamin, M., Thirawuth, V., Young, G.D., Myint, K.S., Ferguson, L.M., Hoke, C.H., Jr., Innis, B.L., Vaughn, D.W.: **An intranasal challenge model for testing Japanese encephalitis vaccines in rhesus monkeys**. *Am J Trop Med Hyg* 1999, **60**:329-37.

176. Rauschenfels, S., Krassmann, M., Al-Masri, A.N., Verhagen, W., Leonhardt, J., Kuebler, J.F., Petersen, C.: **Incidence of hepatotropic viruses in biliary atresia**. *Eur J Pediatr* 2009, **168**:469-76.

177. Reiss, K., Stencel, J.E., Liu, Y., Blaum, B.S., Reiter, D.M., Feizi, T., Dermody, T.S., Stehle, T.: **The GM2 glycan serves as a functional coreceptor for serotype 1 reovirus**. *PLoS Pathog* 2012, **8**:e1003078.

178. Ren, R., Racaniello, V.R.: **Poliovirus spreads from muscle to the central nervous system by neural pathways**. *J Infect Dis* 1992, **166**:747-52.

179. Ren, R.B., Costantini, F., Gorgacz, E.J., Lee, J.J., Racaniello, V.R.: **Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis**. *Cell* 1990, **63**:353-62.

180. Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C.: **The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution**. *Nature* 1995, **375**:291-8.

181. Reynolds, A.J., Bartlett, S.E., Hendry, I.A.: **Molecular mechanisms regulating the retrograde axonal transport of neurotrophins**. *Brain Res Brain Res Rev* 2000, **33**:169-78.

182. Richardson-Burns, S.M., Kominsky, D.J., Tyler, K.L.: **Reovirus-induced neuronal apoptosis is mediated by caspase 3 and is associated with the activation of death receptors**. *Journal of neurovirology* 2002, **8**:365-80.

183. Rodgers, S.E., Barton, E.S., Oberhaus, S.M., Pike, B., Gibson, C.A., Tyler, K.L., Dermody, T.S.: **Reovirus-induced apoptosis of MDCK cells is not linked to viral yield and is blocked by Bcl-2**. *J Virol* 1997, **71**:2540-6.

184. Rubin, D.H., Weiner, D.B., Dworkin, C., Greene, M.I., Maul, G.G., Williams, W.V.: **Receptor utilization by reovirus type 3: distinct binding sites on thymoma and fibroblast cell lines result in differential compartmentalization of virions**. *Microb Pathog* 1992, **12**:351-65.

185. Sabin, A.B., Hennessen, W.A., Winsser, J.: **Studies on variants of poliomyelitis virus. I. Experimental segregation and properties of avirulent variants of three immunologic types**. *The Journal of experimental medicine* 1954, **99**:551-76.

186. Sagot, Y., Rosse, T., Vejsada, R., Perrelet, D., Kato, A.C.: **Differential effects of neurotrophic factors on motoneuron retrograde labeling in a murine model of motoneuron disease**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1998, **18**:1132-41.

187. Saha, N., Kolev, M., Nikolov, D.B.: **Structural features of the Nogo receptor signaling complexes at the neuron/myelin interface**. *Neuroscience research* 2014, **87**:1-7.

188. Salinas, S., Schiavo, G., Kremer, E.J.: **A hitchhiker's guide to the nervous system: the complex journey of viruses and toxins**. *Nat Rev Microbiol* 2010, **8**:645-55.

189. Salk, D.: **Eradication of poliomyelitis in the United States. I. Live virus vaccine-associated and wild poliovirus disease**. *Rev Infect Dis* 1980, **2**:228-42.

190. Salk, D.: **Eradication of poliomyelitis in the United States. II. Experience with killed poliovirus vaccine**. *Rev Infect Dis* 1980, **2**:243-57.

191. Salk, D.: **Eradication of poliomyelitis in the United States. III. Poliovaccines--practical considerations**. *Rev Infect Dis* 1980, **2**:258-73.

192. Salk, J., Salk, D.: **Control of influenza and poliomyelitis with killed virus vaccines**. *Science* 1977, **195**:834-47.

193. Salsman, J., Top, D., Boutilier, J., Duncan, R.: **Extensive syncytium formation mediated by the reovirus FAST proteins triggers apoptosis-induced membrane instability**. *J Virol* 2005, **79**:8090-100.

194. Samuel, M.A., Diamond, M.S.: **Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival**. *J Virol* 2005, **79**:13350-61.

195. Samuel, M.A., Wang, H., Siddharthan, V., Morrey, J.D., Diamond, M.S.: **Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis**. *Proc Natl Acad Sci U S A* 2007, **104**:17140-5.

196. Sawyer, W.A., Lloyd, W.: **The Use of Mice in Tests of Immunity against Yellow Fever**. *The Journal of experimental medicine* 1931, **54**:533-55.

197. Sbai, O., Ferhat, L., Bernard, A., Gueye, Y., Ould-Yahoui, A., Thiolloy, S., Charrat, E., Charton, G., Tremblay, E., Risso, J.J., Chauvin, J.P., Arsanto, J.P., Rivera, S., Khrestchatisky, M.: **Vesicular trafficking and secretion of matrix metalloproteinases-2, -9 and tissue inhibitor of metalloproteinases-1 in neuronal cells**. *Mol Cell Neurosci* 2008, **39**:549-68.

198. Schiavo, G., Greensmith, L., Hafezparast, M., Fisher, E.M.: **Cytoplasmic dynein heavy chain: the servant of many masters**. *Trends in neurosciences* 2013, **36**:641-51.

199. Schlesinger, J.J., Chapman, S., Nestorowicz, A., Rice, C.M., Ginocchio, T.E., Chambers, T.J.: **Replication of yellow fever virus in the mouse central nervous system: comparison of neuroadapted and non-neuroadapted virus and partial sequence analysis of the neuroadapted strain**. *J Gen Virol* 1996, **77 (Pt 6)**:1277-85.

200. Sellards, A.W.: **The Behavior of the Virus of Yellow Fever in Monkeys and Mice**. *Proc Natl Acad Sci U S A* 1931, **17**:339-43.

201. Sherry, B., Torres, J., Blum, M.A.: **Reovirus induction of and sensitivity to beta interferon in cardiac myocyte cultures correlate with induction of myocarditis and are determined by viral core proteins**. *J Virol* 1998, **72**:1314-23.

202. Shi, P.Y., Li, W., Brinton, M.A.: **Cell proteins bind specifically to West Nile virus minus-strand 3' stem-loop RNA**. *J Virol* 1996, **70**:6278-87.

203. Skehel, J.J., Joklik, W.K.: **Studies on the in vitro transcription of reovirus RNA catalyzed by reovirus cores**. *Virology* 1969, **39**:822-31.

204. Song, L., Pachter, J.S.: **Monocyte chemoattractant protein-1 alters expression of tight junction-associated proteins in brain microvascular endothelial cells**. *Microvasc Res* 2004, **67**:78-89.

205. Speck, K., Schneider, B.S., Deashinta, N.: **A rodent model to advance the field treatment of crush muscle injury during earthquakes and other natural disasters**. *Biological research for nursing* 2013, **15**:17-25.

206. Stamatovic, S.M., Shakui, P., Keep, R.F., Moore, B.B., Kunkel, S.L., Van Rooijen, N., Andjelkovic, A.V.: **Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability**. *J Cereb Blood Flow Metab* 2005, **25**:593-606.

207. Staples, J.E., Gershman, M., Fischer, M., Centers for Disease, C., Prevention: **Yellow fever vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP)**. *MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control* 2010, **59**:1-27.

208. Strebel, P.M., Ion-Nedelcu, N., Baughman, A.L., Sutter, R.W., Cochi, S.L.: **Intramuscular injections within 30 days of immunization with oral poliovirus vaccine--a risk factor for vaccine-associated paralytic poliomyelitis**. *The New England journal of medicine* 1995, **332**:500-6.

209. Sturzenbecker, L.J., Nibert, M., Furlong, D., Fields, B.N.: **Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle**. *J Virol* 1987, **61**:2351-61.

210. Tai, J.H., Williams, J.V., Edwards, K.M., Wright, P.F., Crowe, J.E., Jr., Dermody, T.S.: **Prevalence of reovirus-specific antibodies in young children in Nashville, Tennessee**. *J Infect Dis* 2005, **191**:1221-4.

211. Tan, B.H., Fu, J., Sugrue, R.J., Yap, E.H., Chan, Y.C., Tan, Y.H.: **Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity**. *Virology* 1996, **216**:317-25.

212. Tardieu, M., Powers, M.L., Weiner, H.L.: **Age dependent susceptibility to Reovirus type 3 encephalitis: role of viral and host factors**. *Annals of neurology* 1983, **13**:602-7.

213. Theiler, M.: **Susceptibility of White Mice to the Virus of Yellow Fever**. *Science* 1930, **71**:367.

214. Theiler, M., Smith, H.H.: **The Effect of Prolonged Cultivation in Vitro Upon the Pathogenicity of Yellow Fever Virus**. *The Journal of experimental medicine* 1937, **65**:767-86.

215. Theiler, M., Smith, H.H.: **The Use of Yellow Fever Virus Modified by in Vitro Cultivation for Human Immunization**. *The Journal of experimental medicine* 1937, **65**:787-800.

216. Thibodeaux, B.A., Garbino, N.C., Liss, N.M., Piper, J., Blair, C.D., Roehrig, J.T.: **A small animal peripheral challenge model of yellow fever using interferon-receptor deficient mice and the 17D-204 vaccine strain**. *Vaccine* 2012, **30**:3180-7.

217. Tyler, K.L., McPhee, D.A., Fields, B.N.: **Distinct pathways of viral spread in the host determined by reovirus S1 gene segment**. *Science* 1986, **233**:770-4.

218. Tyler, K.L., Squier, M.K., Rodgers, S.E., Schneider, B.E., Oberhaus, S.M., Grdina, T.A., Cohen, J.J., Dermody, T.S.: **Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein sigma 1**. *J Virol* 1995, **69**:6972-9.

219. van der Meer, P., Ulrich, A.M., Gonzalez-Scarano, F., Lavi, E.: **Immunohistochemical analysis of CCR2, CCR3, CCR5, and CXCR4 in the human brain: potential mechanisms for HIV dementia**. *Exp Mol Pathol* 2000, **69**:192-201.

220. van der Schaar, H.M., Rust, M.J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., Smit, J.M.: **Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells**. *PLoS Pathog* 2008, **4**:e1000244.

221. Van Steenwinckel, J., Reaux-Le Goazigo, A., Pommier, B., Mauborgne, A., Dansereau, M.A., Kitabgi, P., Sarret, P., Pohl, M., Melik Parsadaniantz, S.: **CCL2 released from neuronal synaptic vesicles in the spinal cord is a major mediator of local inflammation and pain after peripheral nerve injury**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2011, **31**:5865-75.

222. Verma, S., Kumar, M., Gurjav, U., Lum, S., Nerurkar, V.R.: **Reversal of West Nile virus-induced blood-brain barrier disruption and tight junction proteins degradation by matrix metalloproteinases inhibitor**. *Virology* 2010, **397**:130-8.

223. Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E., Andino, R.: **Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population**. *Nature* 2006, **439**:344-8.

224. Virgin, H.W.t., Bassel-Duby, R., Fields, B.N., Tyler, K.L.: **Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing)**. *J Virol* 1988, **62**:4594-604.

225. Visse, R., Nagase, H.: **Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry**. *Circ Res* 2003, **92**:827-39.

226. Wang, H., Siddharthan, V., Hall, J.O., Morrey, J.D.: **West Nile virus preferentially transports along motor neuron axons after sciatic nerve injection of hamsters**. *Journal of neurovirology* 2009, **15**:293-9.

227. Wang, J.J., Liao, C.L., Chiou, Y.W., Chiou, C.T., Huang, Y.L., Chen, L.K.: **Ultrastructure and localization of E proteins in cultured neuron cells infected with Japanese encephalitis virus**. *Virology* 1997, **238**:30-9.

228. Wang, P., Dai, J., Bai, F., Kong, K.F., Wong, S.J., Montgomery, R.R., Madri, J.A., Fikrig, E.: **Matrix metalloproteinase 9 facilitates West Nile virus entry into the brain**. *J Virol* 2008, **82**:8978-85.

229. Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., Flavell, R.A.: **Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis**. *Nat Med* 2004, **10**:1366-73.

230. Warren, G.L., Summan, M., Gao, X., Chapman, R., Hulderman, T., Simeonova, P.P.: **Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models**. *J Physiol* 2007, **582**:825-41.

231. Weible, M.W., 2nd, Hendry, I.A.: **What is the importance of multivesicular bodies in retrograde axonal transport in vivo?** *J Neurobiol* 2004, **58**:230-43.

232. Weiner, H.L., Drayna, D., Averill, D.R., Jr., Fields, B.N.: **Molecular basis of reovirus virulence: role of the S1 gene**. *Proc Natl Acad Sci U S A* 1977, **74**:5744-8.

233. Weiner, H.L., Powers, M.L., Fields, B.N.: **Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin**. *J Infect Dis* 1980, **141**:609-16.

234. Welgus, H.G., Stricklin, G.P., Eisen, A.Z., Bauer, E.A., Cooney, R.V., Jeffrey, J.J.: **A specific inhibitor of vertebrate collagenase produced by human skin fibroblasts**. *J Biol Chem* 1979, **254**:1938-43.

235. Welsch, S., Miller, S., Romero-Brey, I., Merz, A., Bleck, C.K., Walther, P., Fuller, S.D., Antony, C., Krijnse-Locker, J., Bartenschlager, R.: **Composition and three-dimensional architecture of the dengue virus replication and assembly sites**. *Cell host & microbe* 2009, **5**:365-75.

236. Wengler, G., Wengler, G., Gross, H.J.: **Studies on virus-specific nucleic acids synthesized in vertebrate and mosquito cells infected with flaviviruses**. *Virology* 1978, **89**:423-37.

237. Wenner, H.A., Rabe, E.F.: **The recovery of virus from regional lymph nodes of fatal human cases of poliomyelitis**. *Am J Med Sci* 1951, **222**:292-9.

238. Wyatt, H.V.: **Provocation of poliomyelitis by multiple injections**. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1985, **79**:355-8.

239. Yang, W.X., Terasaki, T., Shiroki, K., Ohka, S., Aoki, J., Tanabe, S., Nomura, T., Terada, E., Sugiyama, Y., Nomoto, A.: **Efficient delivery of circulating poliovirus to the central nervous system independently of poliovirus receptor**. *Virology* 1997, **229**:421-8.

240. Zaitseva, E., Yang, S.T., Melikov, K., Pourmal, S., Chernomordik, L.V.: **Dengue virus ensures its fusion in late endosomes using compartment-specific lipids**. *PLoS Pathog* 2010, **6**:e1001131.

241. Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., Kuhn, R.J.: **Visualization of membrane protein domains by cryo-electron microscopy of dengue virus**. *Nat Struct Biol* 2003, **10**:907-12.

242. Zisman, B., Wheelock, E.F., Allison, A.C.: **Role of macrophages and antibody in resistance of mice against yellow fever virus**. *J Immunol* 1971, **107**:236-43.