

CHAPTER I: INTRODUCTION

THE NEUROTROPIC ARBOVIRUSES

Introduction

The term “arbovirus” describes a mode of viral transmission (arthropod-borne) rather than a taxonomical classification. Accordingly, a heterogeneous group of over 500 viruses falls under this category, 150 of which can cause disease, including neuroinvasive disease, in humans (1). In the United States, fatal epidemics of equine encephalitis were described as early as the 19th century (2) with subsequent isolation of the first etiologic agent from infected horse tissue in 1930 (3). Today, arboviruses are recognized as leading causative agents of viral encephalitis worldwide and important pathogens of the human central nervous system (CNS) (4).

Arboviruses are maintained in nature in enzootic life cycles between small vertebrate hosts and blood feeding arthropods (mosquitoes, psychodids, ceratopogonids, and ticks). Vector inoculation occurs with viral ingestion during a blood meal with subsequent transfer through the saliva to a new host. The particular feeding behavior of the mosquito - short duration with frequent interruption - is ideal for arbovirus transmission (5). Accordingly, mosquitoes are the most common mode of transmission

for a number of arboviral species. Further research on viral dissemination within the mosquito has demonstrated that midgut epithelial cells are: 1) an important initial site of virus replication and 2) refractory to infection with certain species of arbovirus, perhaps due to the innate antiviral function of the RNA interference pathway (6, 7). Therefore, the mosquito mesenteron is likely an important determinant of arbovirus species transmissibility.

Arbovirus transmission is also dependent on a vertebrate host, and birds and small mammals are frequent natural reservoirs. In contrast, large mammals, such as horses and humans, are typically dead-end hosts for the virus, as they fail to develop sufficient viremia to sustain viral transmission. However, exceptions are observed and human-to-human transmission of dengue virus by *Aedes aegypti* is known to occur (8, 9). In the dead-end host, arbovirus infection can clinically manifest. Most frequently, clinical disease is limited to a mild flu-like syndrome. However, infection can result in severe CNS disorder, coma, and death (1).

Arboviruses are widely distributed geographically and have been located on all continents except Antarctica (10). As viral distribution is largely limited by the geographical range of the vector species, arboviruses pose a significant threat of rapid dissemination. Warm climate trends, tropical urbanization patterns, and the high mutation rate intrinsic to an RNA virus all favor arboviral emergence. The 2005-2006 Reunion Island outbreak of Chikungunya virus (CHIKV) perhaps best illustrates this trait; here a single glycoprotein mutation in CHIKV enabled transmission in a novel mosquito species (*Aedes albopictus*) and a novel geographical range, resulting in 266,000

cases of human disease (11). In the United States, there are five highly prevalent mosquito-borne species of arboviruses: West Nile virus (WNV), La Crosse virus (LACV), St. Louis encephalitis virus (SLEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV). These species contribute to a domestic incidence of 150 to 3,000 cases per year and cost 150 million dollars in surveillance and vector control (4, 12). While arboviruses continue to be a serious public health concern, no vaccines are available to target the endemic North American pathogens, and supportive care remains the current therapeutic standard (13).

Arboviruses can be further subdivided by the clinical syndrome that results from human infection, namely rash and arthralgia, hemorrhagic disease, or acute encephalitis. Arboviruses that specifically infect CNS neurons and cause acute encephalitic disease (the neurotropic arboviruses) are the focus of all subsequent discussion and include the families *Togaviridae* (genus *Alphavirus*), *Flaviviridae*, and *Bunyaviridae* (**Table I-1**). All neurotropic arboviruses have single-stranded RNA genomes; however, they differ in complexity. Flaviviruses contain a positive-sense genome that is translated as a single polyprotein and subsequently cleaved. Alphaviruses also contain a positive-sense genome, but segregate production of structural and nonstructural proteins via subgenomic RNA production. Finally, bunyaviruses are the most complex, containing a segmented genome of two negative-sense RNA strands and one ambisense RNA strand.

Table I-1. The neurotropic arboviruses

Family	Genus	Species	Genome
<i>Togaviridae</i>	<i>Alphavirus</i>	<u>Old World (encephalitis in mice)</u> <ul style="list-style-type: none"> • Sindbis virus (SINV) • Semliki Forest virus (SFV) <u>New World (encephalitis in humans)</u> <ul style="list-style-type: none"> • eastern equine encephalitis virus (EEEV) • western equine encephalitis virus (WEEV) • Venezuelan equine encephalitis virus (VEEV) 	positive-ssRNA (subgenome production)
<i>Flaviviridae</i>	<i>Flavivirus</i>	<ul style="list-style-type: none"> • Japanese encephalitis virus (JEV) • St. Louis encephalitis virus (SLEV) • West Nile virus (WNV) 	positive-ssRNA
<i>Bunyaviridae</i>	<i>Orthobunyavirus</i>	<ul style="list-style-type: none"> • California encephalitis virus (CEV) • La Crosse virus (LACV) 	negative-ssRNA (segmented)

Pathogenesis

Human infection with a neurotropic arbovirus begins with peripheral inoculation. Therefore, initial rounds of viral replication occur in the periphery, specifically within skeletal muscle cells as well as Langerhans cells of the skin (14-17). Replication in the periphery typically results in a transient, cell-unassociated viremia (15), with subsequent invasion of the CNS. However, the exact mechanism of CNS invasion has been debated in the literature. Hematogenous seeding of both capillary endothelial cells (15, 18) and choroid plexus epithelial cells (16) have been described. More recently, hematogenous seeding of peripheral nerves within the olfactory neuroepithelium with retrograde axonal transport to the CNS was demonstrated for SLEV as well as the alphaviruses (19-21). Thus the exact mechanism of arbovirus entry into the CNS remains unclear and is likely to be virus-specific and/or multifactorial.

Upon penetrating the CNS, neurons are the primary target cell for arbovirus replication (16, 22-25). Spread through the CNS can occur via neuron-to-neuron transmission (23) or spill over into the cerebrospinal fluid (26). Once inside the target cell, virus propagation is rapid with associated cell death. In response to arbovirus infection *in vitro*, cell death by apoptosis (27, 28), host protein shut-down (29), energy depletion (30), and loss of membrane potential (31) have all been described. Further *in vivo* studies suggest, however, that apoptosis is a prominent mechanism of viral-induced neuronal depletion in the CNS (27, 32). Once the antiviral immune response of the host is activated, neuronal death can also occur via cytotoxic T-cell targeting and bystander injury (1).

Immune Response

Infection with a neurotropic arbovirus leads to a rapid and sustained immune response in the host. The initial phase of this response includes production of type I interferons (IFNs) in cells of the peripheral draining lymph node (33). Mutational analysis has shown that production is dependent upon active viral replication, specifically dsRNA intermediates (34-36), and type I IFN can be detected in the serum within hours following arbovirus inoculation (33). Systemic type I IFN can cross the blood-brain barrier, access CNS neurons, and stimulate gene expression in a STAT1-dependent manner (37, 38). Therefore, peripheral type I IFN production in response to neurotropic arbovirus infection is sufficient to initiate an antiviral response in the CNS. Upon virus invasion, a second wave of intra-CNS type I IFN production ensues; thus neurotropic arbovirus infection of CNS neurons occurs *in vivo* in the context of type I IFN. This early, innate antiviral response is essential to both CNS protection and host survival. Mice deficient in response to type I IFN, due to knockout of the type I IFN receptor, demonstrate increased neurovirulence, viral load, and extraneural viral dissemination upon challenge with a neurotropic arbovirus (39-44).

While the innate arm of the immune response is essential for limiting virus spread and surviving the acute phase of infection, the adaptive arm is necessary for eliminating virus and establishing long-term immunity in the CNS. The adaptive immune response to neurotropic arboviruses has been studied extensively in mice using the model alphavirus and mouse CNS pathogen, Sindbis virus (SINV). In an adult mouse, both humoral and cellular immune responses to SINV can be detected as early as three days following

infection. Specifically, virus-reactive lymphocytes can be isolated from draining lymph nodes, a mononuclear cell infiltrate can be detected in the CNS, and neutralizing antibodies can be detected in the serum (45). The appearance of neutralizing antibody in the serum is coincident with the disappearance of virus, and direct evidence demonstrates that neutralizing antibody can promote alphavirus clearance through the reticuloendothelial system in conjunction with complement (46). However, complement- and phagocytic cell-independent antiviral function has also been described, specifically for the SINV E2 glycoprotein antibody. In primary neurons isolated from embryonic rat dorsal root ganglia and differentiated *in vitro*, E2 antibody treatment was shown to be sufficient for intracellular viral inhibition and termination of mature virion release (47). Furthermore, synergistic antiviral effects were demonstrated with combined E2 antibody and type I IFN treatment (48). Thus the humoral immune response has multiple contributions to anti-arboviral defense in the CNS: 1) neutralization of virus in the serum to prevent further CNS invasion, 2) non-cytolytic viral clearance from infected neurons, and 3) as antibody has been shown to persist in the brain long-term (49), sustained antiviral control in the persistently infected CNS. In contrast to the prominent role of B-cells in establishing anti-SINV immunity, CD8⁺ T-cells are not essential for viral clearance in the CNS, rather this T-cell subset appears to more subtly dictate the kinetics of intracellular viral inhibition (50). Accordingly, IFN- γ production and not cytotoxic T-cell lysis has been elucidated as the contributory mechanism (51).

In summary, both innate and adaptive immune responses are critical to CNS defense against a neurotropic arbovirus. The innate immune response is dominated by

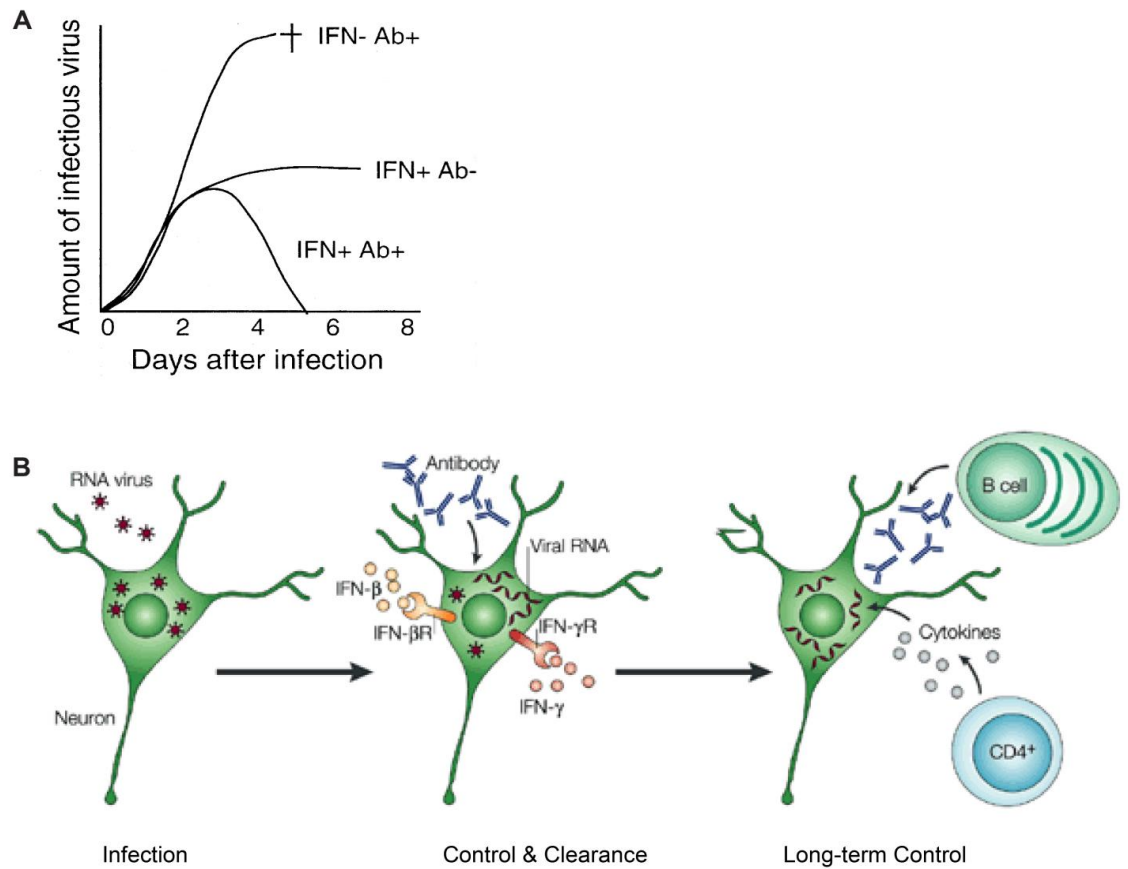


Figure I-1. Immune response to a neurotropic arbovirus

(A) Independent effects of type I IFN (IFN) and antiviral antibody (Ab) on alphavirus replication in tissue. Type I IFN is essential for early control of virus replication. In the absence of type I IFN, infection is often fatal. In contrast, antibody is essential for viral clearance. Adapted from (10). (B) Stepwise immune response to a neurotropic arbovirus infection in a CNS neuron. First, type I IFN controls virus replication. Second, the adaptive immune system clears virus in the absence of neuronal lysis (antiviral antibodies are essential and T-cells further contribute IFN- γ). Finally, a lasting immune presence is established in the CNS. Adapted from (52).

type I IFN production and is essential for limiting virus spread and surviving the acute phase of infection. In contrast, the adaptive immune response is dominated by neutralizing antibody production and is essential for non-cytolytic viral clearance within neurons and maintained antiviral control in the CNS (**Figure I-1**).

Maturation-Dependent Susceptibility

Epidemiologic data collected in the United States during outbreaks of arboviral encephalitis describe an inverse correlation between patient age and disease severity, in terms of overall morbidity as well as the development of long-term neurological sequelae (53-56). Therefore, infected children are frequently at greater risk for developing convulsions, stupor, coma, and seizures in the acute setting in addition to cognitive deficits, paralysis, and seizure disorders as permanent sequelae (13). For SLEV and WNV specifically, heightened susceptibility is also observed in adults over the age of 50 (57, 58).

Age-dependent susceptibility to neurotropic arbovirus infection has been modeled in rodents. Upon challenge with SINV, a dramatic transition from fatal disease in one-week-old mice to full recovery in two-week-old mice has been described (59-61). For Semliki Forest virus (SFV) (23) as well as Japanese encephalitis virus (JEV) (62) this window of susceptibility has been further narrowed to days 13-15 of life. Development of fatal encephalitis in the immature animal correlates with higher viral load in the CNS (61, 63) as well as increased rate and extent of intra-neuronal virus spread (23).

For over four decades, researchers have attempted to elucidate the mechanism of age-dependent susceptibility to neurotropic arbovirus infection. As type I IFN is

essential for survival, differences in type I IFN production were initially explored. Surprisingly, immature mice are not defective in type I IFN production, rather elevated type I IFN levels have been observed in the immature mouse brain following intracranial viral inoculation (63). Additionally, researchers have pursued age-dependent differences in neuroinvasion. However, susceptible mice do not demonstrate significant alterations in the blood-brain barrier that might suggest heightened viral access to the CNS (64). Furthermore, fatal outcome and increased viral load persist in the immature animal following intracranial inoculation (61, 63), suggesting intrinsic age-dependent susceptibility within the CNS rather than increased viral delivery to the CNS. Finally, researchers have explored age-dependent differences in adaptive antiviral immune function. In the context of SINV challenge, however, susceptible mice have been shown to be competent in both cellular and humoral antiviral immune responses (45). Furthermore, mice with severe combined immunodeficiency retain the ability to restrict virus spread in the CNS in a maturation-dependent manner (65). Finally, age-dependent differences in virus titer persist in the absence of an adaptive immune system, in the context of *ex vivo* infection of mouse brain tissue (66). Together these data strongly suggest that adaptive immune differences do not fully explain age-dependent host susceptibility to neurotropic arbovirus infection.

In contrast, age-dependent host susceptibility has been strongly associated with the degree of neuronal maturity in the CNS. First, several animal model systems demonstrate patterned infection of the CNS following neurotropic arbovirus challenge with heightened and sustained viral presence in areas of regional neuronal immaturity

(23, 62). Furthermore, regional neuronal immaturity within the CNS has been directly demonstrated to increase host susceptibility. Upon transplantation of embryonic neurons into the brain of mature JEV-resistant rats, the animals became susceptible to JEV infection with virus specifically localizing to the transplanted cells (62). Finally, neuronal maturation *in vitro* has been demonstrated to be sufficient for enhanced resistance to neurotropic arbovirus replication and viral-induced cell death (67, 68). Thus degree of neuronal maturity importantly dictates neurotropic arbovirus pathogenesis within the CNS. However, several questions remain. First, how does neuronal maturity change with age in the postnatal CNS? This question is addressed in the subsequent background section, “Postnatal Development of the CNS”. Second, by what molecular mechanism does neuronal maturation confer resistance to the pathogenesis of a neurotropic arbovirus? This question underlies the research conducted and will be addressed in the subsequent data chapters, Chapter II and III.

MOUNTING AN ANTIVIRAL RESPONSE IN THE CNS

Introduction

The CNS is the body’s critical control center; it dynamically orchestrates both basic motor programs that are essential for sustaining life and higher cognitive function. This unique organ is composed of multiple cell types including astrocytes, microglia, oligodendrocytes, stromal cells, and neurons. Neurons are by far the predominant cell type, numbering approximately 10^{11} in the human brain (69), consistent with the fact the neuron is the functional subunit of the CNS. Fully developed neurons are maintained in a

postmitotic state and thus to preserve function, the CNS must protect these quiescent cells from injury. Accordingly, the CNS has developed a number of unique immune features including limited lymphatic drainage, low basal MHC class I expression, and limited professional antigen presenting cell presence, dendritic cells in particular, due to the selective function of the blood-brain barrier (52). Restricting adaptive immune function within the CNS has been hypothesized to play a beneficial role evolutionarily by reducing the risk of excessive foreign or self antigen-driven inflammation. In the context of a viral infection, however, this evolutionary compromise increases the CNS's reliance on a swift and effective innate immune response to curtail virus spread and minimize neuronal cell loss.

Mechanisms of Regulation

In the absence of stimulation, the CNS is maintained in an immunologically quiescent state. The blood-brain barrier, composed of a thick basement membrane, an endothelial cell layer, and supporting astrocyte foot processes, limits solute penetration to the CNS as well as transmigration of circulating leukocytes via tight endothelial cell junctions and low adhesion molecule expression (70, 71). Maintenance of the blood-brain barrier has been shown to occur partially through local production of transforming growth factor- β , which is sufficient to downregulate cell homing to the CNS *in vivo* (72). However, routine T-cell surveillance of the CNS has been described (73, 74), suggesting that some degree of blood-brain barrier penetration occurs in the absence of inflammation. Accordingly, additional mechanisms of T-cell inhibition have evolved in the CNS. These include inhibition of T-cell proliferation and interleukin-2 (IL-2)

production by brain gangliosides (75) and direct induction of activated T-cell apoptosis in the brain parenchyma via neuronal FAS ligand expression (76). Healthy neurons can also inhibit microglia activation and aggregation by local production of neurotrophins (77), local production of chemokines such as CX3CL1 (78), and direct interaction of neuronal CD200 with its microglial receptor (79). Finally, neuronal ganglioside production has been shown to inhibit expression of MHC class I and II molecules on the surface of astrocytes (80). Thus immune effector cell presence, antigen presentation, and inflammatory cytokine production are all suppressed in the healthy CNS.

Upon infection with a neurotropic virus, however, adaptive immune function is rapidly upregulated in the CNS. Astrocytes and microglia become activated in response to neuronal damage, driving the production of inflammatory cytokines and chemokines (81-83). Innate inflammatory glial cascades in turn stimulate the upregulation of MHC class I and II expression within the CNS and additionally facilitate disruption of the blood-brain barrier. Specifically, MHC class II molecules are upregulated on the surface of microglia as well as perivascular macrophages (84), promoting antigen presentation in the CNS. Additionally, adhesion molecules are upregulated on the surface of capillary endothelial cells (70, 71, 85), promoting transmigration of activated immune effector cells.

While neurotropic viral infection triggers an immune response in the CNS, it also triggers countermeasures to protect CNS neurons from excessive immune-mediated injury. First, while MHC class I expression is broadly enhanced in the CNS in response to virus, neurons remain relatively refractory to MHC class I upregulation (86), which

may be due, in part, to decreased activation of the NF κ B pathway (87). Additionally, neurons continue to express FAS ligand during infection, and together these mechanisms aid in neuronal evasion of cytotoxic T-cell lysis (88). Finally, apoptotic neurons can induce neighboring microglia to dampen the inflammatory response. Specifically, phosphatidylserine-expressing neurons stimulate the release of neuroprotective agents from microglia and further prevent the release of pro-inflammatory molecules, including nitric oxide and tumor necrosis factor- α (89).

Neurons as Immune-Competent Cells

Neurons have historically been depicted as victims of viral infection with surrounding astrocytes or microglia dominating the initial phase of innate antiviral defense (69). Recent evidence suggests, however, that neurons are immune-competent cells that actively engage in and shape the antiviral response of the CNS (90, 91). First of all, neurons have competent viral pattern-recognition receptor (PRR) pathways and can produce innate immune cytokines in response to viral infection. An antiviral response begins with viral detection, which is dominated at the cellular level by PRR binding to a conserved pathogen moiety. Engagement of a PRR with its ligand stimulates an intracellular signal transduction cascade and ultimately drives cellular production of innate immune cytokines, principally type I IFNs. Both astrocytes and microglia demonstrate robust basal expression of PRRs, specifically the PRR subfamily of Toll-like receptors (TLRs) (92, 93). More recently, however, PRR expression in neurons was described. To date, neurons have been shown to express all 10 members of the human TLR family and to produce IFN- α and - β in response to viral infection and/or viral

mimetics (94-96). Furthermore, neurons have been shown to produce type I IFN *in vivo*. In response to infection with a neurotropic arbovirus specifically, neurons have been shown to produce IFN- α and, to a greater extent, IFN- β (85, 97). Finally, knockout of TLR-3 was shown to enhance WNV replication in cultures of primary cortical mouse neurons (98), suggesting that neuronal PRR expression impacts antiviral response to a CNS pathogen. However, as only 3% of infected neurons were found to be competent in type I IFN production *in vivo* (97), the overall impact of neuronal type I IFN production during viral CNS infection has yet to be elucidated and likely requires the development of neuron-specific PRR knockout mice.

In addition to being competent in viral detection and cytokine production, neurons can potently respond to the local cytokine environment of the CNS to effectively inhibit virus replication. Neuronal exposure to type I IFN *in vitro* induces a robust transcriptional upregulation – of antiviral gene products specifically (86). Furthermore, this transcriptional upregulation is sufficient to confer cell-autonomous antiviral protection, as demonstrated by lower virus titer and increased cell survival in neurons pretreated with type I IFN (86, 99). Additionally, neuronal response to type I IFN has been examined *in vivo*, and transgenic astrocyte-driven expression of IFN- α within the CNS has been shown to stimulate the upregulation of neuronal antiviral inhibitors in a STAT1-dependent manner (100), suggesting that neuronal response to type I IFN is not dampened in the CNS microenvironment. Finally, while systemic cellular response to type I IFN has long been understood as an essential component of host defense against a neurotropic arbovirus (see preceding section, “The Neurotropic Arboviruses: Immune

Response”), recent evidence demonstrates that neurotropic viral pathogenesis is enhanced with nestin-specific knockout of the type I IFN pathway. Upon intranasal challenge with vesicular stomatitis virus, wild-type mice were observed to restrict virus replication to olfactory bulb neurons. In contrast, gross virus replication was observed in the CNS of nestin-specific knockout mice with resulting fatality (101). This finding importantly suggests that intra-neural control of virus replication, as stimulated by type I IFN, is an important component of host defense against a neurotropic pathogen.

The Pros and Cons of Type I IFN

In the context of infection with a neurotropic virus, type I IFN is critical for stimulating antiviral gene expression and curtailing virus spread in the CNS. However, IFN- β specifically has also been shown to play a protective role in the CNS in the context of autoimmune disease. For more than 10 years, IFN- β has been used in the treatment of multiple sclerosis (MS), and clinical data demonstrate that IFN- β is particularly effective in treating the relapsing-remitting course of disease (102). In the animal model of MS, experimental autoimmune encephalitis (EAE), exacerbated disease is observed upon knockout of IFN- β (103), confirming the protective role of IFN- β reported in the clinical literature. While the exact mechanism of neuroprotection has yet to be fully dissected, current research suggests diverse mechanisms of action including reduced T-cell proliferation (104), decreased blood-brain barrier permeability to activated leukocytes (105, 106), altered Th1/Th2 ratios (107), and a shift in favor of anti-inflammatory cytokine production with an upregulation of IL-10 (108) and a downregulation of IL-12

(109) having been specifically described in peripheral blood mononuclear cells treated with IFN- β .

In contrast to the therapeutic action of IFN- β in the CNS, excessive treatment with IFN- α can lead to significant neurotoxicity. Reported neurological side effects in patients include confusion, fatigue, lethargy, psychiatric symptoms, conceptual disorganization, cortical blindness, coma and, rarely, death (110). Transgenic mice constitutively expressing IFN- α in CNS astrocytes, under the control of a glial fibrillary acidic protein (GFAP) promoter, also demonstrate severe structural and functional abnormalities including neurodegeneration, gliosis, and pronounced calcification, in addition to disturbed synaptic plasticity and hippocampal hyperexcitability (111). Surprisingly, STAT1 deficiency aggravates IFN- α -induced pathology in the CNS, suggesting that the neurotoxic effects of IFN- α are mediated by less conventional type I IFN signaling pathways (112). Finally, IFN- α can exacerbate neurotropic viral pathogenesis in the CNS by directly inducing neuronal dysfunction in a glutamate signaling-dependent manner (113). Therefore, while production of type I IFN is critical to host defense against a neurotropic virus, production of IFN- α specifically must be tightly regulated in the CNS during infection.

POSTNATAL DEVELOPMENT OF THE CNS

Introduction to Neural Progenitor Cells (NPCs)

Neural stem cells are defined by their ability to both self-renew and differentiate to form the three neural lineages: neurons, astrocytes, and oligodendrocytes.

Differentiation of neural stem cells occurs *in vitro* in a stepwise fashion through the formation of a lineage-unrestricted progenitor cell (i.e. the progenitor cell retains the ability to differentiate into a neuron, astrocyte, or oligodendrocyte) and subsequently, a lineage-restricted precursor cell (**Figure I-2A**). However, *in vivo* stem and progenitor populations have proved difficult to distinguish owing to overlapping expression of marker genes (114). The term “neural progenitor cell” (NPC) will therefore be used in all subsequent discussion to describe an immature neural cell with stem properties and the multipotent potential to form neurons, astrocytes, and oligodendrocytes in culture (115).

NPCs were first isolated from the embryonic mammalian CNS in the early 1990’s (116-119). More surprisingly, isolation of NPCs from restricted regions within the adult mammalian CNS was additionally accomplished in the early 1990s (120, 121). Subsequent to this groundbreaking work in the mouse, NPC populations were identified in the CNS of primates (122) as well as humans (123, 124). Currently, it is well established that NPCs persist in the adult mammalian brain, predominantly in two neurogenic regions: the subventricular zone (SVZ) adjacent to the lateral ventricle and the subgranular zone (SGZ) of the hippocampus (**Figure I-2B**). Furthermore, active neurogenesis has been described in both the SVZ and the SGZ of the adult mammalian brain, providing direct evidence that neuronal differentiation continues in the postnatal CNS.

Despite the persistence of NPCs in the adult mammalian brain, age-dependent differences in NPC number and function have been described. In the SVZ of aged mice (24-26 months) NPC numbers were found to be reduced by approximately two-fold when

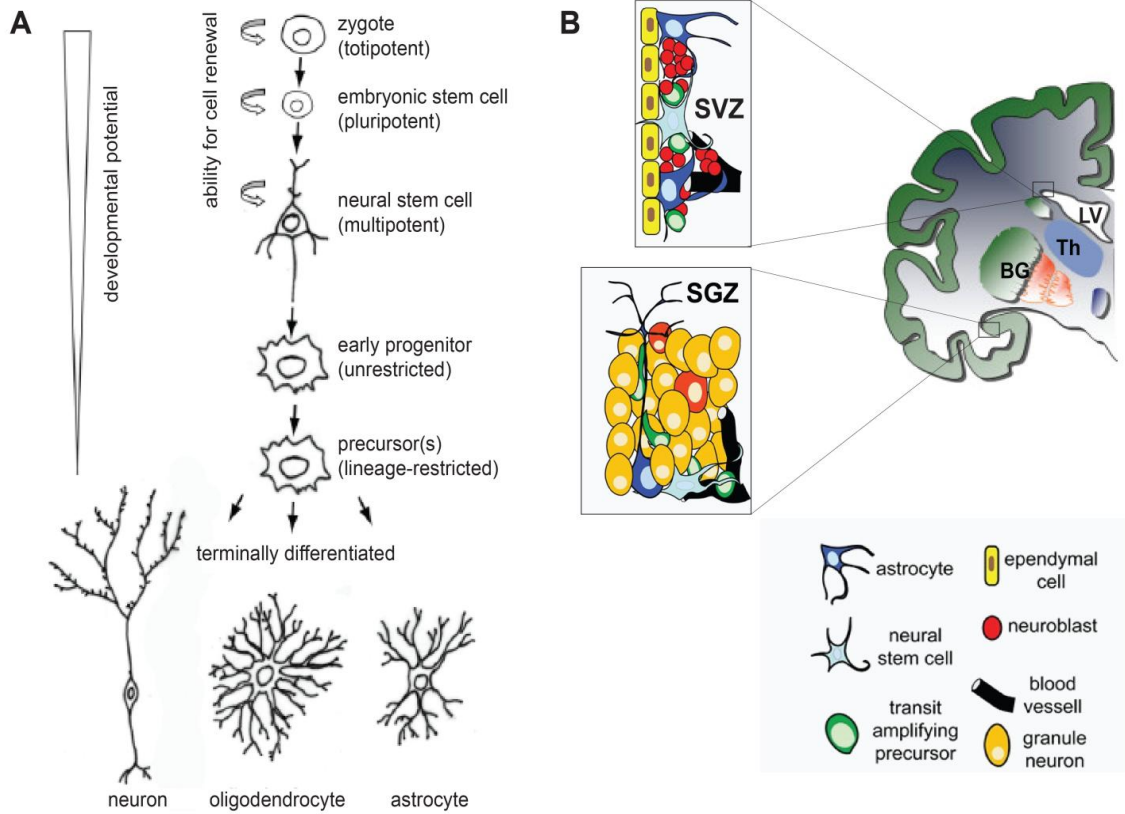


Figure I-2. Neural progenitor cells (NPCs)

(A) NPCs are lineage unrestricted cells with the potential to differentiate into neurons, oligodendrocytes, and astrocytes. *In vivo*, NPCs are indistinguishable from neural stem cells. Adapted from (115). (B) NPCs reside in two neurogenic regions within the adult mammalian brain: the subventricular zone (SVZ) adjacent to the lateral ventricle (LV) and the subgranular zone (SGZ) of the hippocampus. Adapted from (125).

compared to young controls (aged 2-4 months) (126). This dramatic decline in NPC number within the SVZ correlates with maturation of the ependyma, from a pseudostratified columnar epithelium in the fetal brain to a simple cuboidal epithelium in the adult (127). Neurogenesis in the rat brain has also been shown to decline dramatically at approximately 7 months of age, which correlates with decreased NPC numbers in the SGZ as well as diminished dendrite outgrowth following differentiation (128). Finally, age-dependent reduction of the NPC pool has been observed in primates, specifically in macaque monkeys aged greater than 20 years (129). Thus NPC number and neurogenesis potential are age-dependent in the mammalian CNS.

Modeling Human Neuronal Development *in vitro*

While NPCs can be routinely isolated from the mouse CNS for investigation *in vitro*, a similar approach is not sustainable for human NPCs. Accordingly, protocols for deriving NPCs from human embryonic stem cells (hESCs) were first established in 2001 (130, 131). hESCs are pluripotent cells derived from the inner cell mass of a pre-implanted embryo. Accordingly, hESCs are capable of differentiating into all three germ layers: ectoderm, endoderm, and mesoderm. Preliminary protocols for differentiating hESCs into NPCs relied on their spontaneous differentiation potential, allowing the formation of a free-form mass of cells or “embryoid body” (130). Subsequently, neuroectoderm was mechanically dissected away from the other “contaminating” germ layers that had formed, and the isolated neuroectoderm was allowed to expand and differentiate into neurons, astrocytes, and oligodendrocytes (130, 131). However, there are several problems inherent to such an approach, including neuroectoderm

contamination during mechanical isolation in addition to chaotic differentiation of the embryoid body, resulting in exposure of the neuroectoderm to uncontrolled signaling by multiple pathway members. Accordingly, substantial effort has been dedicated over the past decade to developing superior methods of neuronal derivation. However, an optimal protocol has yet to be established and obtaining pure populations of human NPCs and mature human neurons remains an ongoing area of research (132).

Viral Susceptibility of NPCs

Significant post-infectious neurological sequelae have been documented for a number of neurotropic viruses. Specific to the neurotropic arboviruses, permanent cognitive deficits, motor disorders, and seizure disorders have been described (54). Additionally, neurotropic arboviruses have been shown to replicate to higher levels in the immature CNS (61, 63) and localize to regions containing immature neurons (23, 62). Thus with the discovery of NPCs in the postnatal mammalian brain, researchers began to question whether NPCs could in fact be a previously unrecognized target of neurotropic viral infection *in vivo* and if so, whether viral infection of NPCs could result in persistent neurological deficits in the host.

In 2000, murine NPCs were successfully infected with mouse cytomegalovirus (CMV) *in vitro* (133), providing the first conclusive evidence that NPCs are permissive to neurotropic viral infection. NPC permissibility has subsequently been demonstrated for human immunodeficiency virus (HIV) (134, 135), coxsackievirus B3 (CVB3) (136), and the neurotropic arbovirus, JEV (137). For HIV specifically, viral genome has also been

detected in nestin-positive cells within archival pediatric brain tissue (138), suggesting that NPC infection occurs during the natural course of human disease.

In response to NPC infection with a neurotropic virus, significant cellular defects have been described. Most prominently, neurotropic viral infection can inhibit NPC proliferation (133, 135, 137, 139), and G1 phase arrest due to viral-induced activation of cell-cycle check point pathways has been described (137, 140). Neurotropic viral infection can also limit the differentiation potential of NPCs. Infection with CMV was shown to inhibit the derivation of neurofilament-positive cells from NPCs in culture (133), suggesting a specific reduction in the potential of NPCs to form mature neurons. Infection with CMV was also shown to inhibit the migration of developing NPCs in the CNS (133). However, this effect is likely virus-specific as no defect in NPC migration was observed following CVB3 infection *in vivo* (139). Finally, viral-induced apoptosis has been described, both at the NPC stage (137) and upon NPC differentiation to a mature neuron (136). Together these data suggest that neurotropic viral infection of NPCs can have negative and lasting consequences on neurogenesis potential within the postnatal CNS. Accordingly, in a transgenic mouse model of persistent HIV infection, neurogenesis was found to be significantly impaired in the adult brain with reduced total number of newborn neurons and aberrant dendrite formation in the newborn cells (141).

NPCs are permissive to neurotropic viruses. Of greater physiologic relevance, however, NPCs are specifically targeted by neurotropic viruses during the course of an *in vivo* infection. Immunohistochemical data demonstrate that the cortical marginal region and SVZ of the newborn mouse brain are most susceptible to CMV infection (142).

Higher viral load was also qualitatively appreciated in the striatum and SVZ, compared to the cortex, of the suckling mouse brain following JEV infection (137). Finally, CVB3 was shown to specifically distribute to regions of BrDU-positive cells within the neonatal CNS (139). Together these data strongly suggest that susceptibility to neurotropic viral infection is heightened in NPCs versus mature neurons. Accordingly, researchers have begun to investigate possible mechanisms of enhanced NPC susceptibility.

Work on JEV has focused on NPC-specific mechanisms of viral entry (143) and upon infection, adaptive immune activation. However, NPCs were surprisingly shown to be competent in MHC class I expression and pro-inflammatory cytokine production upon challenge with JEV (144), suggesting that decreased activation of the adaptive antiviral immune response cannot account for increased NPC viral susceptibility *in vivo*. Alternatively, JEV entry into NPCs was demonstrated to be lipid raft-dependent (143), and differences in surface moiety composition between NPCs and mature neurons may exist such that NPCs are particularly susceptible neurotropic viral attachment and entry. Additionally, research on CMV pathogenesis has examined whether active cell cycling impacts viral susceptibility. Following cerebral infusion with epidermal growth factor, increased susceptibility of the adult mouse brain was observed, with a significant increase in virus-positive cells within the SVZ (145). These data suggest that active cell cycling enhances CMV infection of NPCs. However, the heightened susceptibility of NPCs to neurotropic virus infection may be multifactorial, and a number of variables have yet to be explored including maturation-dependent changes in innate neuronal immune function.

THE TYPE I INTERFERON (IFN) PATHWAY

Components of the Pathway

In 1957 Isaacs and Lindenmann coined the term “interferon” to describe a secreted substance capable of interfering with the course of a viral infection (146). In the ensuing half century IFNs have been cloned, purified, characterized, and therapeutically tested. Currently, IFNs are grouped under the Class II cytokines and are structurally subdivided into three types according to distinct receptor recognition: the type I IFNs (described in detail below), the type II IFNs (consisting of a single cytokine, IFN- γ), and the type III IFNs (consisting of IFN- λ 1, IFN- λ 2, and IFN- λ 3, also known as interleukin 29 (IL-29), IL-28A, and IL-28B, respectively).

IFN- γ , which functions primarily as an immunomodulatory cytokine, is produced by CD 8+ T cells, CD 4+ T cells, and natural killer cells. In contrast, type I IFNs can be produced *in vitro* by most cell types (147). Cellular production of type I IFN is largely regulated at the transcriptional level and is classically induced by viral stimuli as well as synthetic viral mimetics like poly(I:C). However, type I IFN induction in response to mitogens, cytokines, and microbial products has also been reported (reviewed in (148)). The molecular pathways mediating type I IFN induction have been elucidated with the discovery of cellular pattern recognition receptors (PRRs), which currently include the Toll-like receptors (TLRs), the NOD-like receptors, and the RIG-I-like receptors (149). However, novel PRR families are still being identified. Furthermore, signal transduction downstream of the PRRs is highly complex and has yet to be fully dissected. Therefore,

our understanding of type I IFN induction in response to infectious stimuli remains an evolving area of research.

In humans, more than twenty genes encode the five classes of type I IFN: α , β , ϵ , κ , and ω (150). IFN- α can be further subdivided into 13 different subtypes: $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 10$, $\alpha 13$, $\alpha 14$, $\alpha 16$, $\alpha 17$, and $\alpha 21$. These genes are clustered on chromosome 9 in humans and on chromosome 4 in mice. The variation in part explains the pleiotropic effects of the type I IFNs including antiviral, antibacterial, and antiproliferative activity among many others (151). Despite restricted amino acid sequence similarity, all type I IFNs share a closely related three-dimensional structure and are recognized by a single transmembrane receptor (152).

The type I IFN receptor is a heterodimer, consisting of the IFN- α/β receptor 1 subunit (IFNAR1, formerly subunit α) and the IFN- α/β receptor 2 subunit (IFNAR2, formerly subunit β). These subunits are encoded by distinct genes, which have been localized to chromosome 21 in humans and to chromosome 16 in mice (153, 154). Multiple transcript variants have been identified for *IFNAR1* in accordance with different cell line models. However, only a single *IFNAR1* transcript is identifiable in nontransformed cells (155). In contrast, the human *IFNAR2* gene encodes four transcript variants that are derived from exon skipping, alternative splicing, and differential usage of a polyadenylation site (156). When translated, these four transcript variants result in the production of three distinct protein isoforms designated IFNAR2a, IFNAR2b, and IFNAR2c. IFNAR2b and c are transmembrane proteins, identical in their extracellular and transmembrane domains but differing in the length of their intracellular domain.

Transfection studies have demonstrated that IFNAR1 and IFNAR2c can reconstitute the complete function of the native type I IFN receptor; thus IFNAR2c has been designated the signaling competent isoform (157). In contrast, IFNAR2b may function as a dominant negative regulator (158). IFNAR2a is a soluble protein, lacking the transmembrane domain as the result of a splicing event. In the mouse model, IFNAR2a is capable of binding to type I IFN and has been shown to have both agonistic and antagonistic properties (159).

The type I IFN receptor lacks intrinsic kinase activity. Therefore, downstream signal transduction is dependent on IFNAR's association with the cytoplasmic non-receptor protein tyrosine kinases, Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). Despite the divergent nomenclature, Jak1 and Tyk2 both belong to the Janus family of kinases (Jaks). Jak proteins contain four conserved domains: a catalytically active kinase domain, a regulatory pseudokinase domain, a Src homology 2 (SH2) domain that functions as a binding platform for downstream effectors, and a 4.1, ezrin, radixin, moesin (FERM) domain that mediates a Jak's interaction with its cognate transmembrane receptor. Tyk2 was the first family member identified (160, 161) and the first non-receptor tyrosine kinase found to be associated with type I IFN pathway activation, due to the ability of transfected Tyk2 to restore type I IFN sensitivity in a mutant human cell line (162). Using similar methods, a critical role for Jak1 in type I and II IFN pathway activation was identified (163). Subsequent work further demonstrated that Tyk2 and Jak1 are constitutive and direct binding partners of IFNAR1 and IFNAR2, respectively (164-166).

Tyk2 and Jak1 relay signal from the receptor by phosphorylating downstream effectors, namely signal transducer and activator of transcription (STAT) proteins. In mammalian cells seven distinct STAT proteins have been identified: STAT1, 2, 3, 4, 5A, 5B, and 6. While each STAT is uniquely activated in response to specific extracellular stimuli, the mechanics of STAT activation are relatively conserved. In accordance, several structural and functional domains are conserved among the STAT proteins. These include an amino-terminal dimerization domain, a coil-coil domain involved in protein-protein interactions with non-STAT binding partners like IRF-9, a central DNA-binding domain, and an SH2 domain/tyrosine activation motif. The SH2 domain/tyrosine activation motif is most highly conserved among the STAT proteins, highlighting the critical role of this domain in: 1) STAT recruitment to the cytokine receptor, 2) STAT association with its upstream tyrosine kinase, and 3) STAT homo- and/or heterodimerization (167).

STAT1 and 2 are the founding family members. STAT2 is encoded on human chromosome 12 and on mouse chromosome 10 and is expressed as a single protein isoform of 113 kDa. In contrast, STAT1 is encoded on human chromosome 2 and on mouse chromosome 1 and is expressed as two distinct protein isoforms of 91 (STAT1 α) and 84 (STAT1 β) kDa. These STAT1 isoforms are produced from two differentially spliced mRNA products and are identical except for the 38 carboxy-terminal amino acids, which are absent in STAT1 β . STAT2, STAT1 α , and STAT1 β were first identified along with IRF-9 in an IFN-responsive multiprotein transcription factor complex, designated interferon-stimulated gene factor 3 (ISGF3) (168-170). Within minutes of type I IFN

stimulation, ISGF3 assembly from latently expressed cellular components was observed in the cytoplasm of HeLa cells with subsequent nuclear translocation and site-specific DNA binding. This groundbreaking work by the Darnell lab defined a novel molecular mechanism of ligand-specific gene induction. While further research in the field has demonstrated activation of other STAT family members in response to type I IFN, STAT1 and 2 remain the most critical mediators of type I IFN-dependent gene induction (171). Transfection studies have shown that STAT2, STAT1 α , and, to a lesser extent, STAT1 β can restore type I IFN sensitivity in mutant human cell lines (172, 173). Of further physiological relevance, STAT1 and 2 knockout mice are profoundly defective in their response to type I IFN. Accordingly, these mice are more susceptible to viral infection (174-176).

The third component of the ISGF3 complex, originally designated ISGF3 γ and p48, was ultimately determined to share homology with members of the IFN regulatory factor (IRF) family of proteins and was accordingly renamed IRF-9. In total nine IRF family members have been described, all containing a homologous N-terminal region that is essential for DNA binding. IRF-9 has been shown to play a principal role in forming the DNA contact site of ISGF3 (177), and cells deficient in IRF-9 fail to respond to type I IFN (178) and accordingly lack antiviral activity (179). Furthermore, fusion of IRF-9 to the C-terminal transcription activation domain of STAT2 was sufficient to induce antiviral gene expression independent of type I IFN stimulation *in vitro* (180).

IRFs are frequent binding partners of the STAT proteins; however, IRF-9 demonstrates a unique ability to bind STAT2 in the absence of cellular stimulation and

STAT2 phosphorylation. This association is mediated by the carboxyl tail of IRF-9, specifically amino acids 200-393 (*181, 182*), and dictates the subcellular localization of IRF-9. In contrast to STAT1 and 2, which in an unphosphorylated state are retained in the cytoplasm, IRF-9 contains a nuclear localization signal (NLS) within its DNA-binding domain that is sufficient for nuclear targeting. However, as overexpression of STAT2 can relocalize IRF-9 to the cytoplasm, it follows that STAT2 can override the NLS of IRF-9 (*183*). This subcellular localization pattern likely improves activation kinetics and signal transduction efficiency within the pathway.

Component Expression Levels Impact Response to Type I IFN

In the mouse model, all organs systems, including the brain, have been shown to express IFNAR1 as well as transmembrane and soluble forms of IFNAR2 (*159, 184*). While microarray analysis suggests relatively constant expression of IFNAR1 across multiple cell and tissue types, expression of IFNAR2 appears to be more tightly regulated at the transcriptional level (*184*). Additionally, independent regulation of the IFNAR2 isoforms has been described (*185*) and accordingly, tissue-specific IFNAR2c/IFNAR2a ratios (*159*).

At the cell surface, flow cytometry analysis has revealed varied expression of IFNAR1 and IFNAR2 across multiple cell models, specifically HeLa, 293T, Daudi, and Jurkat cells (*186*). IFNAR1 expression at the plasma membrane is transient unless stabilized by interaction with Tyk2 (*187*). Furthermore, IFNAR1 is rapidly lost from the cell surface following exposure to IFN- α and - β through ubiquitination and subsequent degradation (*188*). In contrast, IFNAR2 demonstrates decreased basal turnover within

the cell. In response to IFN- β treatment, a subtle and cell type-specific degradation of IFNAR2 was observed. In contrast, IFN- α treatment did not result in IFNAR2 degradation, but instead induced IFNAR2 internalization with subsequent recycling to the plasma membrane (186).

Upregulation of IFNAR has been shown to enhance the cellular response to type I IFN. Specifically, IFN- γ treatment of HepG2 cells was shown to increase mRNA levels of IFNAR1 and IFNAR2 and increase 2-5A-synthetase activity in response to type I IFN (189). In contrast, differentiation of peripheral blood monocytes led to post-translational upregulation of the receptor with relocalization of IFNAR1 from intracellular monocyte compartments to the macrophage cell surface. This relocalization did not impact receptor-ligand affinity but rather increased total ligand binding at the macrophage cell surface (190). Finally, as IFNAR is encoded on human chromosome 21, upregulation of IFNAR is observed in Down syndrome. Accordingly, primary cultures of trisomy 21 fibroblasts were found to be three to seven times more antivirally effective than non-trisomy 21 fibroblasts following treatment with type I IFN (191).

Relative expression of the intracellular type I IFN pathway components also dictates response to type I IFN. Decreased IRF-9 expression in hepatocellular carcinoma cells confers resistance to IFN- α therapy (192). Similarly, decreased expression of all three ISGF3 components has been observed in IFN-resistant human melanoma cells (193), while priming with IFN- γ increases expression of IRF-9, STAT1, and STAT2 and enhances cellular response to type I IFN (194).

Together these data suggest that basal expression of type I IFN pathway components denotes cellular response potential to type I IFN. Accordingly, analysis of primary cardiac cells revealed increased basal expression of IFNAR1, Jak1, Tyk2, STAT2, and IRF-9 in fibroblasts compared to myocytes, resulting in greater IFN-induced antiviral protection in the cardiac fibroblast cells. However, as overexpression of individual pathway components does not universally enhance cellular response to type I IFN (e.g. IFNAR1 overexpression (*186*)), it follows that pathway interactions are dynamic and that cell type-specific bottlenecks exist.

Activation of the Pathway

Except in the contrived setting of an artificial extracellular domain (*195*), homodimers of IFNAR1 and IFNAR2 have not been observed (*196*). Furthermore, both receptor subunits have been shown to actively engage in ligand binding, resulting in the formation of a 1/1/1 ternary complex across a wide range of type I IFN subtypes. More specifically, IFNAR2 has been elucidated as the high affinity binder, with a K_d in the nM range. In contrast, IFNAR1 has been elucidated as the low affinity binder, with a K_d in the μ M range (*196*). Within these nM and μ M ranges, however, type I IFN subtypes differ in their relative binding affinity for IFNAR1 and IFNAR2, with IFN- β demonstrating high relative affinity for IFNAR1 and IFN- $\alpha 2$ conversely demonstrating high relative affinity for IFNAR2. Differential receptor subunit affinities were initially correlated with divergent subtype effects, with IFN- β favoring antiproliferation and IFN- $\alpha 2$ conversely favoring ISGF3 formation and antiviral activity (*196*). However, specific type I IFN subtype activities have since been correlated with total ternary complex

binding affinity rather than binding affinity for the individual receptor subunits (197). Finally, receptor subunit affinity does appear to impact the Hill slope of the ligand-induced response. Specifically, high relative IFNAR1/IFNAR2 affinity was correlated with a steeper Hill slope value of antiviral response to encephalomyocarditis virus (198).

In response to ligand binding, IFNAR1 and IFNAR2 dimerize and are rapidly phosphorylated by their associated tyrosine kinases, Tyk2 and Jak1 (199-202). Mutational analysis has shown that phosphorylation of IFNAR1 at Tyr466 creates a critical docking site for latent STAT2 (203), which binds to IFNAR1 via its SH2 domain and is subsequently phosphorylated by Tyk2 at Tyr690. Phosphorylation of STAT2 at Tyr690 in turn creates a docking site for STAT1. STAT1 is subsequently phosphorylated at Tyr701 and forms a heterodimer with STAT2 via reciprocal SH2 domain-tyrosine activation motif interactions. In this manner, phosphorylation of IFNAR1 in response to type I IFN drives STAT activation. However, mutational analyses have also demonstrated that tyrosine phosphorylation within the intracellular domain (residue 337 or 512) of IFNAR2c is required for STAT1 and 2 phosphorylation in U5A cells (204, 205). Furthermore, STAT2 has been shown to associate with the intracellular domain of IFNAR2c in a constitutive, phosphorylation-independent manner (206). This interaction was not originally appreciated as a potential mechanism of type I IFN gene induction (207). However, proteolytic cleavage of IFNAR2c in response to type I IFN, liberating the intracellular domain, has recently been described (208). Furthermore, the cleaved intracellular domain of IFNAR2c has been shown to form a ternary complex with STAT2 and IRF-9, resulting in its nuclear translocation. Association of latent STAT2 with the

intracellular domain of IFNAR2c is therefore a likely novel mechanism type I IFN-stimulated gene induction (209). Accordingly, the exact detail of STAT association with and activation by the type I IFN receptor has yet to be fully dissected.

Once heterodimerized, STAT1 and 2 dissociate from the receptor, exposing a gain of function NLS, which is sufficient to drive nuclear translocation of the STAT complex in an importin receptor-dependent manner (210, 211). As complexes of STAT1/STAT2, STAT2/IRF-9, and STAT1/STAT2/IRF-9 have all been identified in the cytoplasm post-stimulation with type I IFN (212, 213), it follows that some degree of STAT heterodimerization can occur prior to association with IRF-9, however, the exact location and stepwise association of ISGF3 is still only partially understood. Additionally, it is likely that multiple variations of the complex are formed in response to type I IFN. Upon translocation to the nucleus, ISGF3 recognizes and binds to a specific nucleotide sequence. While IRF-9 is known to play a prominent role in DNA binding, phosphatase treatment of ISGF3 was found to reduce its ability to bind DNA, suggesting that the phosphorylated STATs also importantly dictate the DNA interaction (214). Subsequently, phosphorylated STAT1 and IRF-9 were shown to make the precise site-specific DNA contact, while phosphorylated STAT2 interacted more transiently with the DNA, yet provided potent transcriptional activation once the DNA contact was made (177, 215).

Genes that are upregulated in response to IFN are known as IFN-stimulated genes (ISGs). With the development of recombinant human type I IFN in the early 1980s, a list of type I ISGs soon began to emerge (150). Promoter deletion and mutation analyses in

ISG54 (216) and other classic type I ISGs (217) identified a conserved regulatory sequence often present within 100 nucleotides of the transcriptional start site. This conserved sequence (AGTTTCNNTTTCC) was designated the IFN-stimulated response element (ISRE) (216), as functional analysis revealed the ISRE to be necessary for ISGF3 binding and type I IFN induction (reviewed in (217)). In response to type I IFN, hundreds of genes are upregulated within the cell. Pertinent to mounting an effective antiviral response, these genes include components of the type I IFN signaling pathway (e.g. STAT1, STAT2, and IRF-9), components of the viral PRR pathway (e.g. IRF-7), activators of the adaptive antiviral immune response (e.g. MHC I), and direct antiviral effectors (e.g. MxA) (218, 219) (**Figure I-3**).

The Mx GTPase Family of Antiviral Inhibitors

Mx proteins are relatively high molecular weight GTPases with described antiviral function. These proteins contain three distinct structural domains: a highly conserved N-terminal GTPase domain, a central interactive domain (CID), and a relatively divergent C-terminal effector domain with leucine zipper motifs. The CID and amino-terminal portion of the effector domain constitute the Mx “stalk”, which is structurally organized into four-helical bundles (220).

Similar to the dynamin superfamily of high molecular weight GTPases, Mx proteins can both self-assemble and associate with intracellular membranes. Oligomerization is driven by protein-protein interactions within the Mx stalk, specifically three distinct interfaces and one loop, and is critical for protein stability and GTP hydrolysis (221) as well as membrane binding and antiviral activity (220). Thus Mx

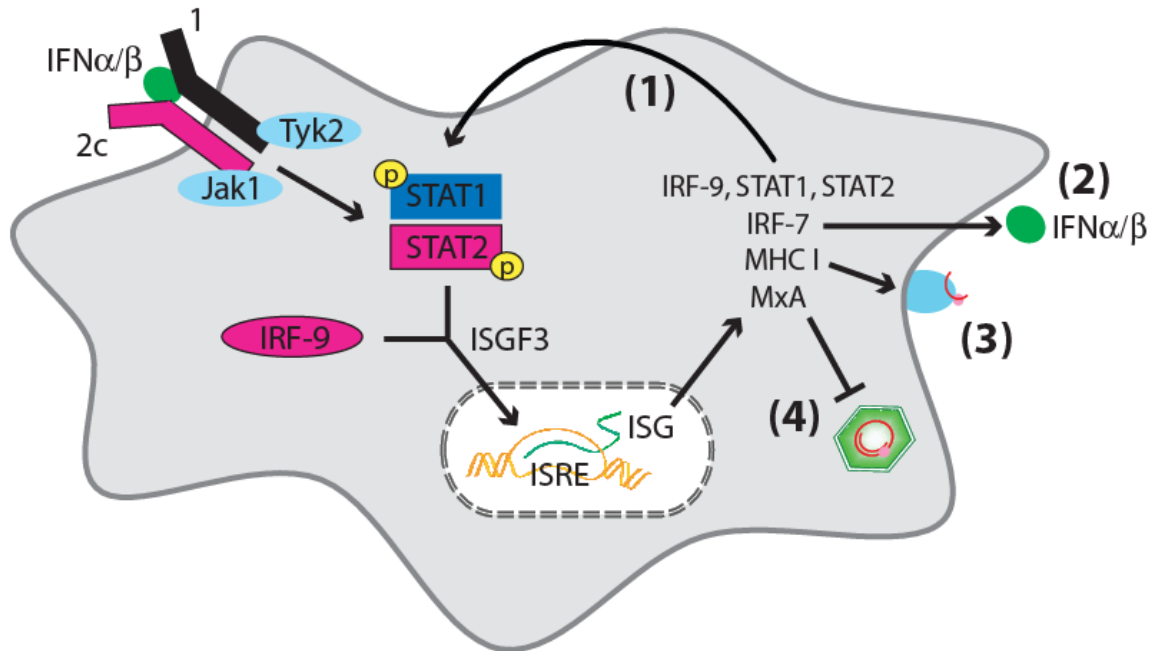


Figure I-3. The canonical type I IFN signaling pathway

Type I IFN induces an antiviral state in human neurons. Ligand is bound at the neuronal cell surface by a receptor heterodimer (IFNAR1 and IFNAR2c), stimulating an intracellular phosphorylation cascade. Signal is transduced to the nucleus by ISGF3, a ternary complex of IRF-9, phosphorylated STAT1, and phosphorylated STAT2. An antiviral state is transcriptionally upregulated in the neuron by production of (1) components of the type I IFN pathway that can potentiate signal transduction, (2) components of the viral pattern recognition receptor pathway that can potentiate production of type I IFN, (3) adaptive immune activators that can engage professional immune cells in the antiviral response, and (4) cell-autonomous antiviral effectors that can directly inhibit viral replication within the cell.

function is dependent on the three-dimensional structure of the multimeric protein complex. Accordingly, an oligomeric ring-like structure has recently been proposed, consisting of: 1) a base ring of 16 Mx dimers linked by interacting stalk regions, 2) an outer ring interface composed of N-terminal GTPase domains, and 3) an inner ring interface composed of stalk loops L2 and L4 (222). These exposed stalk loops are putative sites of viral inhibition as they are theoretically capable of binding negatively charged lipids and ribonucleoproteins.

In mice, two Mx family members have been described: Mx1 and Mx2. The *Mx1* gene (an acronym for orthomyxovirus resistance gene 1) was first identified in an inbred mouse strain that demonstrated profound resistance to viral challenge with influenza A (223). Locus analysis subsequently revealed a second IFN-responsive transcription unit, sharing 90% identity with *Mx1*, that was accordingly named *Mx2* (224). Despite the closely related sequence homology, Mx1 has been localized to the nucleus while Mx2 has been localized to the cell cytoplasm. Accordingly, Mx1 has been shown to have antiviral function against viruses that replicate in the nucleus (e.g. Influenza A and Thogoto virus), while Mx2 has been shown to inhibit viruses that replicate in the cytoplasm (e.g. vesicular stomatitis virus and LACV) (reviewed in (225)). Though a clever evolutionarily-derived antiviral system, most inbred strains of mice contain mutated copies of the *Mx* genes, the probable result of a founder effect, and are thus deficient in Mx production (226).

The human genome encodes homologous Mx proteins, which have been designated MxA and MxB (227, 228). However, the mechanics of Mx-mediated antiviral

defense are quite divergent in humans compared to mice. First of all, both human Mx proteins have been localized to the cell cytoplasm (228, 229). More specifically, MxA localizes to COP-I-positive subdomains of the smooth ER-Golgi-intermediate compartment (230), while MxB localizes to the cytoplasmic-face of the nuclear envelope (231). Furthermore, while MxA has been extensively described as an antiviral effector, capable of inhibiting viruses that replicate both in the nucleus and in the cytoplasm, MxB has to date not been conclusively demonstrated to have antiviral activity (reviewed in (225)).

MxA is a particularly attractive candidate antiviral effector to study in the context of type I IFN-dependent inhibition of neurotropic arboviruses in human neurons. First of all, MxA has previously been shown to inhibit viral replication in the mouse brain (232) as well as in human glioblastoma cells (233). However, the effector function of MxA is known to be cell type-specific (233), and the antiviral breadth of MxA as well as its mechanism of action has yet to be characterized in human neurons. Second, while many antiviral effectors are promiscuously induced during the course of viral infection, MxA induction is tightly regulated by type I IFNs and, to a lesser extent, type III IFNs (234). The MxA promoter contains two perfect ISRE sites, both of which are bound by ISGF3 and required for full induction (235). Finally, MxA has previously been shown to inhibit neurotropic arbovirus replication. Specifically, inhibition of LACV and other related bunyaviruses has been described (232, 236, 237). Additionally, MxA has been shown to inhibit SFV, an Old World alphavirus (232, 238). However, MxA inhibition of New World alphaviruses, including VEEV and WEEV, has yet to be described. Additionally,

the mechanism by which MxA inhibits alphavirus replication has yet to be dissected. Accordingly, more detailed analyses, particularly within the Alphavirus genus, are required to fully elucidate the antiviral competency of MxA in the context of human neuronal infection with a neurotropic arbovirus.

CONCLUSION

Neurotropic arboviruses specifically infect CNS neurons (16, 22-25) to cause acute encephalitic disease and permanent neurological sequelae in humans, frequently in an age-dependent manner (53-56). Decreased susceptibility to neurotropic arbovirus infection within the mature host has been correlated with relative maturation of CNS neurons (23, 62, 67, 68). However, the mechanism by which neuronal maturation confers enhanced protection against neurotropic arbovirus pathogenesis has yet to be dissected. In the subsequent data chapters, we provide evidence to suggest that the innate immune function of human neurons is enhanced with maturation. In Chapter II, an *in vitro* model of human neuronal development is introduced, which uses hESCs to derive enriched populations of human NPCs and mature neurons. Using the stem cell model in conjunction with human neuronal cells and primary cultures of rodent neurons, novel maturation-dependent changes in the neuronal type I IFN signaling pathway are identified that confer enhanced resistance to neurotropic arbovirus replication. In Chapter III, a downstream mechanism of type I IFN-stimulated antiviral defense is further dissected by elucidating MxA as a functional inhibitor of neurotropic arbovirus replication in human neuronal cells. Together these data demonstrate that antiviral type I

IFN pathway activity increases with human neuronal differentiation, with maturation of human NPCs specifically, resulting in enhanced defense against a neurotropic arbovirus. In Chapter IV, the significance of this work is considered, including the scientific potential of the hESC model and the larger implications of maturation-dependent innate neuronal immune function on host susceptibility to a neurotropic arbovirus.

CHAPTER II:

HUMAN NEURONAL DIFFERENTIATION MODULATES TYPE I INTERFERON PATHWAY ACTIVITY AND SUSCEPTIBILITY TO NEUROTROPIC ARBOVIRUS INFECTION¹

Neurotropic arboviruses specifically infect central nervous system (CNS) neurons to cause acute encephalitis and permanent neurological sequelae in humans, with particular severity in the pediatric population. Preferential development of neurological sequelae in the less developed CNS may correlate with *in vivo* susceptibility of neural progenitor cells (NPCs). However, mechanism(s) underlying enhanced susceptibility to neurotropic arbovirus infection in NPCs compared to mature neurons have yet to be fully described. Using human embryonic stem cells (hESCs), we derived enriched populations of NPCs and mature neurons and comparatively analyzed innate type I IFN pathway function. With differentiation of NPCs, we observed increased type I IFN pathway component expression, including increased surface expression of the IFN- α/β receptor 2 subunit (IFNAR2), and enhanced type I IFN-dependent inhibition of neurotropic arbovirus replication. Increased type I IFN pathway activity was additionally observed with *in vitro* differentiation of primary cortical rat neurons and human neuronal cells.

¹ The data from this chapter are planned for publication as “Farmer, J.R., Altschaefl, K.M., Peltier, D.C., O’Shea, K.S., and Miller, D.J. Human neuronal differentiation modulates type I interferon pathway activity and susceptibility to neurotropic arbovirus infection.”

Furthermore, overexpression of IFNAR2 in immature human neuronal cells was sufficient for increased type I IFN-dependent inhibition of neurotropic arbovirus replication, suggesting that IFNAR2 mediates enhanced antiviral response to type I IFN in mature human neurons. We conclude that human NPCs are more susceptible to neurotropic arbovirus infection than mature neurons due, in part, to differences in innate type I IFN pathway function.

INTRODUCTION

Arthropod-borne viruses (arboviruses) are leading causative agents of viral encephalitis worldwide (4). Humans are incidental, dead-end hosts that fail to develop sufficient viremia to sustain virus transmission. In humans, the clinical consequences of arbovirus infection are typically limited to mild flu-like disease. However, infection can result in severe CNS disorders, coma, and death (1). Arboviruses that specifically infect human CNS neurons to cause acute encephalitic disease are known as the neurotropic arboviruses and include the bunyaviruses (e.g. La Crosse virus), flaviviruses (e.g. Japanese encephalitis virus), and alphaviruses (e.g. western and Venezuelan equine encephalitis viruses).

The innate immune response to neurotropic arboviruses is dominated by production of type I IFN and is essential for host survival and acute curtailment of virus spread both in the periphery and in the CNS (39-44, 239). Accordingly, mice deficient in response to type I IFN (IFNAR^{-/-}) are more susceptible to neurotropic arbovirus infection and have more severe CNS pathology (152). Within the CNS, type I IFN elicits an

antiviral response in multiple cell types, including neurons (97, 100). Neurons have historically been depicted as immunologically quiescent cells. However, growing evidence indicates that neurons actively shape the antiviral response of the CNS (69, 85, 91, 240). Stimulating neurons with type I IFN both *in vitro* (241) and *in vivo* (100) induces a dramatic transcriptional upregulation of antiviral genes, which is sufficient for cell-autonomous inhibition of virus replication and protection from virus-induced cell death (68, 242). Furthermore, recent evidence demonstrates that susceptibility to neurotropic virus infection is conferred with nestin-specific knockout of the type I IFN pathway (101), suggesting that intra-neural control of virus replication is an important component of host defense against a neurotropic pathogen.

At the cellular level, activation of the type I IFN pathway is initiated by cytokine binding to a receptor heterodimer, IFN- α/β receptor 1 and 2 subunits (IFNAR1 and IFNAR2), which activates the associated tyrosine kinases, Tyk2 and Jak1, respectively, and stimulates an intracellular phosphorylation cascade, involving STAT1 and STAT2. Phosphorylated STAT1 and 2 heterodimerize and associate with IFN regulatory factor 9 (IRF-9) to form the multi-protein transcription factor complex, IFN-stimulated gene factor 3 (ISGF3). Inside the nucleus, ISGF3 binds to the *cis*-acting IFN-stimulated response element (ISRE), located upstream of most type I IFN-stimulated genes, ultimately driving the transcriptional upregulation of numerous antiviral inhibitors as well as a number of innate and adaptive immune mediators.

Susceptibility to neurotropic arbovirus infection has also been inversely correlated with host age (53-56). The pediatric population is frequently at increased risk for

developing permanent post-infectious neurological sequelae, such as cognitive deficits, paralysis, and seizure disorders (13). Preferential development of neurological sequelae in the less developed CNS may correlate with *in vivo* susceptibility of neural progenitor cells (NPCs) (137).

NPCs are self-renewing, multipotent precursors of astrocytes, oligodendrocytes, and neurons. Within the postnatal mammalian CNS, NPCs principally reside within two neurogenic regions: the subventricular zone (SVZ) adjacent to the lateral ventricle and the subgranular zone (SGZ) of the hippocampus. NPCs are both permissive to neurotropic viruses (133-136) and preferentially targeted during the course of an *in vivo* infection (139, 142). Specific to the neurotropic arboviruses, NPCs were recently demonstrated to be highly susceptible to *in vivo* infection with Japanese encephalitis virus (JEV), resulting in loss of the proliferative NPC pool from the SVZ of the early postnatal mouse brain (137). Additional evidence demonstrates that infection of NPCs can have negative and lasting consequences on neurogenesis potential within the postnatal CNS (135, 137, 139-141), emphasizing the need to more thoroughly understand mechanisms of NPC susceptibility to neurotropic arbovirus infection.

Due to the critical role of type I IFN in anti-arbovirus defense within the CNS, we chose to directly examine type I IFN pathway function, and resulting susceptibility to neurotropic arboviruses, in the context of human neuronal differentiation. Using human embryonic stem cells (hESCs) we were able to derive enriched populations of human NPCs and more mature human neurons, and here we demonstrate enhanced antiviral type I IFN pathway activity in mature neurons compared to NPCs. We additionally dissect the

molecular detail of this enhanced response, demonstrating that overexpression of IFNAR2 in immature human neuronal cells is sufficient for increased type I IFN-dependent inhibition of neurotropic arbovirus replication.

EXPERIMENTAL PROCEDURES

Reagents

Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) with the following exceptions: brain-derived neurotrophic factor (BDNF) from Prospec (Rehovot, Israel), bovine growth serum from HyClone (Logan, UT), laminin and poly-D-lysine from Sigma (St. Louis, MO), and noggin from R&D Systems (Minneapolis, MN). Recombinant human IFN α -A/D and rat IFN- α were purchased from PBL Biomedical Laboratories (Piscataway, NJ) and stored as single use aliquots at -80°C.

Antibodies used in the analysis of human cells were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) with exception of antibodies directed against: actin, GABA, GFAP, and NF200 (Sigma), NeuN, PSA-NCAM, and VGLUT2 (Millipore, Billerica, MA), IFNAR2 (PBL Biomedical Laboratories), IRF-7 (Cell Signaling Technology, Danvers, MA), IRF-9 (BD Transduction Laboratories, San Jose, CA), nestin (R&D Systems), MHC I (BioLegend, San Diego, CA), TH (Immunostar, Hudson, WI), and MxA (243). Antibody directed against Sox3 was generously donated by Dr. Michael Klymkowsky (University of Colorado at Boulder, Boulder, Colorado). Antibodies used in the analysis of rodent cells were purchased from Santa Cruz Biotechnology with

exception of the antibody directed against β -tubulin (Developmental Studies Hybridoma Bank, NICHD, University of Iowa).

Cell lines

The Be(2)-c human neuroblastoma cell line and the Vero African green monkey kidney cell line were obtained from the American Type Culture Collection (Manassas, VA). Be(2)-c and Vero cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in high glucose (4.5 g/L) formulation Dulbecco's modified Eagle's medium (DMEM) supplemented with 4mM L-glutamine, 5% bovine growth serum, penicillin at 10 units/mL, and streptomycin at 10 μ g/mL (complete DMEM).

Neuronal Differentiation

Be(2)-c cells were routinely differentiated over the course of 3 weeks in complete DMEM supplemented with 10 μ M all-*trans* retinoic acid as previously described (68).

Cultures of primary rat neurons were prepared from embryonic day 18 Sprague-Dawley rat cortices (BrainBits LLC, Springfield, IL) as previously described (95). In brief, cortices were digested with papain and plated as single cell suspensions at 1×10^5 cells/cm² on poly-D-lysine coated wells in NeurobasalTM media supplemented with 2% B-27 and 500 μ M L-glutamine. Media was changed daily. Undifferentiated cultures were analyzed at day 3 post-plating. Differentiated cultures were analyzed at day 14 post-plating, at which point the cultures demonstrated mature neuronal morphology, marker expression, and functional sensitivity to glutamate (95).

To model human neuronal development *in vitro*, H7 hESCs were obtained from the WiCell Research Institute (Madison, WI) at passage 25 and were cultured on feeder layers of irradiated mouse embryonic fibroblasts (GlobalStem, Rockville, MD) with daily changes of media consisting of DMEM/F12 supplemented with 20% knockout serum replacement, 1 mM glutamax, 0.1 mM non-essential amino acids, 0.1 mM β -mercaptoethanol, and 4 ng/mL human basic fibroblast growth factor (bFGF). To initiate NPC differentiation, H7 colonies were mechanically isolated from the feeder layer and grown in low attachment plates in NPC media (DMEM/F12, 2% B-27 (without vitamin A), 20 ng/mL bFGF) supplemented with 500 ng/mL noggin. After three weeks in suspension culture, neurospheres were clearly visualized and triturated by pipette to smaller cell aggregates, which were plated on poly-D-lysine (50 μ g/mL) and laminin (20 μ g/mL) coated dishes and allowed to expand as single cell cultures for one week in NPC media. To initiate neuronal differentiation, NPC cultures were grown for an additional two weeks in NeurobasalTM media supplemented with 1% N-2, 2% B-27, 0.1 mM non-essential amino acids, and 10 ng/mL human BDNF. All hESC protocols were approved by the University of Michigan Human Pluripotent Stem Cell Research Oversight (HPSCRO) Committee. hESC maintenance and differentiation was carried out at the A. Alfred Taubman Consortium for Stem Cell Therapies Laboratory at the University of Michigan, Ann Arbor.

Immunoblot Analysis

Cell supernatants were removed and cells were washed twice with phosphate-buffered saline (PBS) prior to lysis. Cells were lysed directly in tissue culture plates with

reducing SDS-PAGE sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 14.4 mM 2-mercaptoethanol, 0.02% bromophenol blue). Antigen detection was accomplished using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and enhanced chemiluminescence reagent solution containing 100 mM Tris (pH 8.5), 1.25 mM luminol, 0.2 mM *p*-coumaric acid, and 0.001% hydrogen peroxide. Digital chemiluminescent images were obtained using an Alpha Innotech Fluorchem 8900 (Cell Biosciences, Santa Clara, CA). Band intensities were quantitated using AlphaEaseFC software. Final images were prepared using Adobe Photoshop software.

Immunocytochemistry Analysis

Be(2)-c cells were plated on Lab-Tek II CC2 eight-well chamber slides (Nalge Nunc, Rochester, NY) prior to staining. hESC-derived neurons were stained directly in tissue culture plates. Cells were fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, blocked in 10% goat serum, and incubated overnight at 4°C in primary antibody, diluted in 1% goat serum. The following day, cells were washed and incubated with Texas Red- or FITC-conjugated secondary antibody (Jackson ImmunoResearch), diluted as above. Finally, cells were washed and incubated in 0.5 µg/mL 4,6-diamidino-2-phenylindole (DAPI) to visualize the nucleus. Slides were coverslipped using ProLong® Gold (Invitrogen) and stored in the dark for 24 hours prior to analysis. Plates were analyzed immediately. Analysis was carried out using an Olympus IX70 inverted microscope, and final images were assembled using MetaMorph Premier software.

Flow Cytometry Analysis

Cells were detached in 0.05% Trypsin-EDTA, filtered using a 70 μm nylon mesh, and labeled at approximately 10^5 cells. For intracellular staining, cells were fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, and all other steps were carried out in 1% bovine serum albumin (BSA) at 4°C. For extracellular staining, all steps were carried out at 4°C in PBS supplemented with 2% fetal bovine serum, 1% HEPES, and 1% sodium azide. Cells were incubated for at least 20 minutes with primary antibody. Rabbit isotype control was purchased from Jackson ImmunoResearch; mouse isotype control was purchased from Sigma. Cells were incubated as above with the appropriate FITC-conjugated secondary reagent (Jackson ImmunoResearch). For IFNAR2 and MHC I labeling, an additional amplification step was performed using a biotin-conjugated secondary reagent (Jackson ImmunoResearch) in conjunction with Alexa Fluor® 488 streptavidin (Invitrogen). For extracellular staining, live cells were identified using 7-amino-actinomycin D exclusion (Calbiochem). Cells were analyzed on a BD FACSCanto, and final histograms were assembled using FlowJo version 7.2.5.

Constructs and Overexpression

Overexpression plasmids encoding human IRF-9 and STAT2 were purchased from OriGene (Rockville, MD). An overexpression plasmid encoding human IFNAR2c with a C-terminal HA-tag (HA-IFNAR2) was generously donated by John Krolewski (University of California, Irvine, CA). IRF-9, STAT2, and HA-IFNAR2 cDNAs were subcloned into vector pTRE2hyg (BD Biosciences, San Jose, CA) to achieve

doxycycline-inducible expression. The pTet-On plasmid was purchased from BD Biosciences. The reporter plasmid encoding secreted alkaline phosphatase under the control of the human ISG-56K promoter (pISRE-SEAP) was purchased from InvivoGen (San Diego, CA). shRNA targeting human IRF-9 (NM_006084) in vector pGIPZ was obtained from the shRNA Core Facility (University of Michigan, Ann Arbor).

Cells were transfected using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Stable cell lines were generated by serial clonal selection. Conditional overexpression was achieved with 1 µg/mL doxycycline treatment for 36 hours.

Secreted Alkaline Phosphatase (SEAP) Analysis

Quanti-Blue substrate was prepared according to the manufacturer's instruction (InvivoGen). SEAP was analyzed in the tissue culture supernatant at 24 hours post-treatment with type I IFN. Triplicate cell supernatant samples of 20 µL were incubated with 200 µL of Quanti-Blue reagent in 96-well plates at 37°C for a minimum of 8 hours. Absorbance was measured at 620 nm.

Cell Viability Analysis

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cell viability. Cells were incubated with MTT at 0.5 mg/mL for 3 hours at 37°C. The reaction was stopped using 10% Triton X-100 and 0.1 N hydrochloric acid to lyse cells and solubilize formazan crystal. Following crystal solubilization, absorbance was measured in triplicate at 570 nm.

Viruses

Fort Morgan virus (FMV) strain CM4-146 was purchased from the American Type Culture Collection. Venezuelan equine encephalitis virus (VEEV) strain TC-83, a live attenuated vaccine strain derived from serial passage of the equine-virulent, epizootic Trinidad donkey strain (244), was generously donated by Robert Tesh (University of Texas Medical Branch, Galveston, TX). Infectious western equine encephalitis virus (WEEV) was generated from full-length WEEV cDNA clone pWE2000 as previously described (68). This clone derives from WEEV strain Cba 87, an epizootic strain from Argentina that is both neurovirulent and neuroinvasive in mice and cynomolgus macaques.

Virus Purification and Infection

Infection of hESC-derived neurons was accomplished using sucrose gradient-purified virions. Vero cells were infected at a low multiplicity of infection (MOI), harvested at 48 hours post-infection, centrifuged at 1,000 x g for 5 minutes to pellet cellular debris, and virions were precipitated from precleared tissue culture supernatant by addition of polyethylene glycol and sodium chloride to 7% and 2.3% final concentrations, respectively, and gentle stirring overnight at 4°C. Virions were recovered by centrifugation at 3500 x g for 20 minutes, resuspended in Hank's balanced salt solution (HBSS), loaded onto 15-45% linear sucrose step gradients, and centrifuged at 40,000 x g for 90 min. Visible virion bands were collected, diluted in HBSS, pelleted at 35,000 x g for 60 min, resuspended in HBSS, and stored at -80°C in single use aliquots.

Cells were infected at an MOI of 0.01. WEEV infections were conducted in the Biosafety Level 3 facility at the University of Michigan, Ann Arbor. All other infections were conducted in regular tissue culture facilities using the necessary Biosafety Level 2 precautions.

Plaque Analysis

Cell supernatants were collected at the indicated time post-infection and serially diluted in HBSS supplemented with 0.2% BSA. Viral dilutions were added to subconfluent Vero cell monolayers and allowed to attach for 90 minutes at 37°C. Post-viral attachment, cells were overlaid with complete DMEM containing 1.2% SeaPlaque agarose (Cambrex, Rockland, ME) heated to 50°C. The agarose overlay was allowed to solidify at room temperature for at least 15 minutes. Cells were then incubated at 37°C until plaques could be clearly visualized by light microscope (usually 36 to 48 hours). Cells were fixed and virus inactivated by addition of formaldehyde to 10% for 60 minutes. The agarose overlay was removed using a gentle stream of water, and monolayers were stained with 0.1% crystal violet in 20% methanol for 5 minutes.

Statistical Analysis

Unless otherwise specified, comparative statistical analyses were conducted using a two-tailed Student *t* test assuming equal variance where a p value of <0.05 was considered significant.

RESULTS

Enriched populations of human NPCs and mature human neurons can be derived from hESCs

Derivation of NPCs from hESCs, with subsequent derivation of mature neurons, astrocytes, and oligodendrocytes, has previously been described (130, 131, 245). However, obtaining pure populations of human NPCs and mature human neurons remains an ongoing area of research (132). Using the bone morphogenic protein (BMP) antagonist, noggin, hESCs were differentiated to enriched cultures of NPCs following an established technique (246). Immature cultures of hESC-derived cells were harvested at day 28 of differentiation, at which point the cells displayed morphological characteristics of NPCs with moderately-sized perikaryon and small, largely unbranched neurites (**Figure II-1A, bottom left panel**). Characterization by immunocytochemistry indicated uniform expression of NPC markers, the transcription factor Sox3 and the intermediate filament nestin (**Figure II-1B, left panel**). Characterization by flow cytometry (**Figure II-1C, grey lines**) further indicated that $99.7 \pm 0.2\%$ of cells were neural (PSA-NCAM-positive), with a predominance of NPCs ($74.7 \pm 4.9\%$ Sox3-positive), and a minority population of more mature neurons ($44.5 \pm 6.4\%$ NeuN-positive). Immature cultures of NPCs were differentiated over the course of two weeks by modifying previously published techniques (130, 247, 248), and mature cultures of hESC-derived cells were harvested at day 42 of differentiation, at which point the cells displayed morphological characteristics of mature neurons with small perikaryon and an extensive network of branched processes (**Figure II-1A, bottom right panel**). Characterization by

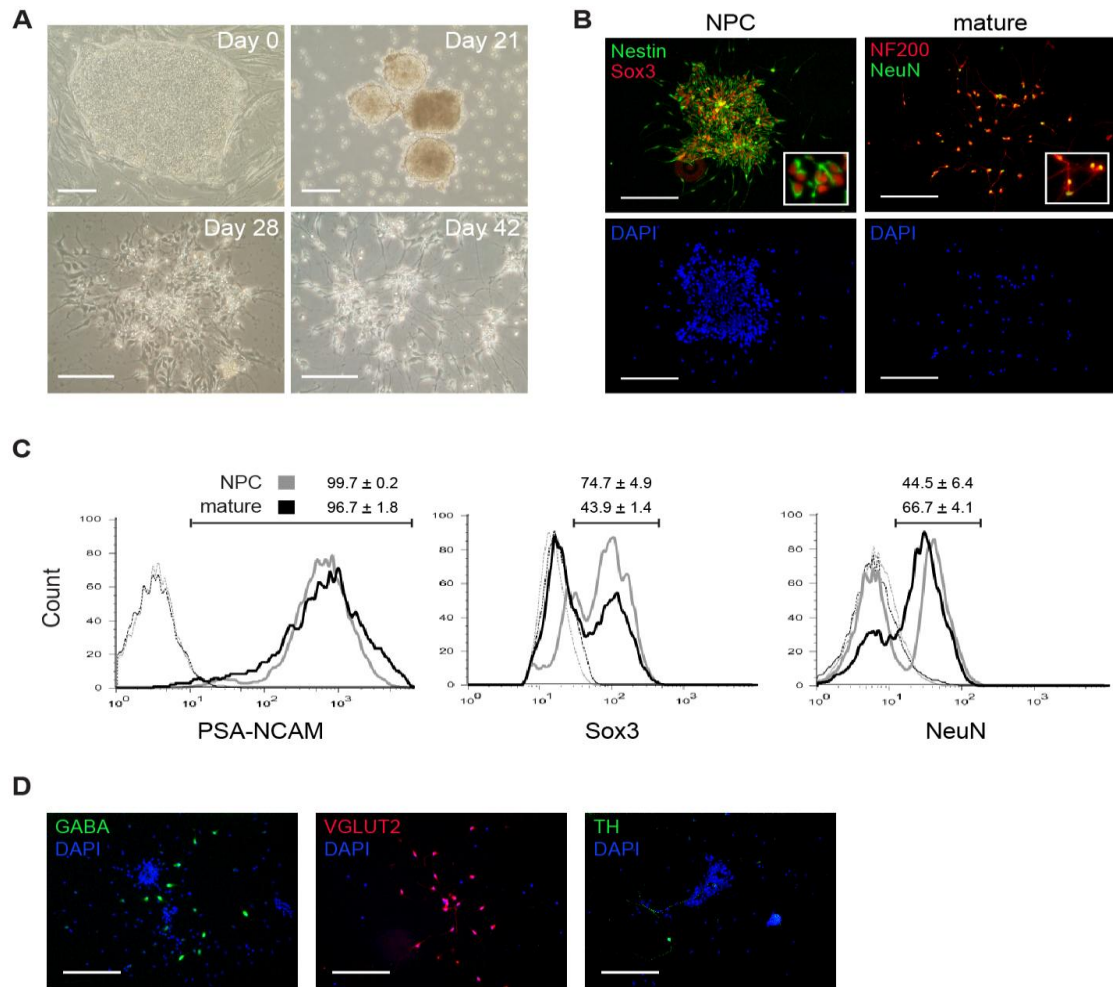


Figure II-1. Enriched populations of NPCs and mature neurons can be derived from hESCs

(A) Light micrographs depicting neuronal differentiation of hESCs: pluripotent hESC colony growing on a layer of irradiated mouse embryonic fibroblasts (top, left), neurospheres in suspension culture (top, right), adherent NPCs (bottom, left), adherent mature neurons (bottom, right); scale bar = 200 μ m. (B) Immunocytochemistry analysis of NPC and mature neuronal cultures. Expression of NPC markers (top, left) or mature neuronal markers (top, right) is shown as an overlay image; scale bar = 200 μ m; inlay = 4x magnification. DAPI staining identifies all cells in the field (bottom panel). (C) Flow cytometry analysis of NPC and mature neuronal cultures. Cell surface PSA-NCAM labeling was used to assess neural purity. Nuclear Sox3 and NeuN labeling was used to assess NPC and mature neuronal purity, respectively. Mean percentage positive cells relative to isotype control is shown with SEM (n=2). (D) Immunocytochemistry analysis of mature neuronal subtypes. Expression of the indicated GABAergic (GABA), glutamatergic (vGLUT2), and dopaminergic (TH) marker is shown as an overlay image with DAPI staining identifying all cells in the field; scale bar = 200 μ m.

immunocytochemistry indicated uniform expression of mature neuronal markers, the transcription factor NeuN and the heavy neurofilament NF200 (**Figure II-1B, right panel**). Characterization by flow cytometry (**Figure II-1C, black lines**) further indicated that $96.7 \pm 1.8\%$ of cells were neural (PSA-NCAM-positive), with a predominance of mature neurons ($66.7 \pm 4.1\%$ NeuN-positive), and a minority population of residual NPCs ($43.9 \pm 1.4\%$ Sox3-positive). Finally, mature neuronal cultures were analyzed by immunocytochemistry to characterize potential neuronal subtypes. This analysis demonstrated a predominance of GABAergic (GABA-positive) and glutamatergic (VGLUT2-positive) cells, with a minority presence of dopaminergic (TH-positive) cells (**Figure II-1D**), consistent with the production of mature forebrain neurons. Overall, these results demonstrate that highly enriched populations of NPCs and mature forebrain neurons can be reliably generated from hESCs for subsequent immunological and virological analyses.

Type I IFN pathway component expression and function is enhanced with differentiation of human NPCs to mature neurons

NPCs are susceptible to infection with neurotropic viruses (*133-136*), including infection with the neurotropic arbovirus, JEV (*137*). This heightened susceptibility may be due, in part, to maturation-dependent differences in cellular proliferation (*145*), surface moiety composition (*143*), or anti-apoptotic gene expression (*249, 250*). To determine if NPCs are more susceptible than mature neurons to infection with neurotropic viruses due to maturation-dependent differences in cell intrinsic innate immune function, we directly probed type I IFN pathway component expression and

function in hESC-derived NPCs and mature neurons. Immunoblot analysis demonstrated increased basal expression of IRF-9 in mature neurons compared to NPCs, with a fold increase of 3.09 ± 0.20 (**Figure II-2A**). In contrast, no significant maturation-dependent difference in basal expression of STAT1 or STAT2 was observed. Flow cytometry analysis additionally demonstrated increased surface expression of IFNAR2 in mature neurons compared to NPCs, with a fold increase of 1.56 ± 0.25 (**Figure II-2B**). These data indicate that multiple type I IFN signaling pathway components are upregulated with differentiation of NPCs to mature neurons, specifically IRF-9 and IFNAR2.

To test whether increased basal type I IFN pathway component expression in mature neurons confers enhanced pathway response, hESC-derived NPCs and mature neurons were stimulated with IFN α -A/D, a hybrid universal human type I IFN (251). At 24 hours post-treatment, whole cell lysates were harvested and analyzed by immunoblot for expression of STAT1, a known type I IFN-stimulated gene. While STAT1 expression was similar in unstimulated NPCs and mature neurons (**Figure II-2A**), STAT1 expression increased dramatically in mature neurons relative to NPCs in response to type I IFN treatment (**Figure II-2C**). To assess a downstream type I IFN-simulated antiviral response, hESC-derived NPCs and mature neurons were challenged with the neurotropic arbovirus, Venezuelan equine encephalitis virus (VEEV). In the absence of type I IFN priming, a 0.8 log reduction in VEEV titer was observed in mature neurons relative to NPCs (**Figure II-2D, left panel**), suggesting that type I IFN-independent innate immune function increases with differentiation of NPCs to mature neurons. However, in the presence of type I IFN priming, a 2.6 log reduction in VEEV titer was observed in mature

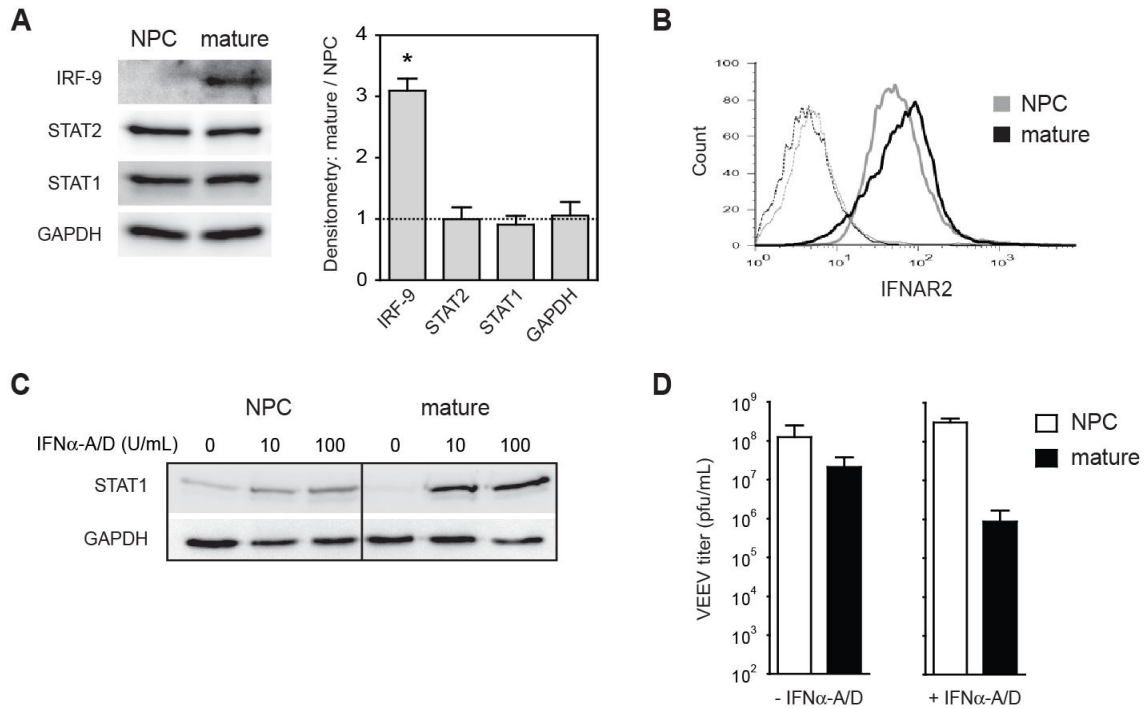


Figure II-2. Type I IFN pathway component expression and function is enhanced with differentiation of human NPCs to mature neurons

(A) Basal IRF-9 expression increases with differentiation. Immunoblot analysis of the designated type I IFN pathway component or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Quantitation of the immunoblot data is shown in the right panel. Results represent mean densitometry ratios of mature neurons over NPCs \pm SEM (n=3; IRF-9, n=2). *p<0.05, relative to GAPDH. (B) Basal surface IFNAR2 expression increases with differentiation; flow cytometry analysis. (C) IFN-stimulated STAT1 expression increases with differentiation. Immunoblot analysis at 24 hours post-treatment. (D) IFN-stimulated antiviral response increases with differentiation. Cells were pretreated or not for 24 hours with 50 U/mL IFN α -A/D. Cell supernatants were collected at 72 hours post-infection and analyzed for VEEV virions by plaque assay. Results are shown as means \pm SEM (-IFN α -A/D, n=2; +IFN α -A/D, n=3).

neurons relative to NPCs, a difference which now approached statistical significance ($p = 0.06$) (**Figure II-2D, right panel**). These data suggest that antiviral type I IFN pathway function increases with human neuronal differentiation, resulting in enhanced defense against a neurotropic arbovirus in mature human neurons compared to human NPCs.

Type I IFN pathway component expression and function is enhanced with differentiation of primary cortical rat neurons

In vivo analysis of NPC susceptibility to virus infection has been studied almost exclusively in the rodent model (133, 136, 137, 139, 141). To test whether neuronal type I IFN pathway function changes with maturation in rodents, we generated primary neuronal cultures from embryonic day 18 rat cortices. Cells were differentiated for 3 or 14 days *in vitro* to obtain populations of relatively immature (synaptophysin-negative) and mature (synaptophysin-positive) neuronal cultures as previously described (95). Analysis of basal type I IFN pathway component expression by immunoblot demonstrated increased expression of IRF-9 in day 14 compared to day 3 differentiated neurons, with a fold increase of 3.47 ± 0.48 (**Figure II-3A**). To probe type I IFN pathway function, primary neuronal cultures were stimulated with rat IFN- α . Immunoblot analysis at 24 and 48 hours post-treatment demonstrated a preferential upregulation of STAT1 in day 14 compared to day 3 differentiated neurons that reached statistical significance over three independent trials ($p = 0.039$) (**Figure II-3B**). Together these data demonstrate that type I IFN pathway activity is enhanced with maturation in primary rodent neurons.

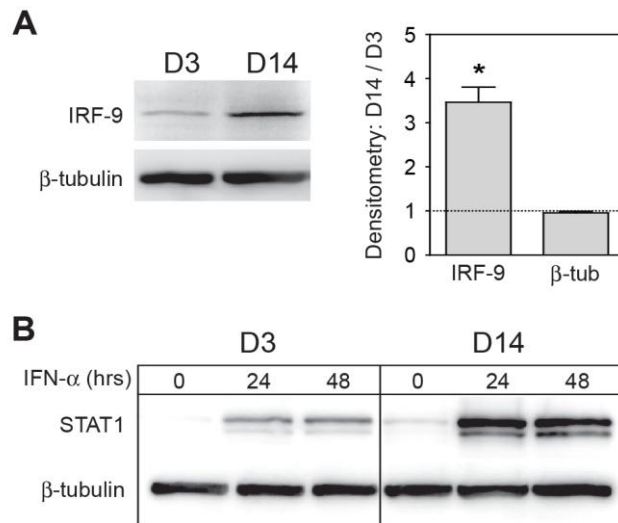


Figure II-3. Type I IFN pathway component expression and function is enhanced with differentiation of primary cortical rat neurons

(A) Basal IRF-9 expression increases with differentiation. Immunoblot analysis; β -tubulin was analyzed as a loading control. Quantitation of the immunoblot data is shown in the right panel. Results represent mean densitometry ratios of day 14 differentiated over day 3 differentiated neurons \pm SEM (n=3). *p<0.05, relative to β -tubulin. (B) IFN-stimulated STAT1 expression increases with neuronal differentiation. Immunoblot analysis post-treatment with 50 U/mL rat IFN- α .

Expression of type I IFN signaling pathway components increases with differentiation of human neuronal cells

In the previous sections we demonstrate enhanced type I IFN pathway activity in response to maturation of hESC-derived NPCs and primary cortical rat neurons. To dissect the underlying molecular mechanism(s), we asked whether this maturation-dependent innate immune difference could be recapitulated using a human neuronal cell line. Previously, our laboratory investigated maturation-dependent changes in innate neuronal antiviral immunity using the human neuroblastoma cell line, Be(2)-c. In response to Be(2)-c cell differentiation with retinoic acid, we observed enhanced competency of viral pattern recognition receptor pathways (252) and enhanced type I IFN-mediated inhibition of a neurotropic arbovirus (68). Thus we chose to directly probe type I IFN pathway function in this model system. Populations of undifferentiated (Be(2)-c) and differentiated (Be(2)-c/m) cells were analyzed by immunoblot for expression of the following type I IFN pathway components: IRF-9, STAT2, STAT1, Tyk2, and Jak1. This analysis demonstrated a significant increase in basal IRF-9 and STAT2 expression in response to neuronal differentiation, with fold increases of 4.05 ± 0.59 and 1.44 ± 0.11 , respectively (**Figure II-4A**). In contrast, no differentiation-dependent downregulation was observed. IRF-9 expression was further analyzed by immunocytochemistry, which localized IRF-9 predominately to the nucleus of both cell types and, consistent with the immunoblot data, demonstrated increased expression with neuronal differentiation (**Figure II-4B**).

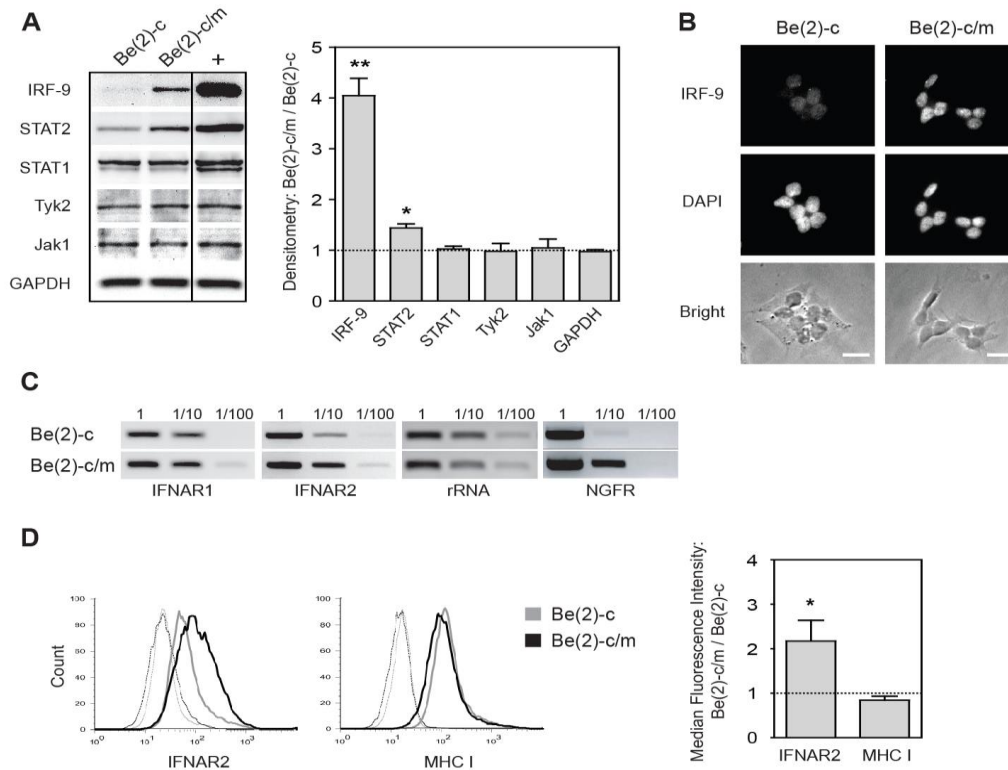


Figure II-4. Expression of type I IFN signaling pathway components increases with differentiation of human neuronal cells

Basal type I IFN pathway component expression was analyzed in undifferentiated (Be(2)-c) and differentiated (Be(2)-c/m) human neuroblastoma cells. (A) IRF-9 and STAT2 expression increase with neuronal differentiation. Immunoblot analysis of the designated type I IFN pathway component or GAPDH as a loading control. IFN α -A/D-treated, differentiated cell lysate (+) was used as a positive control for detection of the IFN-inducible components: IRF-9, STAT2, and STAT1. Quantitation of the immunoblot data is shown in the right panel. Results represent mean densitometry ratios of differentiated over undifferentiated cells \pm SEM (n=3). *p<0.05, **p<0.005, relative to GAPDH. (B) IRF-9 expression increases with neuronal differentiation; immunocytochemistry analysis. DAPI staining confirmed nuclear localization. Brightfield images depict distinct cellular morphologies; scale bar = 25 μ m. (C) IFNAR2 expression increase with neuronal differentiation. Semi-quantitative RT-PCR analysis, 10-fold serial dilutions of the cDNA are shown. Ribosomal RNA (rRNA) and was used as a loading control. Nerve growth factor receptor (NGFR) was used as a positive control for differentiation. (D) Surface IFNAR2 expression increases with neuronal differentiation; flow cytometry analysis. Surface MHC class I expression was analyzed as a technical control. Quantitation of the flow cytometry data is shown in the right panel. Results represent median fluorescence intensity ratios of differentiated over undifferentiated cells \pm SEM (n=5). *p<0.05, relative to MHC I.

We were unable to reliably detect basal IFNAR expression by immunoblot analysis (data not shown), but probed a transcriptional upregulation of IFNAR1 and 2 by semi-quantitative RT-PCR. Four transcript variants of IFNAR2 have been identified (156), which result in the production of three distinct protein isoforms, each with unique signaling competency (157-159) and occasionally, differential expression (185). To probe expression of mRNA transcripts encoding all three isoforms, two primer sets were designed for IFNAR2. Primer set 1 spanned the conserved extracellular domain, amplifying all transcript variants as a single molecular weight product. Primer set 2 spanned a differentially spliced region, amplifying each transcript variant as a unique molecular weight product (**Figure II-5A**). RT-PCR analysis with primer set 1 indicated a global upregulation of IFNAR2 in response to human neuronal differentiation, with a fold increase of 1.97 ± 0.33 (**Figure II-4C**). Analysis with primer set 2 further indicated a transcriptional upregulation of all three IFNAR2 isoforms, including the signaling competent transmembrane isoform, IFNAR2c (**Figure II-5B**). In contrast, we observed a modest increase in IFNAR1 that did not reach statistical significance ($p = 0.085$) (**Figure II-4C**). To determine whether a transcriptional upregulation of IFNAR2 could be functionally relevant, IFNAR2 expression was analyzed by flow cytometry. This analysis demonstrated a significant increase in IFNAR2 surface expression in response to human neuronal differentiation, with a fold increase of 2.17 ± 0.94 (**Figure II-4D**).

Together these data demonstrate an analogous phenotype to that observed with the hESC-derived neurons: multiple type I IFN signaling pathway components are upregulated in response to human neuronal differentiation. Specifically, upregulation of

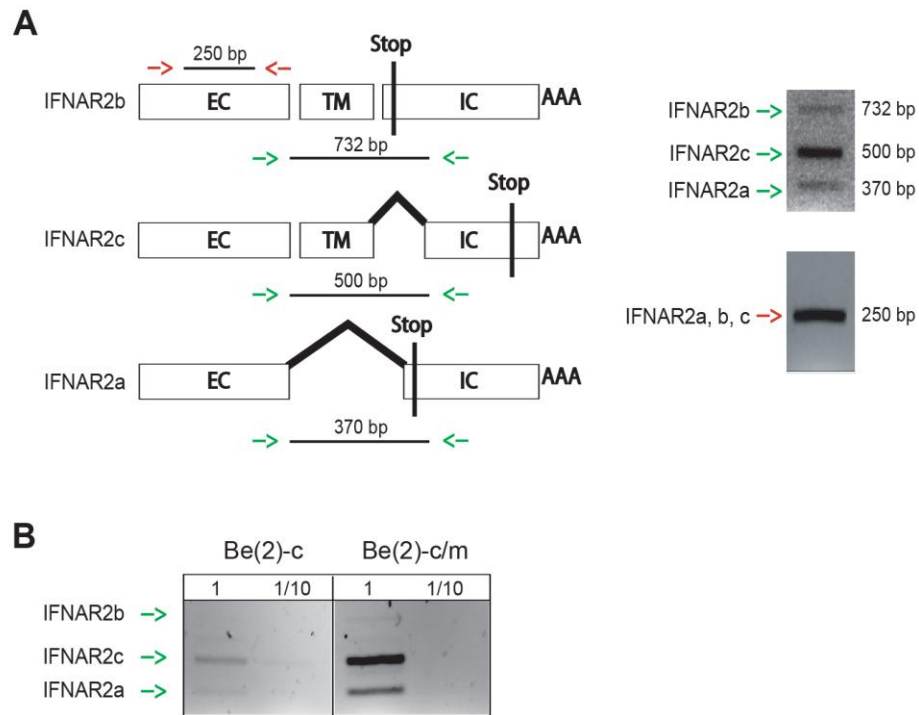


Figure II-5. Analysis of global and isoform-specific IFNAR2 transcript levels

(A) IFNAR2 primer set 1 (red) spans the conserved extracellular domain and amplifies all IFNAR2 transcripts as a single molecular weight product. IFNAR2 primer set 2 (green) spans a differentially spliced region and amplifies each isoform-specific transcript as a unique molecular weight product. (B) IFNAR2 isoforms are all transcriptionally upregulated with neuronal differentiation. Semi-quantitative RT-PCR analysis, 10-fold serial dilutions of the cDNA are shown.

IRF-9 and IFNAR2 were observed with maturation of NPCs as well as human neuroblastoma cells. In contrast, upregulation of STAT2 was limited to the human neuroblastoma cell model.

Activation of the type I IFN pathway increases with differentiation of human neuronal cells

In response to Be(2)-c cell differentiation, enhanced type I IFN-mediated inhibition of neurotropic arbovirus replication was previously described (68). Type I IFNs classically induce a cellular antiviral state through the sequential phosphorylation of STAT2 at tyrosine 690 and STAT1 at tyrosine 701. The phosphorylated STATs associate with IRF-9 in the ISGF3 transcription factor complex and drive the transcription of antiviral genes containing upstream ISREs. Although alternative signal transducers, transcription factor complexes, and promoter elements have been described ((209), and reviewed in (171, 253)), we explored differential activation of the canonical type I IFN signaling pathway. Upstream pathway activation was analyzed in the Be(2)-c cell model by monitoring STAT phosphorylation in response to treatment with IFN α -A/D. Immunoblot analysis at 30 minutes post-treatment revealed a significant increase in phosphorylated species of STAT1 (**Figure II-6A, top panel**) and STAT2 (**Figure II-6A, bottom panel**) in differentiated compared to undifferentiated cells.

Downstream pathway activation was analyzed in the Be(2)-c cell model by monitoring antiviral gene induction in response to treatment with IFN α -A/D. Our selection of antiviral genes included a direct antiviral effector (MxA), a component of the viral pattern recognition receptor pathway (IRF-7), a component of the type I IFN

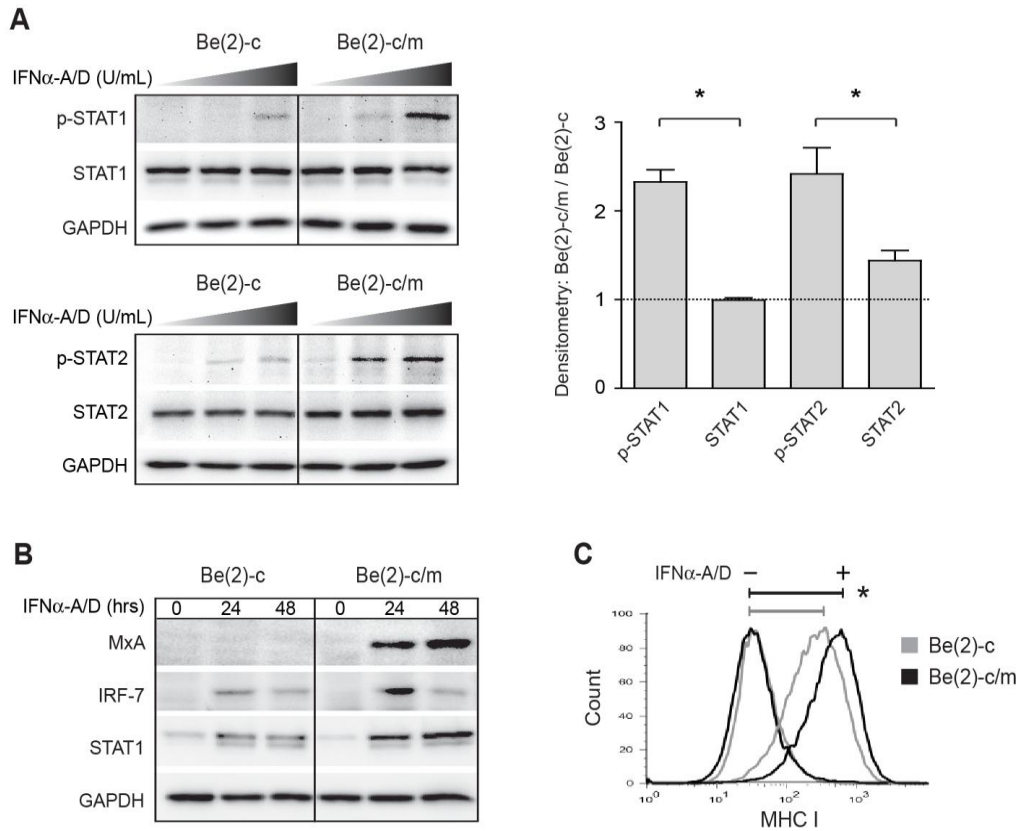


Figure II-6. Activation of the type I IFN pathway increases with differentiation of human neuronal cells

Type I IFN pathway activation was analyzed in undifferentiated (Be(2)-c) and differentiated (Be(2)-c/m) human neuroblastoma cells. (A) IFN-stimulated STAT phosphorylation increases with neuronal differentiation. Immunoblot analysis of Tyr701-phosphorylated STAT1 (p-STAT1), total STAT1, Tyr690-phosphorylated STAT2 (p-STAT2), and total STAT2. Cells were treated with 5, 50, or 500 U/mL IFN α -A/D and harvested at 30 minutes post-treatment. Quantitation of the immunoblot data at 500 U/mL IFN α -A/D treatment is shown in the right panel. Results represent mean densitometry ratios of differentiated over undifferentiated cells \pm SEM ($n \geq 3$). * $p < 0.05$, as indicated. (B) IFN-stimulated antiviral gene expression increases with differentiation. Immunoblot analysis of MxA, IRF-7, and STAT1. Cells were treated with 500 U/mL IFN α -A/D and harvested as indicated. GAPDH was analyzed as a loading control. (C) IFN-stimulated surface MHC I expression increases with neuronal differentiation. Flow cytometry analysis of cells treated or not with 50 U/mL IFN α -A/D for 48 hours. * $p < 0.05$, relative to the shift observed in the undifferentiated cells.

signaling pathway (STAT1), and an adaptive immune activator (MHC I). MxA induction was of particular interest due to the fact that MxA has described antiviral effector function against neurotropic arboviruses (232, 238) and a strict promoter requirement for ISGF3 (234, 235). Immunoblot analysis at 24 and 48 hours post-treatment with type I IFN revealed increased expression of MxA in differentiated compared to undifferentiated cells. A similar, but transient, preferential upregulation of IRF-7 was also observed. Finally, there was a subtle preferential upregulation of STAT1 in the differentiated cells; however, this relative increase did not reach statistical significance ($p = 0.22$) (**Figure II-6B**). Flow cytometry analysis at 48 hours post-treatment with type I IFN additionally demonstrated increased surface expression of MHC I in differentiated compared to undifferentiated cells (**Figure II-6C**).

Together these data demonstrate an analogous phenotype to that observed with the hESC-derived neurons: activation of the type I IFN pathway is enhanced with human neuronal differentiation. With differentiation, we observed increased activation of the canonical type I IFN signaling pathway at 30 minutes post-treatment that was sustained as increased antiviral gene expression at 24 and 48 hours post-treatment. In contrast, incremental differences in type I IFN response (change in IFN-stimulated gene expression over time) were not observed between undifferentiated and differentiated cells over a 72 hour time course (**Figure II-7**). These data suggest that differentiated neurons respond to type I IFN with greater magnitude but similar kinetics.

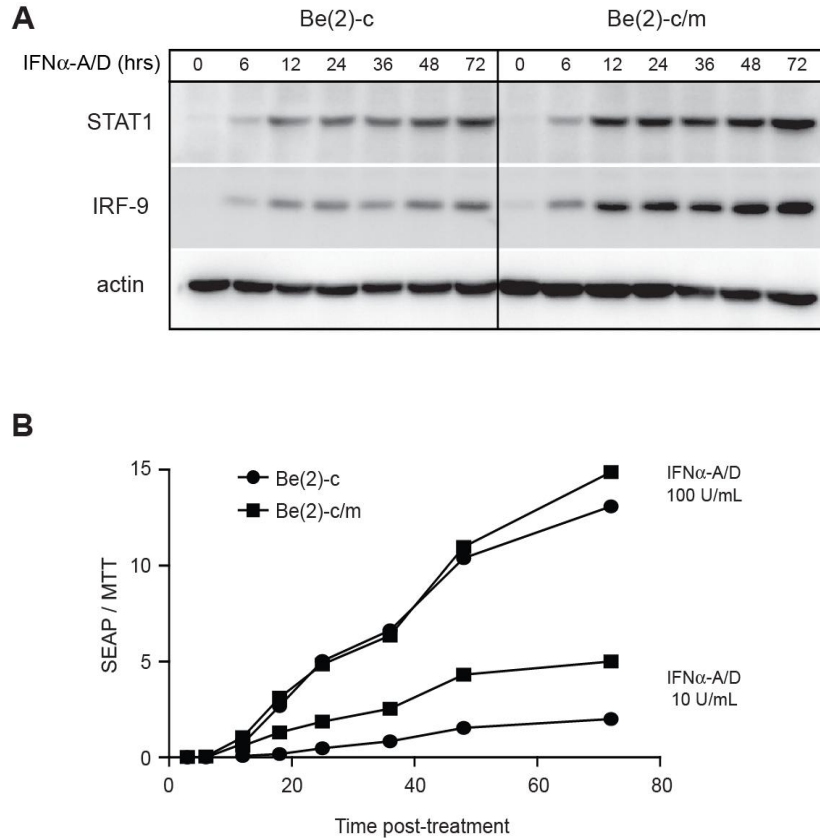


Figure II-7. Kinetics of type I IFN pathway activation are unaltered with differentiation of human neuronal cells

Type I IFN pathway activation was analyzed in undifferentiated (Be(2)-c) and differentiated (Be(2)-c/m) human neuroblastoma cells. (A) Kinetics of IFN-stimulated gene expression are unaltered with neuronal differentiation. Cells were treated with 50 U/mL of IFN α -A/D, lysates were harvested at the indicated time post-treatment, and IFN-stimulated STAT1 and IRF-9 levels were analyzed by immunoblot. Actin was analyzed as a loading control. (B) Kinetics of IFN-stimulated reporter gene expression are unaltered with neuronal differentiation. Response to type I IFN, as measured by secreted alkaline phosphatase (SEAP) production, was analyzed in a stable ISRE-SEAP human neuroblastoma reporter cell line. The y-axis represents ratio of type I IFN response (SEAP)/cell viability (MTT). Two independent trials are shown at type I IFN treatment doses that produced equal (100 U/mL IFN α -A/D) and unequal (10 U/mL IFN α -A/D) magnitude SEAP responses in Be(2)-c and Be(2)-c/m cells.

Differentiation-dependent changes in neuronal type I IFN pathway function can be recapitulated with overexpression of IFNAR2 and STAT2

To dissect the mechanism(s) whereby neuronal differentiation results in enhanced response to type I IFN, we probed the effects of IRF-9, IFNAR2, and/or STAT2 overexpression on IFN-stimulated gene expression in undifferentiated Be(2)-c cells. We analyzed global type I IFN-stimulated gene expression using a stable human neuroblastoma reporter cell line, in which production of secreted alkaline phosphatase (SEAP) was driven by an ISRE. A Tet-ON expression system was also stably introduced to achieve conditional overexpression of IRF-9, IFNAR2, and STAT2. With the stable Tet-ON system, we sought to more closely mimic levels of type I IFN pathway component expression observed in the differentiated cells.

Using an ISRE-SEAP human neuroblastoma reporter cell line, our laboratory previously demonstrated distinct type I IFN dose-response curves in differentiated compared to undifferentiated cells (68). In the newly generated Tet-ON/ISRE-SEAP cell line, neuronal differentiation was similarly associated with an approximate half log reduction in the EC_{50} value (75.2 ± 5.6 vs. 35.9 ± 4.8 U/mL) and a shift in the Hill slope value (2.4 ± 0.2 vs. 1.3 ± 0.1) in favor of mass action type I IFN signal transduction (**Figure II-8A, comparing solid symbols**). Overall, these results indicate increased efficiency in the cellular response to type I IFN with differentiation. To probe whether basal upregulation of IRF-9, IFNAR2, and STAT2 is sufficient to similarly shift the type I IFN dose-response curve, all three pathway components were overexpressed in undifferentiated Be(2)-c cells. While transfection of the empty vector control did not significantly impact response to type I IFN in the undifferentiated cells (**Figure II-8A,**

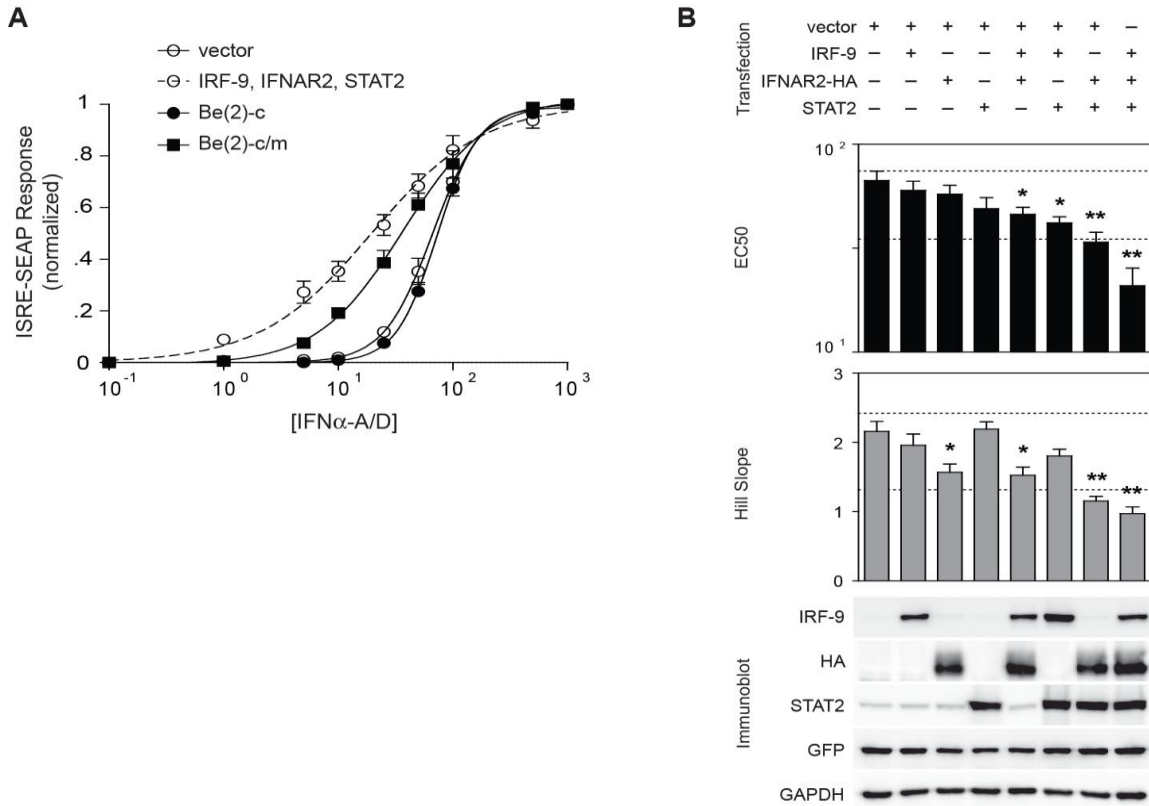


Figure II-8. Differentiation-dependent changes in neuronal type I IFN pathway function can be recapitulated with overexpression of IFNAR2 and STAT2

Global response to type I IFN was analyzed in a stable ISRE-SEAP human neuroblastoma reporter cell line. IRF-9, IFNAR2, and/or STAT2 were conditionally overexpressed using a stable Tet-ON system. (A) Type I IFN dose-response curves were comparatively analyzed in human neuroblastoma cells: undifferentiated (Be(2)-c), differentiated (Be(2)-c/m), and undifferentiated transfected with empty vector control (vector) or IRF-9, IFNAR2, and STAT2 (IRF-9, IFNAR2, STAT2). Normalized SEAP responses were fit using a variable slope nonlinear regression, which is shown with SEM (n=4). (C) Dose-response curve properties were comparatively analyzed in undifferentiated human neuroblastoma cells transfected with empty vector control or the indicated combination of type I IFN signaling pathway components. Data sets were independently fit as described in (A). Mean EC₅₀ and Hill slope values were calculated using GraphPad Prism 5 software and are shown with SEM (n=4). *p<0.05, **p<0.005, relative to empty vector control; dotted lines indicate the level of response observed in undifferentiated (upper) and differentiated (lower) human neuroblastoma cells. Immunoblot analysis confirming component overexpression is shown below; GFP and GAPDH were analyzed as transfection and loading controls, respectively.

comparing solid circle to open circle), overexpression of IRF-9, IFNAR2, and STAT2 was sufficient to recapitulate the shift in both EC₅₀ and Hill slope values observed with neuronal differentiation (**Figure II-8A, comparing open symbols**). To determine the minimal components necessary to enhance type I IFN responsiveness in the undifferentiated cells, we performed detailed overexpression studies of the individual pathway components and all combinations thereof. Overexpression of at least two pathway components was necessary to shift the EC₅₀ value (**Figure II-8B, upper panel**). In contrast, the Hill slope value was significantly shifted in response to overexpression of IFNAR2 alone or in combination with IRF-9 and/or STAT2 (**Figure II-8B, lower panel**). Furthermore, combined overexpression of IFNAR2 and STAT2 produced similar EC₅₀ ($p = 0.075$) and Hill slope ($p = 0.17$) value shifts as compared to the triple overexpression, indicating that basal upregulation of IFNAR2 and STAT2 in immature human neuronal cells is sufficient to recapitulate the mature neuronal response to type I IFN at the level of IFN-stimulated gene expression.

Overexpression of IFNAR2 in immature human neuronal cells is sufficient for increased inhibition of alphavirus replication

To pursue a downstream antiviral response, the impact of IRF-9, IFNAR2, and/or STAT2 overexpression on type I IFN-stimulated inhibition of neurotropic arbovirus replication was tested in undifferentiated Be(2)-c cells. Antiviral response to type I IFN was examined using three model alphaviruses that display various levels of virulence: the CM4-146 strain of Fort Morgan virus (FMV), an avirulent member of the WEE complex (254, 255), the Cba-87 strain of WEEV, an epizootic isolate that is highly

virulent in both rodents (254-256) and primates (257), and the TC-83 vaccine strain of VEEV, an attenuated and lower virulence derivative of the Trinidad donkey epizootic 1AB isolate that has increased sensitivity to type I IFN *in vivo* and *in vitro* (258). Type I IFN priming in Be(2)-c cells transfected with empty vector control reduced FMV, WEEV, and VEEV titers by 4.8, 1.3, and 2.1 logs, respectively (**Figure II-9A-C, comparing white and black vector control bars**), demonstrating varying levels of viral sensitivity to type I IFN. For FMV, this type I IFN-stimulated inhibition was enhanced by approximately two logs in Be(2)-c cells overexpressing IFNAR2 and all combinations thereof (**Figure II-9A**). For WEEV and VEEV, which were less sensitive to type I IFN priming, a similar phenotype was observed, albeit to a lower magnitude. Type I IFN-stimulated WEEV inhibition was enhanced by approximately one log in response to IFNAR2 overexpression in combination with IRF-9 and/or STAT2, but not overexpression of IFNAR2 alone (**Figure II-9B**). Type I IFN-stimulated VEEV inhibition was additionally enhanced by approximately one log in response to IFNAR2 overexpression in combination with IRF-9 or STAT2 (**Figure II-9C**). Although overexpression of either IFNAR2 alone or IFNAR2 in combination with IRF-9 and STAT2 trended towards accentuated VEEV inhibition, the differences only approached statistical significance ($p = 0.056$ and 0.075 , respectively). Finally, in the absence of type I IFN priming, pathway component overexpression did not stimulate an enhanced antiviral response in the Be(2)-c cells (**Figure II-9A-C, comparing white bars**), indicating that the effect of IFNAR2 overexpression on alphavirus inhibition was type I IFN-dependent. These data demonstrate that basal IFNAR2 upregulation in response to

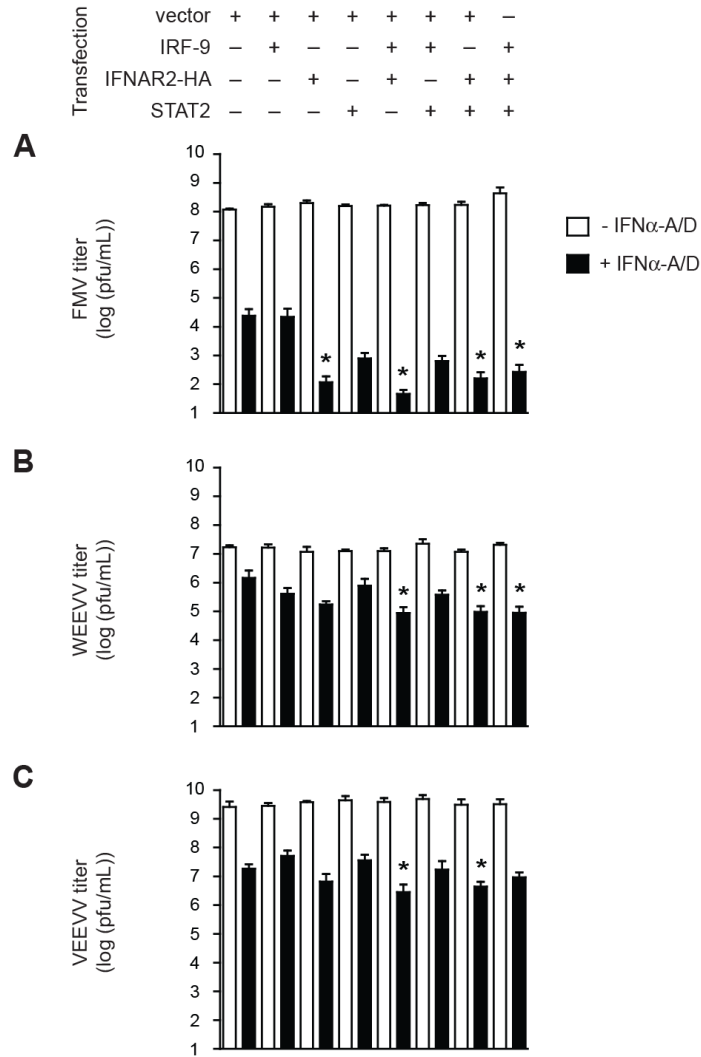


Figure II-9. Overexpression of IFNAR2 in immature human neuronal cells is sufficient for increased inhibition of alphavirus replication

Undifferentiated human neuroblastoma cells were transfected with empty vector control or the indicated combination of type I IFN pathway components. Cells were unprimed or primed with 500 U/mL IFN α -A/D for 24 hours prior to infection with FMV (A), WEEV (B), or VEEV (C). At 72 (A), 36 (B), or 24 (C) hours post-infection, virions present in the cell supernatant were analyzed by plaque assay. Results are shown as means \pm SEM ($n \geq 3$). * $p < 0.05$, relative to the effect of type I IFN priming in the empty vector control transfected cells (comparative statistical analyses were conducted using a two-tailed Student *t* test assuming unequal variance where a *p* value of < 0.05 was considered significant).

neuronal differentiation critically mediates enhanced innate immune response to neurotropic arboviruses. Together with the previous observation that basal IFNAR2 expression increases with differentiation of hESC-derived NPCs to mature neurons (**Figure II-2B**), these results suggest that mature human neurons are protected from neurotropic arbovirus pathogenesis due, in part, to increased surface IFNAR2 expression driving increased antiviral type I IFN pathway activity.

DISCUSSION

The present study identifies maturation-dependent differences in neuronal type I IFN pathway function as a novel mechanism to explain the heightened susceptibility of human NPC to neurotropic arbovirus infection. In accordance with previous research in the mouse model (259, 260), we demonstrate that human NPCs express the type I IFN receptor and are competent in type I IFN pathway signaling. Here we additionally demonstrate, however, that type I IFN pathway function changes in response to human neuronal differentiation such that antiviral pathway activity is less robust in human NPCs compared to mature neurons.

NPC susceptibility has been demonstrated for a number of neurotropic viruses, including CMV (133), HIV (134, 135), and CV3B (136). More recently, *in vivo* NPC infection with a neurotropic arbovirus was described (137), and here we additionally demonstrate that human NPCs are permissive to infection with an alphavirus *in vitro*. In the absence of type I IFN, there was an innate difference in VEEV titer in NPCs compared to mature neurons. As epidermal growth factor infusion to the right lateral

ventricle was recently shown to enhance neurotropic viral infection in the SVZ (145), it is likely that active NPC proliferation promotes viral replication. Additionally, increased neurotropic arbovirus production in immature compared to mature neurons has been previously described for JEV (62) as well as the alphaviruses (61, 63, 65). Therefore, higher NPC titer in the absence of type I IFN is not unexpected. In the presence of type I IFN, however, the maturation-dependent difference in VEEV titer became more pronounced, due to preferential IFN-stimulated viral inhibition in mature neurons. In light of these data, the heightened susceptibility of NPCs to neurotropic arbovirus infection is likely multifactorial due, in part, to maturation-dependent changes in innate neuronal type I IFN pathway function.

It may seem evolutionarily unsound for the CNS's multipotent progenitor pool to have decreased innate antiviral function. However, type I IFNs are known to induce the upregulation of multiple functional gene classes outside of the antiviral inhibitors, including inducers of apoptosis. Moreover, treatment of murine neuronal precursors with IFN- β has been shown to decrease the proliferation and maturation potential of these cells (260), suggesting that decreased type I IFN pathway function in NPCs may be evolutionarily favorable. Additionally, decreased proliferation of NPCs in response to treatment with IFN- γ (261) and TNF- α (262) has been described. In light of the present data, these findings could suggest maturation-dependent changes in a number of neuronal cytokine response pathways. Finally, with maturation, neurons may develop enhanced mechanisms of tailoring type I IFN pathway activation. Our laboratory has shown that human neuronal cell differentiation results not only in the upregulation of type I IFN

signaling pathway components, but suppressors of the type I IFN pathway as well (252). In the present study we found that overexpression of IRF-9, IFNAR2 and STAT2 in undifferentiated human neuroblastoma cells enhanced type I IFN-stimulated gene expression beyond the level observed in the differentiated cells (**Figure II-8A, comparing solid square to open circle dotted line**). These data could be explained by excessive overexpression of type I IFN pathway components or alternatively, insufficient native expression of type I IFN pathway suppressors in the undifferentiated cells. Therefore, with maturation neurons may develop the capacity to modify intracellular type I IFN pathway responses.

In the present study we demonstrate upregulation of IFNAR2 in response to differentiation of NPCs to mature neurons. While maturation-dependent post-translational upregulation of the type I IFN receptor has previously been described (263), our results suggest a transcriptional upregulation of IFNAR2 in response to human neuronal differentiation. Additionally, we demonstrate that IFNAR2 overexpression in immature human neuronal cells can functionally enhance neuronal type I IFN-dependent gene expression and viral inhibition. These data are consistent with a recent report in primary cardiac cells, demonstrating that cell type-specific differences in basal surface IFNAR expression correlate with magnitude of type I IFN-dependent antiviral response (264). In contrast, while a dramatic upregulation of IRF-9 was observed with differentiation of NPCs, overexpression of IRF-9 in immature human neuronal cells did not significantly enhance type I IFN-dependent gene expression or viral inhibition. These data are consistent with at least two molecular mechanisms. First, IRF-9 is not a

limiting pathway component in immature neuronal cells. This mechanism is supported by the subcellular localization pattern of IRF-9. In resting cells, IRF-9 is found in a binary complex with STAT2 (212), which results in the relocalization of IRF-9 to the cell cytoplasm on account of the dominance of the STAT2 nuclear export signal (183). Thus baseline nuclear localization of IRF-9 in the immature cells suggests a high IRF-9/STAT2 ratio. Second, type I IFN pathway activity in the human neuroblastoma cell line could be predominantly IRF-9-independent. This molecular mechanism is less likely. While type I IFN-stimulated signal transduction independent of IRF-9 has been described (reviewed in (253)), type I IFN-stimulated antiviral activity in particular correlates strongly with ISGF3 formation (196). Additionally, shRNA-mediated knockdown of IRF-9 in Be(2)-c and Be(2)-c/m cells suppressed IFN-stimulated gene expression (**Figure II-10**), suggesting that IRF-9 is an important transducer of type I IFN signal in the human neuroblastoma cell line. Finally, we observed a maturation-dependent upregulation of STAT2 that was limited to the human neuroblastoma cell model. Overexpression of STAT2 in immature neuronal cells functionally enhanced neuronal type I IFN-dependent gene expression, yet did not significantly affect type I IFN-dependent inhibition of alphavirus replication. Based on these data, we propose that increased basal IFNAR2 expression in mature human neurons compared to human NPCs results in enhanced antiviral type I IFN pathway activity and decreased susceptibility to infection with a neurotropic arbovirus.

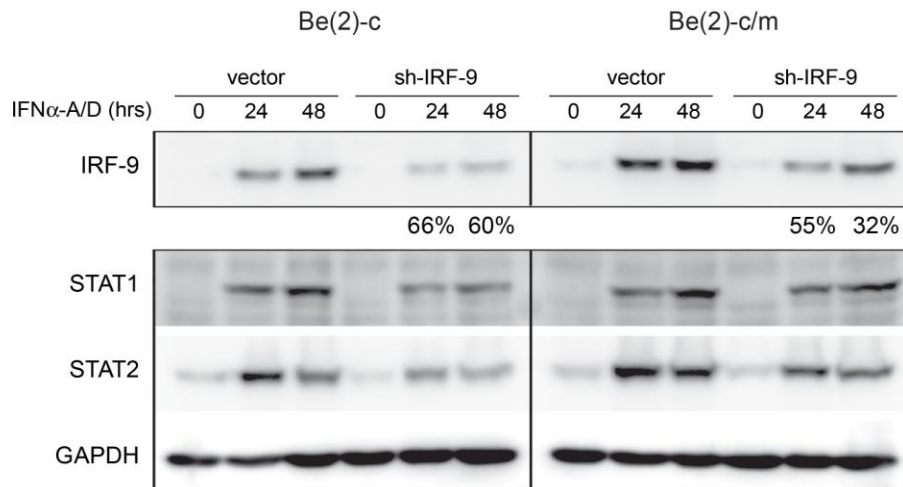


Figure II-10. Stable shRNA-mediated knockdown of IRF-9 decreases type I IFN pathway activity in human neuronal cells

Plasmid encoding shRNA targeting IRF-9 (sh-IRF-9) or empty vector control plasmid (vector) was transfected into undifferentiated human neuroblastoma cells (Be(2)-c), and stable cell lines were generated through clonal selection. Cells were subsequently differentiated (Be(2)-c/m), treated with 500 U/mL IFN α -A/D, and examined for IRF-9 knockdown (top panel, IRF-9) and resulting effects on type I IFN-stimulated gene expression (bottom panel, STAT1 and STAT2) by immunoblot. GAPDH was analyzed as a loading control. Percent IRF-9 knockdown in comparison to empty vector control is shown for the indicated times post-treatment. Several cell lines were tested and representative blots from cell line shRNA 1.1 are shown.

CHAPTER III:

MXA IS A FUNCTIONAL ANTIVIRAL EFFECTOR IN HUMAN NEURONAL CELLS WITH PREFERENTIAL ACTIVITY AGAINST BUNYAVIRUSES COMPARED TO NEW WORLD ALPHAVIRUSES²

Neurotropic arboviruses are significant pathogens of human central nervous system (CNS) neurons and include the bunyaviruses, flaviviruses, and alphaviruses. Type I interferons (IFNs) are critical to host defense against neurotropic arboviruses. Furthermore, the type I IFN-induced antiviral effector, MxA, has been elucidated as a potent inhibitor of bunyavirus replication. However, MxA inhibition has yet to be characterized for the full range of neurotropic arboviruses, specifically the New World alphaviruses. Additionally, MxA function has yet to be characterized in the *in vivo* target cell of the virus, the human neuron. We investigated the effects of MxA overexpression on bunyavirus and New World alphavirus replication in human neuronal cells. Additionally, we investigated antiviral function of the related GTPase, MxB. Here we demonstrate that MxA overexpression is sufficient to inhibit the replication of neurotropic arboviruses in human neuronal cells. Additionally, we demonstrate that

² The data from this chapter are planned for publication as “Farmer, J.R.*, Brownlee, J.W.*, Altschaefl, K.M., Peng, W., and Miller, D.J. MxA is a functional antiviral effector in human neuronal cells with preferential activity against bunyaviruses compared to New World alphaviruses.” (*co-first authors)

MxA preferentially inhibits the replication of bunyaviruses compared to New World alphaviruses. This type of differential MxA-mediated inhibition may underlie differences in innate control of virus replication within the CNS.

INTRODUCTION

Arthropod-borne viruses (arboviruses) are leading causative agents of viral encephalitis worldwide (4). These pathogens are maintained in nature in enzootic life-cycles and transmitted to humans by blood-feeding arthropods. A subset of arboviruses can cause severe pathogenesis within the human CNS by specifically infecting neurons. These neurotropic arboviruses have single-stranded RNA genomes and include the families *Bunyaviridae*, *Flaviviridae*, and *Togaviridae* (genus Alphavirus). While neurotropic arboviruses continue to be a serious public health concern due to their pathogenesis in humans and potential for widespread dissemination in the arthropod vector (265), we currently lack effective antiviral therapeutics (1). Thus a greater understanding of mechanisms of arbovirus inhibition, particularly within human neurons, is required.

Transgenic mouse models demonstrate that type I IFN-mediated responses are essential to surviving an acute neurotropic arbovirus infection (39-44). Type I IFNs are innate immune cytokines that are produced upon detection of conserved viral moieties, such as dsRNA intermediates intrinsic to the life-cycle of a single-stranded RNA virus. Type I IFNs induce a cellular antiviral state through activation of intracellular signal transduction cascades that drive the upregulation of antiviral inhibitors. Following

infection with a neurotropic arbovirus, type I IFNs can be detected in the serum (33) and the CNS (85). Within the CNS, neuronal response is further evidenced by upregulation of type I IFN-stimulated genes, including the Mx GTPase family of antiviral effectors (97, 100).

The majority of Mx proteins are innate, cell-autonomous inhibitors of virus replication. Mx1 is the founding family member, identified in an inbred mouse strain that demonstrated innate resistance to challenge with influenza A (223). Subsequent to this work, Mx proteins have been identified across multiple species (reviewed in (225)). In humans, two Mx genes have been described, *MxA* and *MxB* (228). These genes localize to human chromosome 21 and encode for proteins of 76 (MxA) and 73 (MxB) kDa. Both MxA and MxB were originally localized to the cytoplasm (227, 228). More recently, MxA has been localized to COP-I-positive subdomains of the smooth ER-Golgi-intermediate compartment (230), while MxB has been localized to the cytoplasmic-face of the nuclear envelope (231). These subcellular localization patterns have been associated with distinct protein functions.

MxB has not been shown to function as an antiviral inhibitor, rather MxB activity has been associated with cell-cycle maintenance and regulation of nuclear import (231). In contrast, MxA has been widely described as an antiviral inhibitor (reviewed in (222)). Specific to the neurotropic arboviruses, MxA has been shown to inhibit the replication of bunyaviruses (e.g. La Crosse virus (LACV)) and Old World alphaviruses (e.g. Semliki Forest virus (SFV)). However, the degree of antiviral protection conferred by MxA overexpression varies with virus species and host cell type. For example, MxA

overexpression is sufficient to inhibit replication of LACV across multiple model systems, including vertebrate cells (236), mosquito cells (237), and transgenic mouse models (232). In contrast, more selective MxA-mediated inhibition has been described for the Old World alphaviruses. For example, MxA overexpression is sufficient to inhibit the replication of SFV in human HEp-2 and U937 cells (238), but not murine 3T3 cells (229). Furthermore, MxA overexpression in mosquito cells does not confer resistance to the related Old World alphavirus, Sindbis virus (SINV) (237). However, in transgenic mice overexpressing MxA, SFV replication is inhibited, albeit to a lesser extent than LACV replication (232). Finally, MxA inhibition of New World alphaviruses, including Venezuelan equine encephalitis (VEEV) and the endemic United States pathogen, western equine encephalitis virus (WEEV), has yet to be described. Therefore, more detailed analyses, specifically within the Alphavirus genus, are required to fully elucidate the antiviral competency of MxA in the context of neurotropic arbovirus infection.

Although MxA has been shown to inhibit the replication of neurotropic arboviruses, its function has not been characterized in the *in vivo* target cell of the virus, the human neuron. This is an important research direction due to the fact that cell type-specific mechanisms of MxA inhibition have been described. For example, MxA was shown to attenuate measles virus transcription in human glioblastoma cells (233), while inhibition of measles virus glycoprotein synthesis, independent of a transcriptional attenuation, was described in human monocytes (266). These results are consistent with the diverse mechanisms of action that have been reported for MxA including inhibition of primary transcription (267), secondary transcription (268), genome amplification (238),

and accumulation of viral structural proteins (266). Therefore, a detailed analysis of MxA activity within human neurons is required in order to fully elucidate the inhibitory capacity of MxA against neurotropic arboviruses.

In the present study, we directly examined the antiviral function of MxA in human neuronal cells using four different neurotropic arboviruses. We selected known human pathogens including two California serogroup bunyaviruses, LACV and California encephalitis virus (CEV), and two New World alphaviruses, VEEV and WEEV. In addition, we analyzed the antiviral function of the related GTPase, MxB. For the first time we show evidence of neurotropic arbovirus inhibition by MxA in human neuronal cells. Additionally, we demonstrate differential inhibition of the bunyaviruses compared to the New World alphaviruses. Finally, we extend our findings to dissect the mechanism of differential MxA-mediated inhibition.

EXPERIMENTAL PROCEDURES

Reagents

Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) with the exception of bovine growth serum (HyClone, Logan, UT). Recombinant human IFN α -A/D was purchased from PBL Biomedical Laboratories (Piscataway, NJ) and stored as single use aliquots at -80°C.

Antibodies were purchased as follows: anti-VEEV nucleocapsid, anti-VEEV glycoprotein, and anti-CEV from the American Type Culture Collection (Manassas, VA),

anti-GAPDH and anti-HA from Santa Cruz Biotechnology (Santa Cruz, CA), anti-v5-epitope from GeneTex (Irvine, CA), and anti-MxA from Dr. George Kochs (243).

Cell lines

The Be(2)-c human neuroblastoma cell line and the Vero African green monkey kidney cell line were obtained from the American Type Culture Collection. Be(2)-c and Vero cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in high glucose (4.5 g/L) formulation Dulbecco's modified Eagle's medium (DMEM) supplemented with 4mM L-glutamine, 5% bovine growth serum, penicillin at 10 units/mL, and streptomycin at 10 µg/mL (complete DMEM).

Viruses

California encephalitis virus (CEV) strain BFS-283 and Fort Morgan virus (FMV) strain CM4-146 were purchased from the American Type Culture Collection. CEV BFS-283 was first isolated from *Aedes dorsalis* in 1952 and subsequently shown to be neuroinvasive and neurovirulent in mice (269). La Crosse virus (LACV) strain 1960/human and Venezuelan equine encephalitis virus (VEEV) strain TC-83 were generously donated by Robert Tesh (University of Texas Medical Branch, Galveston, TX). VEEV TC-83 is a live attenuated vaccine strain derived from serial passage of the equine-virulent, epizootic Trinidad donkey strain (244). LACV 1960/human is a human-virulent strain, isolated from post-mortem brain tissue and passaged in C6/36 cells (270). Infectious western equine encephalitis virus (WEEV) was generated from full-length WEEV cDNA clone pWE2000 as previously described (68). This clone derives from

WEEV strain Cba 87, an epizootic strain from Argentina that is both neurovirulent and neuroinvasive in mice and cynomolgus macaques. All WEEV infections were carried out in the Biosafety Level 3 facility at the University of Michigan, Ann Arbor. All other infections were conducted in regular tissue culture facilities using the necessary Biosafety Level 2 precautions.

Constructs and Overexpression

MxA was amplified from IFN-treated Be(2)-c cells using high fidelity Taq polymerase (Invitrogen). MxA was cloned into vector pUNO1 using AgeI and NheI restriction sites. Two MxA clones were fully sequenced in vector pUNO1 and no amino acid changes were noted in alignment with the reported NCBI sequence (NM_001144925). C-terminally v5-epitope and histidine tagged MxB in vector pcDNA3.1/GS was generously donated from Dr. Megan King (231). Constructs were transiently transfected into Be(2)-c cells using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Transfection efficiency was determined to be greater than 75% by *in situ* β -gal staining.

Immunoblot Analysis

Cell supernatants were removed and cells were washed twice with phosphate-buffered saline (PBS) prior to lysis. Cells were lysed directly in tissue culture plates with reducing SDS-PAGE sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 14.4 mM 2-mercaptoethanol, 0.02% bromophenol blue). Antigen detection was accomplished using peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West

Grove, PA) and enhanced chemiluminescence reagent solution containing 100 mM Tris (pH 8.5), 1.25 mM luminol, 0.2 mM *p*-coumaric acid, and 0.001% hydrogen peroxide. Digital chemiluminescent images were obtained using an Alpha Innotech Fluorchem 8900 (Cell Biosciences, Santa Clara, CA). Band intensities were quantitated using AlphaEaseFC software. Final images were prepared using Adobe Photoshop software.

Immunocytochemistry Analysis

Cells were fixed directly in tissue culture plates in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, blocked in 10% goat serum, and incubated overnight at 4°C in primary antibody, diluted in 1% goat serum. The following day, cells were washed and incubated with Texas Red- or FITC-conjugated secondary antibody (Jackson ImmunoResearch), diluted as above. Finally, cells were washed and incubated in 0.5 µg/mL 4,6-diamidino-2-phenylindole (DAPI) to visualize the nucleus. Analysis was carried out using an Olympus IX70 inverted microscope, and final images were assembled using MetaMorph Premier software.

Cell Viability Analysis

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cell viability in response to virus infection. Mock and virus infected cells were incubated with MTT at 0.5 mg/mL for 3 hours at 37°C. The reaction was stopped using 10% Triton X-100 and 0.1 N hydrochloric acid to lyse cells, inactivate virus, and solubilize formazan crystal. Following crystal solubilization, absorbance was

measured in triplicate at 570 nm. Cell viability was calculated as percent A_{570} in infected/uninfected cells.

Plaque Analysis

Cell supernatants were collected at the indicated time post-infection and serially diluted in Hank's Balanced Salt Solution (HBSS) supplemented with 0.2% bovine serum albumin. Viral dilutions were added to subconfluent Vero cell monolayers and allowed to attach for 90 minutes at 37°C. Post-viral attachment, cells were overlaid with complete DMEM containing 1.2% SeaPlaque agarose (Cambrex, Rockland, ME) heated to 50°C. The agarose overlay was allowed to solidify at room temperature for at least 15 minutes. Cells were then incubated at 37°C until plaques could be clearly visualized by light microscope (usually 36 to 48 hours). Cells were fixed and virus inactivated by addition of formaldehyde to 10% for 60 minutes. The agarose overlay was removed using a gentle stream of water and monolayers were stained with 0.1% crystal violet in 20% methanol for 5 minutes.

Statistical Analysis

Comparative statistical analyses were conducted using a two-tailed Student *t* test assuming unequal variance where a *p* value of <0.05 was considered significant.

RESULTS

Mx expression and localization in human neuronal cells

Mx proteins, like the dynamin family of high molecular weight GTPases, have been shown to self-assemble into multiprotein complexes and associate with intracellular membranes. Recent evidence demonstrates that MxA oligomerization is essential to both membrane-association and antiviral function (220). Accordingly, we chose to characterize the expression as well as the subcellular localization of MxA and MxB in the human neuroblastoma cell line, Be(2)-c. In Be(2)-c cells, we previously demonstrated type I IFN-induced antiviral immunity against a neurotropic arbovirus (68). Additionally, we demonstrated that MxA expression in Be(2)-c cells is undetectable by immunoblot in the absence of type I IFN, but robustly induced at 24 and 48 hours post-treatment (**Figure II-6B**). Thus the inhibitory effects of MxA overexpression can be analyzed in the Be(2)-c cell model with limited confounding effects from basal MxA expression. Finally, we demonstrated that Be(2)-c cells do not produce levels of IFN- α and - β detectable by ELISA following infection with WEEV (68), which indicates that the isolated antiviral effector function of MxA can be analyzed in the Be(2)-c cell model in the absence of confounding effects from other type I IFN-stimulated inhibitors.

MxA and MxB proteins could be detected in Be(2)-c cells by 24 hours post-transfection and expression was sustained at 48 hours post-transfection (**Figure III-1A**). MxA was detected as a band of approximately 76 kDa, consistent with previous reports. The MxA antibody described by Flohr et al. (243) additionally demonstrated some cross-

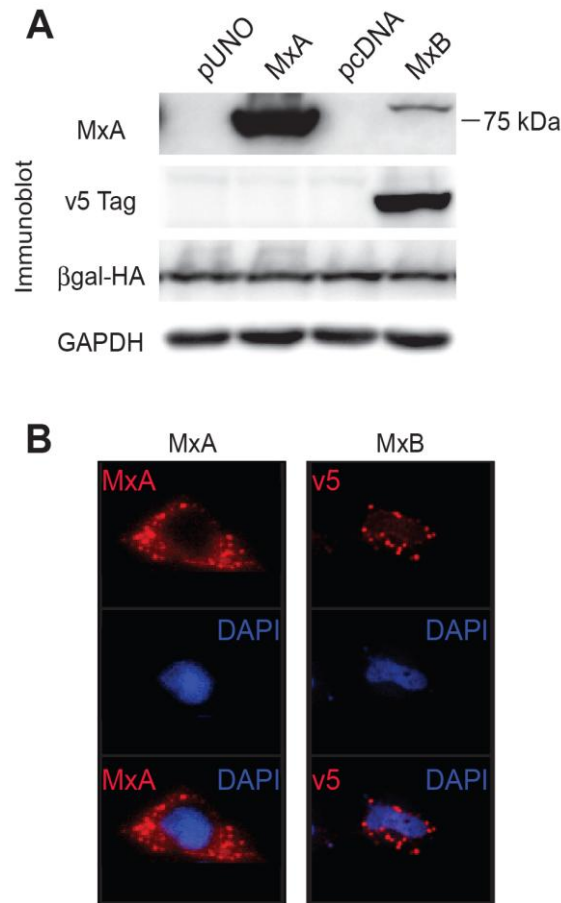


Figure III-1. Mx expression and localization in human neuronal cells

pUNO-MxA, pcDNA-v5-MxB, and empty vector controls were transfected into human neuroblastoma cells. (A) Lysates were harvested at 48 hours post-transfection, and Mx expression was analyzed by immunoblot using the indicated antibodies. HA tagged β -gal was co-transfected as a control. GAPDH was analyzed as a loading control. (B) Mx proteins were localized in human neuroblastoma cells by immunocytochemistry, probing for MxA or the v5-epitope tag as indicated. DAPI staining localized the nucleus.

reactivity with MxB, which migrated slightly higher than the reported 73 kDa, likely due to the C-terminal v5-epitope and histidine tags. MxB was also robustly detected using an anti-v5-epitope antibody.

Analysis of Mx localization by immunocytochemistry revealed punctate expression of MxA and MxB in Be(2)-c cells. MxA puncta were diffusely localized in the cell cytoplasm (**Figure III-1B, left panel**), consistent with the previous report of smooth ER-Golgi-intermediate compartment localization in Vero and Huh-7 cells (230). In contrast, MxB was observed in ring-like puncta around the nucleus (**Figure III-1B, right panel**), similar to the previous report of MxB localization to the cytoplasmic-face of the nuclear envelope in HeLa cells (231).

MxA is antivirally effective against bunyaviruses in human neuronal cells

MxA has been extensively described as an inhibitor of LACV replication in vertebrate and invertebrate cells (236, 237). Additionally, MxA overexpression was sufficient to inhibit LACV replication and pathogenesis in inbred mice naturally deficient in Mx production and transgenically deficient in response to type I IFN (232). However, LACV was introduced peripherally in the mice via intraperitoneal inoculation and LACV titers were subsequently not detected in the mouse brain six days post-inoculation, suggesting that MxA inhibited CNS invasion. The anti-LACV function of MxA has therefore not been investigated within the CNS, specifically within human neurons. We investigated MxA inhibition of LACV and the related California serogroup bunyavirus, CEV, in human neuronal cells. Additionally, we analyzed the inhibitory function of the related GTPase, MxB.

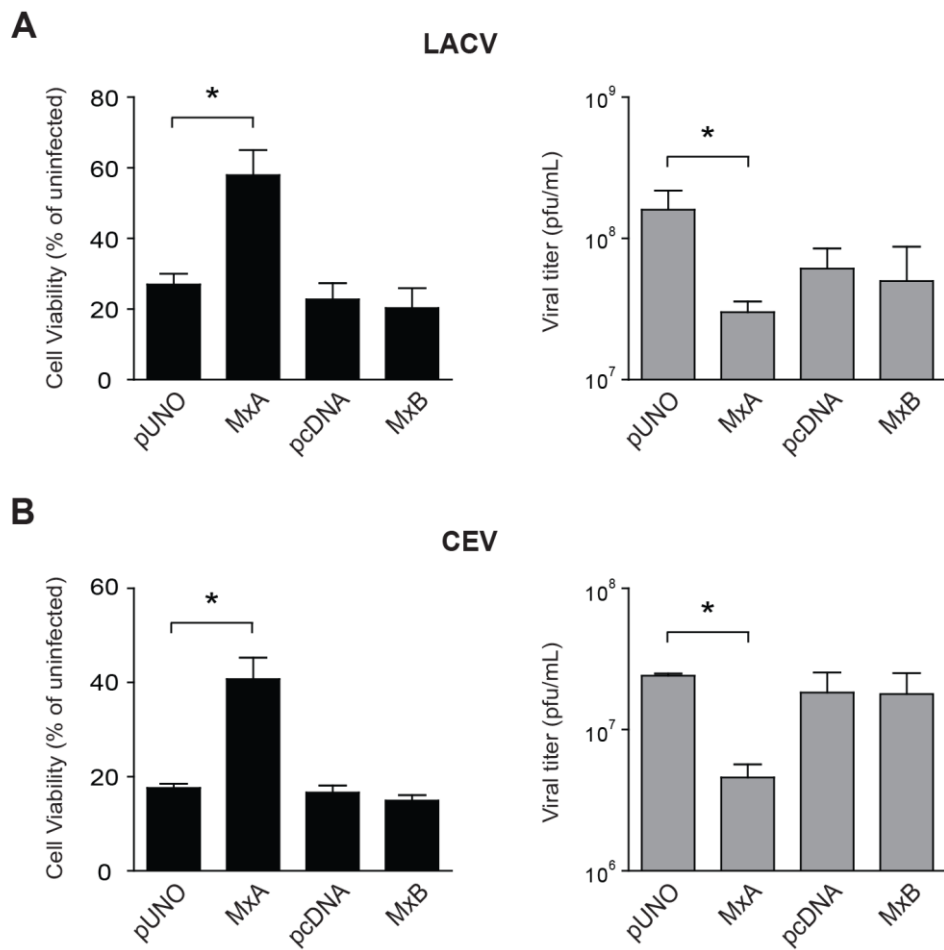


Figure III-2. MxA is antivirally effective against bunyaviruses in human neuronal cells

pUNO-MxA, pcDNA-v5-MxB, and empty vector controls were transfected into human neuroblastoma cells. At 24 hours post-transfection, cells were infected with LACV at an MOI of 0.001 (A) or CEV at an MOI of 0.1 (B). At 36 (A) and 48 (B) HPI, cell viability was analyzed by MTT, and cell supernatants were harvested for virion quantification by plaque assay. Results are shown as means \pm SEM (pUNO, MxA $n \geq 3$; pcDNA, MxB $n \geq 2$). * $p < 0.05$.

Overexpression of MxA but not MxB protected human neuronal cells from virus-induced cell death (**Figure III-2A and B, left panels**). For LACV, MxA overexpression increased cell viability post-challenge by $31.0 \pm 7.3\%$ compared to the empty vector control. For CEV, MxA overexpression increased cell viability post-challenge by $23.1 \pm 5.3\%$ compared to the empty vector control. These data indicate that MxA overexpression doubles neuronal cell viability post-challenge with a California-serogroup bunyavirus. In contrast, no survival benefit was conferred by the overexpression of MxB. Furthermore, increased neuronal cell viability in response to MxA overexpression correlated with decreased virus titer (**Figure III-2A and B, right panels**). For LACV as well as CEV, MxA overexpression resulted in an approximate one log reduction in virion recovery in comparison to the empty vector control, suggesting direct inhibition of virus replication by MxA. These data provide compelling evidence that MxA functions as an antiviral effector in human neurons. Furthermore, overexpression of MxA is sufficient, in the absence of other type I IFN-stimulated effectors, to inhibit bunyavirus replication, specifically LACV and CEV.

MxA can inhibit WEEV-mediated cytotoxicity, but not virion production in human neuronal cells

In the previous section we demonstrate that MxA overexpression is sufficient to inhibit bunyavirus replication in human neuronal cells. Next, we asked whether MxA is sufficient to inhibit other classes of neurotropic arboviruses, specifically New World alphaviruses for which MxA antiviral activity has yet to be characterized. Type I IFN-mediated inhibition of the New World alphavirus, WEEV, was previously demonstrated

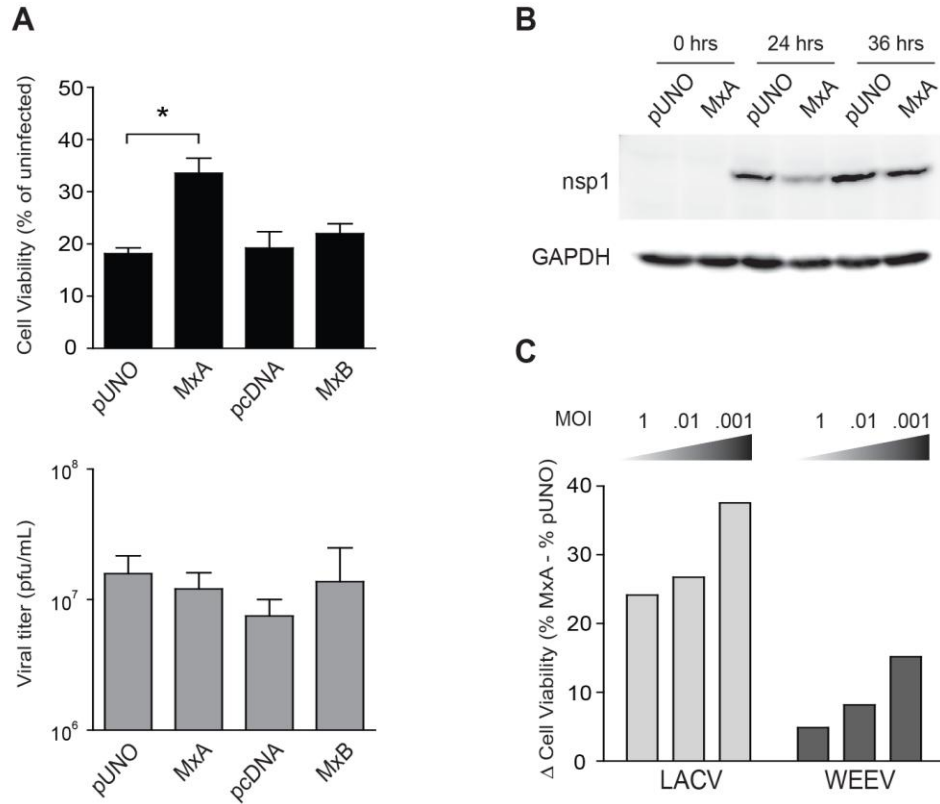


Figure III-3. MxA can inhibit WEEV-mediated cytotoxicity, but not virion production in human neuronal cells

pUNO-MxA, pcDNA-v5-MxB, and empty vector controls were transfected into human neuroblastoma cells. (A) At 24 hours post-transfection, cells were infected with WEEV at an MOI of 0.001. At 36 HPI, cell viability was analyzed by MTT, and cell supernatants were harvested for virion quantification by plaque assay. Results are shown as means \pm SEM (pUNO, MxA $n \geq 3$; pcDNA, MxB $n \geq 2$). * $p < 0.05$. (B) At 24 hours post-transfection, cells were infected with WEEV at an MOI of 0.001. Whole cell lysates were harvested at 0, 24, and 36 HPI and probed by immunoblot for expression of WEEV nonstructural protein 1 (nsp1) or GAPDH as a loading control. (C) At 24 hours post-transfection, cells were infected with WEEV or LACV at the indicated MOI. Cell viability was analyzed at 15 (LACV, MOI 1), 24 (WEEV, MOI 1) and 36 HPI (MOI 0.01 and 0.001). Results are shown as mean % viability from duplicate wells of a single trial.

in Be(2)-c cells (68), and we chose to examine the effects of MxA overexpression on WEEV pathogenesis in this model system.

In response to overexpression of MxA but not MxB, enhanced neuronal protection from WEEV-mediated cytotoxicity was observed (**Figure III-3A, upper panel**). MxA overexpression conferred a $15.4 \pm 4.4\%$ survival benefit in comparison to the empty vector control, indicating that MxA overexpression approximately doubles neuronal cell viability post-challenge with WEEV. Analysis of WEEV nonstructural protein 1 (nsp1) accumulation further indicated an antiviral effect of MxA overexpression at 24 and, to a lesser extent, 36 HPI (**Figure III-3B**). However, increased neuronal cell viability and decreased nsp1 accumulation in response to MxA overexpression did not correlate with decreased WEEV titers at 24 (data not shown) or 36 hours post-infection (HPI) (**Figure III-3A, lower panel**). Finally, in contrast to LACV, the survival benefit conferred by MxA overexpression was largely lost at high MOI WEEV infections (**Figure III-3C**). Together these data suggest that at low MOI infections, MxA overexpression is sufficient to confer protection against WEEV-mediated cytotoxicity in human neurons. While MxA can inhibit bunyavirus replication at the level of virion production, the inhibitory effects of MxA on WEEV replication are limited to protein accumulation at early times post-infection.

MxA is not antivirally effective against VEEV in human neuronal cells

WEEV is sensitive to 50 U/mL of type I IFN treatment in Be(2)-c cells (68). To investigate the type I IFN sensitivity of VEEV in this model system, Be(2)-c cells were pretreated for 24 hours with IFN α -A/D, a hybrid universal human type I IFN (251), and

challenged with VEEV or Fort Morgan virus (FMV) as a control. FMV is a close relative of WEEV, a member of the western equine encephalitis antigenic complex, but is nonpathogenic in humans (271). Additionally, FMV has been used previously to investigate mechanisms of alphavirus inhibition in Be(2)-c cells (272). At 25 U/mL of type I IFN pretreatment, Be(2)-c cells were fully protected from FMV-mediated cytotoxicity. In contrast, VEEV-mediated cytotoxicity persisted at 500 U/mL of type I IFN pretreatment (**Figure III-4A**). These data suggest that VEEV is rather insensitive to the antiviral effects of type I IFN in Be(2)-c cells. We chose to further investigate MxA-mediated inhibition of VEEV in this model system, hypothesizing that VEEV might demonstrate enhanced resistance to MxA activity.

Overexpression of MxA or MxB in human neuronal cells did not confer protection from VEEV-induced cell death (**Figure III-4B, top panel**). A subtle trend was observed with MxA overexpression; however, it only approached statistical significance. Furthermore, MxA overexpression did not limit mature virion production and release, as evidenced by similar VEEV titer in Mx and empty vector control transfected Be(2)-c cells at 48 HPI (**Figure III-4B, bottom panel**). Finally, MxA overexpression did not inhibit the accumulation of VEEV structural proteins, specifically E1 and E2 glycoprotein at 24 HPI and VEEV nucleocapsid at 12 HPI (**Figure III-4C**). These data suggest that MxA is not a functional antiviral effector against VEEV in human neurons.

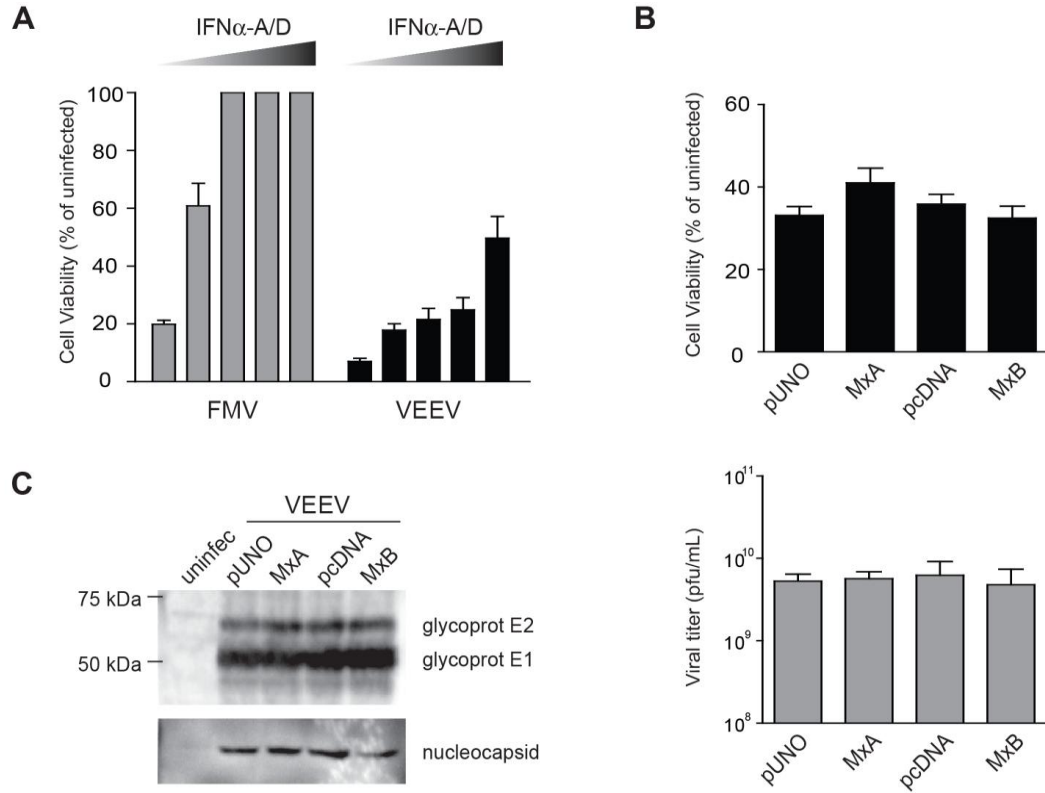


Figure III-4. MxA is not antivirally effective against VEEV in human neuronal cells

(A) Analysis of VEEV sensitivity to type I IFN in Be(2)-c cells using Fort Morgan virus (FMV) as a comparison. Be(2)-c cells were pretreated for 24 hours with 0, 10, 25, 50, or 500 U/mL IFN α -A/D and infected at an MOI of 0.01. Cell viability was analyzed by MTT at 72 HPI. Results are shown as means \pm SEM (n=2). pUNO-MxA, pcDNA-v5-MxB, and empty vector controls were transfected into human neuroblastoma cells. At 24 hours post-transfection, cells were infected with VEEV at an MOI of 0.001. (B) Cell viability was analyzed by MTT at 48 HPI (top panel); cell supernatants were harvested at 48 HPI, and virions were quantified by plaque assay (bottom panel). Results are shown as means \pm SEM (pUNO, MxA n \geq 3; pcDNA, MxB n \geq 2). (C) Whole cell lysates were harvested at 12 and 24 HPI and probed by immunoblot for expression of VEEV glycoproteins E1 and E2 (top panel, 24 HPI) and VEEV nucleocapsid protein (bottom panel, 12 HPI).

Mechanism of differential MxA-mediated inhibition of neurotropic arboviruses in human neuronal cells

In the present study we demonstrate that MxA inhibits bunyavirus replication to a greater extent than New World alphavirus replication in human neuronal cells. Next, we sought to dissect the mechanism of differential inhibition. Previously, MxA was shown to co-localize with the bunyavirus nucleocapsid in perinuclear complexes, and direct protein interaction was demonstrated through co-immunoprecipitation (273). These data led the authors to hypothesize that MxA sequesters bunyavirus nucleocapsid away from sites of virus replication and assembly (273). MxA-mediated sequestration of viral nucleocapsid is conserved across distantly related species, including influenza virus (274) and Thogoto virus (275). Furthermore, in strains of influenza demonstrating differential sensitivity to MxA, inhibition was shown to be nucleocapsid-specific (276). Based on these data, we hypothesized that MxA may differ in its ability to bind and sequester the bunyavirus versus New World alphavirus nucleocapsid. As VEEV demonstrated complete resistance to the inhibitory effects of MxA, we chose to specifically examine MxA-mediated nucleocapsid sequestration for VEEV versus CEV.

At 24 HPI, we analyzed viral nucleocapsid localization by immunocytochemistry in MxA overexpressing cells compared to empty vector control transfected cells. We probed VEEV nucleocapsid subcellular localization using nucleocapsid-specific polyclonal goat antiserum. Additionally, we probed CEV nucleocapsid subcellular localization using antiserum from mice immunized against whole CEV. Unfortunately, VEEV nucleocapsid was extremely difficult to localize due to non-specific antibody

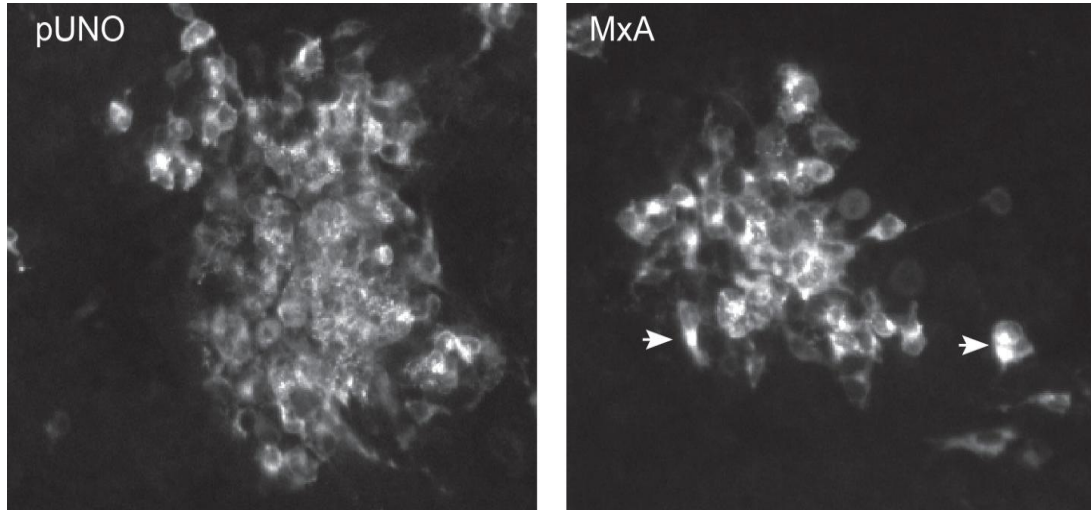


Figure III-5. Effect of MxA overexpression on subcellular localization of CEV antigen in human neuronal cells

Be(2)-c cells were transfected with pUNO or pUNO-MxA. At 24 hours post-transfection, cells were infected with CEV at an MOI of 0.1. At 24 HPI, cells were fixed and CEV antigen was localized by immunocytochemistry. Representative 10x images are shown; arrowheads denote large perinuclear puncta.

cross-reactivity in uninfected cells. In contrast, CEV antigen could be localized as cytoplasmic puncta at 12 (data not shown) and 24 HPI (**Figure III-5**). In response to MxA overexpression, there was a subtle increase in number of large perinuclear puncta. However, the effect was not significant. We conclude that more specific nucleocapsid antibodies are required to definitively analyze the differential impact of MxA on neurotropic arbovirus nucleocapsid localization in human neuronal cells.

DISCUSSION

Prior to this work, anti-neurotropic arbovirus function had been described for the type I IFN-induced antiviral effector, MxA. Most prominently, MxA had been shown to inhibit bunyavirus replication through sequestration of the viral nucleocapsid (273). However, MxA function can be cell type-specific (233), and inhibition of neurotropic arbovirus replication by MxA had not been investigated in human neurons, the *in vivo* target cell of the virus. Therefore, we tested the inhibitory function of MxA using the human neuroblastoma cell line, Be(2)-c. Be(2)-c cells provided an ideal environment for examining the isolated effector function of MxA for several reasons. First, we had previously shown that MxA expression is undetectable by immunoblot in untreated Be(2)-c cells (**Figure II-6B**). Second, we had previously shown that Be(2)-c cells do not produce detectable levels of type I IFN in response to infection with WEEV (68). Therefore, in the Be(2)-c cell model, we were able to examine MxA function with limited confounding effects from basal MxA expression as well as induced expression of other type I IFN-stimulated inhibitors.

In other *in vitro* models, MxA was previously shown to inhibit bunyavirus replication (236, 237). To determine whether MxA functions as a bunyavirus inhibitor in human neurons, we overexpressed MxA in Be(2)-c cells and challenged cells with the known human neuronal pathogens, LACV and CEV. In response to MxA overexpression, we observed increased cell survival and decreased virus titer. These data indicate that MxA is a functional antiviral inhibitor of California serogroup bunyavirus replication in human neurons. In contrast, we did not observe an antiviral effect following overexpression the related GTPase, MxB, consistent with previous reports in other cell systems that MxB is not a functional antiviral effector (236).

While MxA had been consistently described as a bunyavirus inhibitor, more selective and cell type-specific MxA activity had been described for the Old World alphaviruses (229, 237, 238), and MxA-mediated inhibition of the New World alphaviruses had not been characterized. We chose to examine MxA activity against two New World alphaviruses: WEEV, a human pathogenic strain with known sensitivity to type I IFN in the Be(2)-c cell model (68), and VEEV TC-83, a vaccine strain with relative insensitivity to type I IFN in the Be(2)-c cell model (**Figure III-4A**). In response to MxA overexpression, we observed decreased WEEV mediated-cytotoxicity and decreased nonstructural viral protein accumulation at early times post-infection, yet did not observe decreased virus titer. These data suggest that MxA can inhibit WEEV replication, but to a lesser extent than LACV and CEV. Additionally, MxA overexpression did not protect human neuronal cells from VEEV-mediated cytotoxicity and did not inhibit VEEV replication. Together these data demonstrate that MxA is a

more robust inhibitor of bunyavirus replication compared to New World alphavirus replication in human neurons.

Limited inhibition of New World alphavirus replication by MxA within human neurons could enhance viral pathogenesis within the CNS. The innate immune response to a neurotropic arbovirus is dominated by production of type I IFN, which is essential for limiting virus spread and surviving the acute phase of infection (39-44). Neurons respond to type I IFN by upregulating antiviral genes, and a robust upregulation of neuronal Mx has been described (97, 100). Decreased intra-neuronal control of alphavirus replication by MxA could therefore result in increased CNS dissemination. Consistent with this hypothesis, mice transgenically overexpressing MxA are more susceptible to infection with SFV compared to LACV and demonstrate heightened neuropathology following SFV infection (232). However, this experiment was conducted using mice deficient in response to type I IFN (IFNAR^{-/-}). In a more natural host, neurons could upregulate alternative type I IFN-induced antiviral effectors with anti-alphavirus function. To this end, alphavirus inhibition by a type I IFN-dependent, yet Mx-independent mechanism was recently described (277).

In this study, we additionally sought to dissect the mechanism of differential MxA-mediated inhibition. Previously, MxA was shown to inhibit bunyavirus replication through direct binding to the viral nucleocapsid, sequestering it away from sites of virus replication and assembly in perinuclear complexes (273). We hypothesized that MxA may preferentially bind and sequester the bunyavirus versus alphavirus nucleocapsid, thus conferring its differential inhibition. Accordingly, we attempted to observe

increased relocalization of the CEV versus VEEV nucleocapsid in response to MxA overexpression. While a subtle relocalization was observed for CEV following MxA overexpression, the immunocytochemistry data were difficult to interpret due to a lack of robust and selective antibody binding. Specifically, the CEV antiserum cross reacted with multiple viral proteins. In future experiments, nucleocapsid-specific antibodies will be required to more definitively examine MxA-mediated relocalization. Furthermore, we were limited by our supply of MxA antibody, and therefore we were unable to probe the counter observation: does MxA relocalizes to perinuclear complexes with CEV but not VEEV infection? A tagged MxA construct would allow us to more clearly visualize MxA subcellular localization and is a superior future approach. Finally, these experiments were conducted following transient MxA overexpression, as MxA proved slightly toxic to the Be(2)-c cell cultures, limiting the potential to develop a stable cell line. In the future, a stable Tet-ON cell line in which MxA could be conditionally overexpressed would be ideal in terms of limiting cellular toxicity and maintaining consistent MxA overexpression.

Despite the diverse viral outcomes that have been observed following MxA overexpression, a conserved mechanism of action has been hypothesized in light of the recent crystallization of the MxA stalk region (220). Specifically, a ring-like oligomeric structure with sites available for binding viral ribonucleoprotein at the inner ring interface has been proposed (222). MxA-mediated depletion of viral proteins at various stages in the viral life-cycle is therefore a likely conserved mechanism. Among the bunyaviruses, including species known to be inhibited by MxA through nucleocapsid sequestration

(LACV, Bunyamwera virus (BUNV), and Rift Valley Fever virus (RVFV)), the nucleocapsid sequence is highly conserved (**Figure III-6**). This analysis suggests that CEV may be inhibited by MxA through a similar mechanism. While the exact location of MxA binding on the bunyavirus nucleocapsid has yet to be described, the alphavirus nucleocapsid sequence is highly divergent from the bunyavirus nucleocapsid sequence (**Figure III-6**). In the absence of a conserved binding motif, it is likely that this structural protein goes unrecognized by MxA during alphavirus infection. Accordingly, in a SFV replicon system, structural protein-independent MxA inhibition was described (238). Therefore, MxA may mediate more subtle inhibition of the alphaviruses by recognizing alternative viral targets such as the nonstructural proteins. Consistent with this hypothesis, WEEV nsp1 decreased initially following MxA overexpression (**Figure III-3B**).

We conclude that MxA is a functional inhibitor of neurotropic arboviruses in human neuronal cells, suggesting that MxA mediates innate type I IFN-dependent antiviral defense against neurotropic arboviruses in infected human neurons. Additionally, our data demonstrate that MxA is a more robust inhibitor of bunyaviruses compared to New World alphaviruses, suggesting that type I IFN-induced expression of MxA in human neurons may be a critical component of host defense against a California serogroup bunyavirus. Future investigation will elucidate the mechanism of differential MxA-mediated inhibition by determining the specificity of MxA for the bunyavirus versus New World alphavirus nucleocapsid and identifying alternative MxA targets during alphavirus replication.

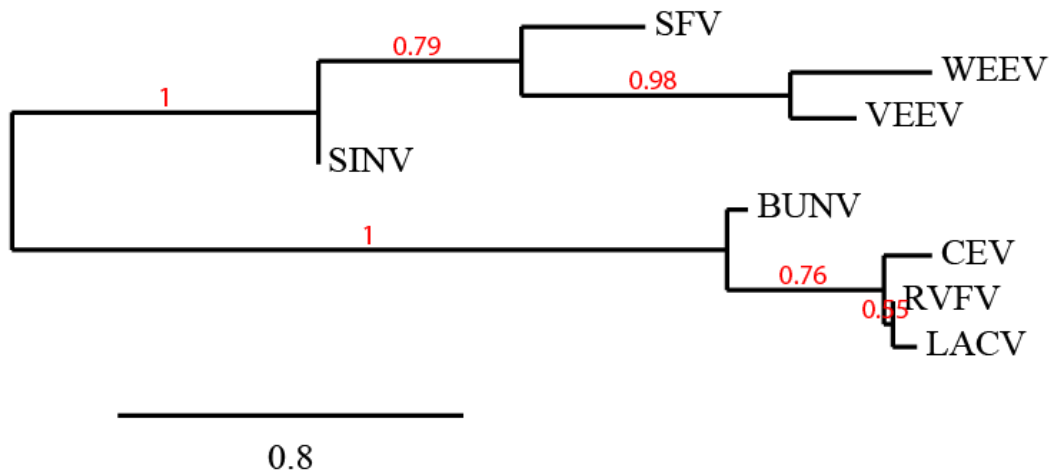


Figure III-6. Phylogram depicting relatedness of viral nucleocapsid proteins

Phylogram was assembled from amino acid sequences using the web server, Phylogeny.fr (278). Branch support values are shown in red; legend indicates relative branch length. MxA sequestration of viral nucleocapsid has previously been described for LACV, Bunyamwera virus (BUNV), and Rift Valley Fever virus (RVFV) (273).

CHAPTER IV:

DISCUSSION

OVERVIEW

Neurotropic arboviruses are significant pathogens of the human central nervous system (CNS) that specifically infect neurons (16, 22-25) to cause acute encephalitic disease and life-long neurological complications, with particular severity in the pediatric population (53-56). While age-dependent host susceptibility to neurotropic arboviruses remains only partially understood, decreased pathogenesis has been correlated with an intact type I interferon (IFN) pathway (39-44) and relative neuronal maturity (23, 62, 67, 68). Therefore, we set out to test the following hypothesis: antiviral type I IFN pathway activity increases with human neuronal differentiation, resulting in enhanced defense against neurotropic arboviruses in mature human neurons compared to human neural progenitor cells (NPCs). We addressed this hypothesis through three specific aims: 1) we modeled human neuronal differentiation *in vitro* using human embryonic stem cells (hESCs), 2) we defined maturation-dependent changes in the neuronal type I IFN pathway, and 3) we assessed the antiviral competency of the type I IFN-induced effector, MxA, against neurotropic arboviruses in human neuronal cells. For each aim, we achieved success, encountered limitations, and unveiled interesting future directions.

Together our data demonstrate that antiviral type I IFN pathway activity increases with human neuronal differentiation, with maturation of human NPCs specifically, and that this enhanced response confers protection against neurotropic arbovirus pathogenesis *in vitro*.

HUMAN NEURONAL DIFFERENTIATION CAN BE MODELED USING STEM CELLS

Implications

We were successful in establishing a protocol for the routine derivation of NPCs and mature neurons from hESCs. Specifically, we are now able to obtain populations that are over 95% pure for PSA-NCAM, a marker of the neuronal lineage, and relatively enriched in NPCs (approximately 70% Sox3-positive) or mature neurons (approximately 70% NeuN-positive). This technological advance is extremely important. First, while much information can be gained from working with human neuronal cell lines, it is difficult to correlate relative stages of *in vitro* maturation with actual stages of *in vivo* development. In contrast, using hESCs we are able to produce neuronal populations that have been identified in the postnatal human CNS. Conclusions derived using the hESC model therefore have direct implications regarding the *in vivo* immune potential of a human neuron. Second, human neuronal cell lines, such as the Be(2)-c cell line, are frequently derived from tumor specimens. These human cells are therefore dedifferentiated in their “immature” state and redifferentiated in their “mature” state. In contrast, hESC-derived NPCs are truly immature neural cells that can be used to assess

maturation-dependent changes in innate neuronal immune function in the absence of confounding effects from cell transformation and/or injury. Finally, using an *in vitro* model in which greater than 95% of cells express a neuron-specific marker, we are able to examine neuron-specific innate immune function. In contrast to astrocytes and microglia that have long been elucidated as CNS cells with potent innate immune activity, many inflammatory responses are more subtly upregulated in neurons. Accordingly, neuronal immune function has historically been more difficult to assess *in vivo* and more widely debated in the literature (69). *In vitro* analyses in highly pure, physiologic populations of human neurons will greatly advance our understanding of innate neuronal immune function.

Limitations

While a power tool, the hESC model of neuronal differentiation does have limitations. First of all, we are currently unable to obtain pure populations of NPCs and mature neurons. In the NPC cultures, radial differentiation was observed following neurosphere attachment that was difficult to inhibit, even with daily changes of bFGF-supplemented media. As a result, we observed approximately 40% NeuN-positive cells in the NPC cultures by flow cytometry, suggesting a substantial minority population of mature neurons. In the mature neuronal cultures, the opposite phenotype was observed: cells located on the periphery of the rosette were fully differentiated at the end of the two week protocol, while cells located in the center of the rosette retained Sox3 signal. Accordingly, the mature neuronal cultures were approximately 40% Sox3-positive by flow cytometry, suggesting a substantial minority population of residual NPCs.

Persistent immaturity at the rosette center suggests that neuronal maturation is being contact inhibited, perhaps through activation of the Notch signaling pathway, which promotes survival of neural stem/progenitor cells both *in vitro* and *in vivo* (279). In subsequent protocols, we could attempt to reduce contact-mediated inhibition by antagonizing the Notch signaling pathway (e.g. treat with γ -secretase inhibitors). Persistent immaturity at the rosette center could also be reduced using longer differentiation periods; however, astrocytes were identified as early as three weeks post-differentiation. Therefore, using the currently established culture conditions, we are limited to a two week protocol. In populations of enriched NPCs and mature neurons, we were able to observe a number of innate immune differences. However, it is likely that these phenotypes would become more pronounced in pure populations of NPCs and mature neurons, due to the fact that overlapping populations of differentiating neural cells are currently contributing to similar innate immune responses in the NPC and mature neuronal cultures. Eliminating these overlapping populations would allow us to more accurately assess the innate immune potential of a human NPC compared to a mature neuron.

In contrast to human neuronal cells, hESC-derived neurons are extremely sensitive to plating density. Specific to the neurosphere attachment phase of the protocol, mechanical as opposed to enzymatic dissection was required to maintain viable populations of NPCs post-plating. Accordingly, cell clumps rather than single cell suspensions were added to tissue culture wells, resulting in highly variable cell densities per well. In the stem cell field, researchers frequently eliminate the requirement for

plating consistency by using single cell analyses such as immunocytochemistry and patch clamping. However, our work requires analysis of viral spread through a neuronal population. Accordingly, plating inconsistencies will continue to be a substantial concern while working with the hESC model. In subsequent protocols, we could attempt to minimize contact-dependent NPC survival by treating with Notch ligands, which have been shown to promote neural stem/progenitor cell survival post-plating (279).

Finally, hESCs require substantially more time, money, and effort to maintain than human neuronal cell lines or primary cultures of rodent neurons. These factors limit the ability to perform in depth analyses in the hESC model. To functionally analyze type I IFN pathway components, we created a stable human neuroblastoma cell line that was doxycycline responsive (Tet-ON) and alkaline phosphatase secreting in response to type I IFN (ISRE-SEAP). Such methods do not readily translate to hESCs. However, the laboratory of Dr. O'Shea at the University of Michigan, Ann Arbor, recently achieved success in generating a stable Tet-ON hESC line. Such technical advances will enable future functional investigations in hESC-derived cells at the University of Michigan.

Future Directions

In our matured hESC-derived cultures, we observed several neuronal subtypes including GABAergic (GABA-positive), glutamatergic (VGLUT2-positive), and dopaminergic (TH-positive) cells. We were interested in examining the innate immune function of NPCs relative to mature neurons. Therefore, in our differentiation protocol, we did not select for a certain subtype of mature neuron. However, such methods have been described for the derivation of GABAergic, dopaminergic, serotonergic and motor

neurons from ES cells (reviewed in (132)). Accordingly, we could adapt the hESC model to examine innate immune function and viral susceptibility within specific subtype populations of mature neurons. This analysis is of particular interest in the context of a virus with known neuronal selectivity. For example, Japanese encephalitis virus (JEV) causes Parkinson's disease-like symptoms by targeting the basal ganglia (280, 281), an area highly enriched in GABAergic neurons. In contrast, Tick-borne encephalitis causes shoulder weakness and atrophy by targeting motor neurons within the cervical spinal cord (282, 283).

Additionally, we identified astrocytes (GFAP-positive) in our mature neuronal cultures at three weeks post-differentiation. Methods for deriving enriched populations of astrocytes and oligodendrocytes from ES cells have also been described (reviewed in (132)). Therefore, we could adapt the hESC model to establish co-cultures of multiple CNS cell types. Using co-culture systems, we can begin to mimic the *in vivo* innate immune response to virus infection. For example, we could examine viral susceptibility in neurons versus astrocytes in addition to cytokine production and cytokine response. Such experiments would greatly enhance our understanding of the innate immune interplay between neurons and neighboring CNS cell types.

Upon differentiating hESCs, we produced mixed populations of NPCs and mature neurons, ranging from approximately 70% NPCs at day 28 of differentiation to 70% mature neurons at day 42 of differentiation as previously described. As NPCs and mature neurons are located in close proximity within the brain, it would be interesting to investigate differential viral susceptibility at the cellular level of this co-culture system.

Research suggests that *in vivo* NPCs are particularly susceptible to infection with neurotropic viruses (137, 139, 142). However, there are multiple *in vivo* factors that may confer this susceptibility. Thus *in vitro*, are NPCs more susceptible to viral infection than mature neurons? If so, NPC susceptibility is conferred at the cellular level. At the cellular level, there are multiple factors that may further dictate NPC susceptibility including decreased type I IFN pathway function, active cell cycling, unique surface moiety composition, and/or decreased anti-apoptotic gene expression. In the co-culture system, each susceptibility factor could be independently explored. For example, we could treat with mitotic inhibitors and examine the effects of cell cycle arrest on viral antigen localization in NPCs compared to mature neurons. Additionally, we could treat with type I IFN and examine maturation-dependent competition for the ligand and resulting effects on NPC susceptibility. Finally, the co-culture system could be used to investigate the innate immune interplay between NPCs and mature neurons. Specifically, it would be interesting to assess whether type I IFN treatment stimulates additional cytokine and/or chemokine production in the hESC-derived cultures. While neurons are only recently being elucidated as competent cytokine-producing cells (97), research in the Be(2)-c cell model demonstrates that type I IFN induces a sustained upregulation of ISGs (**Figure II-7**), which can be inhibited by periodic media changes (data not shown). These results suggest that Be(2)-c cells produce soluble factors in response to type I IFN treatment, which can potentiate activation of the type I IFN pathway. Therefore, it would be of great interest to examine type I IFN-induced cytokine and chemokine production in NPCs compared to mature neurons.

Finally, our laboratory has previously demonstrated upregulation of pattern recognition receptor pathways in response to human neuroblastoma cell differentiation, resulting in increased IFN- β transcript production in Be(2)-c/m compared to Be(2)-c cells (95). We could additionally probe maturation-dependent type I IFN production in response to virus infection in NPCs compared to mature neurons. Type I IFNs not only induce the upregulation of antiviral genes, but additionally impact global innate and adaptive immune responses within the host. Specifically, type I IFNs have been shown to regulate the function of natural killer cells, stimulate dendritic cell maturation, and contribute to the activation of T- and B-cells (reviewed in (284)). As activated T- and B-cells are critical to neurotropic arbovirus clearance within the CNS, it would be interesting to explore maturation-dependent type I IFN production in response to neurotropic arbovirus infection in cultures of hESC-derived neurons. Preferentially increased type I IFN production in mature neurons relative to NPCs could suggest increased adaptive immune system activation and ultimately, enhanced viral clearance. Additionally, maturation-dependent differences in type I IFN subtype production may exist. *In vivo* mature (NeuN-positive) neurons have been shown to produce both IFN- α and - β in response to neurotropic viral challenge (97). However, NPCs may favor production of IFN- α in response to neurotropic arbovirus infection, while mature neurons may favor production of IFN- β . Increased IFN- α production in the immature versus mature CNS could result in preferential neurotoxicity and neuropathology following neurotropic arbovirus challenge.

Therapeutic Potential

Infection with a neurotropic arbovirus can result in life-long complications including cognitive deficits, motor disorders, and seizure disorders (54). This has led researchers to question whether NPCs are significantly lost and/or altered during virus infection. Accordingly, a number of NPC abnormalities have been observed in response to infection with neurotropic viruses including inhibition of NPC proliferation (133, 135, 137, 139), reduced NPC potential to form mature neurons and migrate in the CNS (133), and direct loss of NPCs due to viral-induced apoptosis (137).

Stem cell-based therapy is not a potential during acute neurotropic arbovirus infection. Enhancement of the NPC pool via cerebral infusion with epidermal growth factor resulted in increased CNS susceptibility to cytomegalovirus (CMV) (145), suggesting that NPC transplant during acute infection may only exacerbate CNS pathology. However, stem cell-based therapy has potential in the treatment of post-infectious neurological sequelae for several reasons. First, ES cells have widespread therapeutic potential due to their ability to develop into most CNS subtypes *in vitro* including spinal cord motor neurons, spinal cord interneurons, midbrain dopaminergic neurons, cerebellar Purkinje and granule cells, and cortical pyramidal neurons (reviewed in (285)). Second, transplanted NPCs can restore CNS function. Embryonic NPCs, isolated from mouse cortex, were shown to reestablish damaged motor pathways when grafted into the adult brain (286). Furthermore, ES cell-derived NPCs and matured neurons were shown to form axonal projections to the thalamus, midbrain, and cortex when grafted into the frontal lobe of a mouse neonate (287). With the development of

induced pluripotent stem cells (iPSCs) (288, 289), patient-specific NPCs could be derived. Recently, transplantation of iPSC-derived dopaminergic neurons into the adult rat brain was shown to improve Parkinson's disease-like behavior (290). However, significant concerns continue to surround the iPSC field including oncogenic risk and limited differentiation potential (291). Accordingly, substantial research is required before stem cell-based therapy is a clinical reality in the CNS, but our growing understanding of how to differentiate hESCs into NPCs in culture is bringing us ever closer to ultimately achieving this therapeutic goal.

ANTIVIRAL TYPE I IFN PATHWAY ACTIVITY IS ENHANCED WITH HUMAN NEURONAL DIFFERENTIATION

Implications

Many neurotropic arboviruses are known to cause more severe disease in pediatric compared to adult patients (53-56). Infected children are frequently at increased risk for developing acute encephalitis in addition to permanent post-infectious neurological sequelae. Age-dependent susceptibility to neurotropic arboviruses has been modeled for over four decades in rodents, where transition from fatal disease to full recovery has been observed between week one and two of life (59-61). Transition to full recovery does not correlate with increased type I IFN production (63), decreased permeability of the blood-brain barrier (64), or even increased adaptive immune response to virus (45, 65). However, transition to full recovery does correlate with decreased viral

load in the CNS (61, 63), decreased rate and extent of intra-neuronal virus spread (23), and relative neuronal maturity (23, 62, 67, 68).

Prior to our work, the mechanism by which neuronal maturation confers decreased susceptibility to neurotropic arbovirus infection had yet to be fully dissected. As neurons mature, they upregulate anti-apoptotic genes (292), and work in the alphavirus field demonstrated cellular protection from virus-induced lysis in response to bcl-2 overexpression (249). Therefore, anti-apoptotic gene upregulation is likely an important mechanism of mature neuronal survival in the face of alphavirus infection. However, it does not explain the increased antiviral response - decreased alphavirus titer - that has been observed with neuronal maturation (67). Research specifically focused on elucidating the viral susceptibility of NPCs has further demonstrated that active cellular proliferation within the subventricular zone (SVZ) enhances CMV pathogenesis, resulting in increased virus titer and CMV-positive cells in the CNS (145). Thus active cell cycling in immature versus mature neurons likely promotes viral replication during *in vivo* infection. However, innate antiviral immune differences between mature and immature neurons, NPCs specifically, had not been explored.

Here we significantly enhance understanding of age-dependent neuronal susceptibility to neurotropic arboviruses by demonstrating that innate antiviral immune function increases with human neuronal differentiation. More specifically, we provide evidence to suggest enhanced antiviral type I IFN pathway activity in mature human neurons compared to human NPCs. The type I IFN pathway is known to be essential for acutely curtailing neurotropic arbovirus spread and maintaining host survival (39-44).

Therefore, our results suggest that the transition from fatal disease to full recovery in mice challenged with a neurotropic arbovirus does not correlate with type I IFN production in the CNS (63), but alternatively type I IFN response. Our data are supported by several *in vivo* observations. First, analysis of CNS gene expression in response to Sindbis virus (SINV) infection in neonatal versus weanling mice demonstrated preferential upregulation of a single inflammatory gene in the mature mouse brain, IFN-stimulated gene 12 (ISG12). Enforced neuronal expression of ISG12 in the neonatal mice was further demonstrated to significantly delay SINV-induced cell death (293). Second, in mice with an intact type I IFN pathway, age-dependent fatality is observed following subcutaneous inoculation as well as intracranial inoculation (61, 63). In contrast, in mice with type I IFN receptor deficiency (IFNAR^{-/-}), age-dependent survival differences remain in the context of subcutaneous inoculation, but are lost upon intracerebral challenge (294). Therefore, the mature CNS is no longer protected against neurotropic arbovirus infection in the absence of an intact type I IFN signaling pathway. In conjunction with our data, these findings suggest that maturation-dependent differences in neuronal type I IFN pathway function are an important determinant of neurotropic arbovirus pathogenesis within the CNS.

Limitations

In the Be(2)-c human neuroblastoma cell model, we demonstrated increased surface expression of the IFN- α/β receptor 2 subunit (IFNAR2) with neuronal maturation. Furthermore, we demonstrated that IFNAR2 overexpression in immature neuronal cells is sufficient for increased type I IFN-dependent inhibition of alphavirus replication,

specifically Fort Morgan virus (FMV), western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV). In the hESC model, we similarly demonstrated increased surface expression of IFNAR2 with differentiation of NPCs to mature neurons. Additionally, we demonstrated enhanced type I IFN-dependent inhibition of alphavirus replication with neuronal maturation, specifically Venezuelan equine encephalitis virus (VEEV). Together our data suggest that mature human neurons are more antivirally competent against alphaviruses relative to human NPCs due to increased surface IFNAR2 expression. However, we were not able to directly examine the functional impact of IFNAR2 overexpression in the hESC model on account of this experiment's technical difficulty. Therefore, while we observed increased antiviral type I IFN pathway activity with maturation of hESC-derived NPCs, we cannot explicitly confirm that this enhanced response is due to basal upregulation of IFNAR2. Alternative hypotheses exist, including decreased active cell cycling in the mature hESC-derived cultures, resulting in slower virus spread and increased type I IFN effectiveness.

In the hESC model, neuronal maturation resulted in a 2.6 log reduction in VEEV titer in the context of type I IFN treatment, theoretically due to increased surface expression of IFNAR2 in the matured cells. In contrast, IFNAR2 overexpression in immature Be(2)-c cells resulted in a more subtle one log reduction in VEEV titer in the context of type I IFN treatment. These results are consistent with the finding that VEEV is rather insensitive to the antiviral effects of type I IFN in the Be(2)-c cell model. Insensitivity to type I IFN in Be(2)-c cells compared to hESC-derived neurons correlates with increased VEEV replication in the human neuroblastoma cells. Accordingly, no

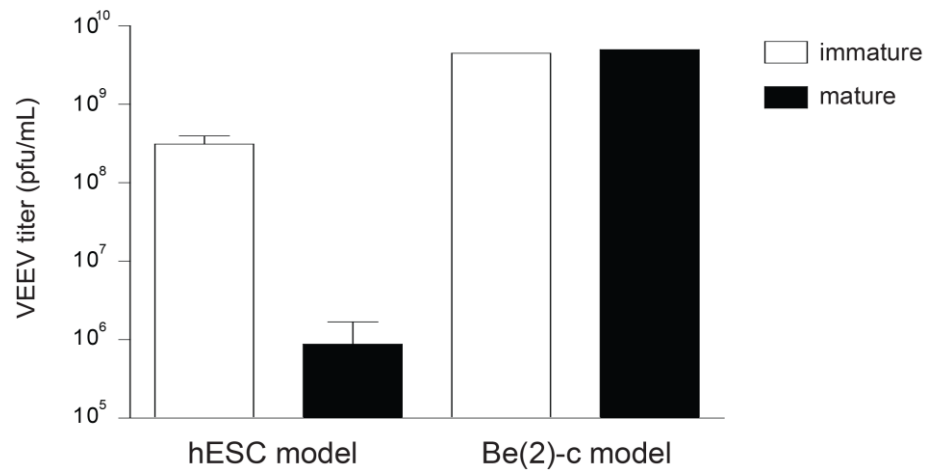


Figure IV-1. VEEV is less sensitive to type I IFN in the human neuroblastoma cell model

hESC-derived NPCs (immature) and mature neurons (mature) compared to undifferentiated (immature) and differentiated (mature) human neuroblastoma Be(2)-c cells were pretreated for 24 hours with 50 U/mL of IFN α -A/D and infected with VEEV TC-83 at an estimated MOI of 0.01. Cell supernatants were harvested at 72 hours post-infection (HPI) and analyzed for VEEV virions by plaque assay. For the hESC model, results are shown as means \pm SEM (n=3). For the Be(2)-c cell model, data from a single experimental trial are shown (similar results were obtained at 48 HPI, data not shown).

maturation-dependent difference in VEEV titer was observed at 50 U/mL of type I IFN treatment in Be(2)-c cells, where as a substantial effect was observed at this treatment dose in hESC-derived neurons (**Figure IV-1**). These results indicate differences in innate antiviral immune function between the two model systems.

Future Directions

NPC susceptibility has been described for a number of neurotropic viruses (133-136), including the neurotropic arbovirus, JEV (137). Our data additionally demonstrate that human NPCs are more susceptible to *in vitro* alphavirus infection than mature neurons. However, NPC susceptibility to alphavirus infection has yet to be conclusively demonstrated *in vivo*. In collaboration with Dr. David Irani at the University of Michigan, Ann Arbor, we were able to grossly co-localize SINV replication with the NPC marker, nestin, in the SVZ of the infected mouse brain (**Figure IV-2**). However, further analysis is required to examine preferential targeting of NPCs by alphaviruses *in vivo*. First, alphavirus localization must be examined in both neurogenic regions of the postnatal mammalian CNS: the SVZ as well as the subgranular zone (SGZ) of the hippocampus. Additionally, this analysis should be extended to human encephalitic pathogens, including WEEV and VEEV. Finally, this analysis could be extended to IFNAR^{-/-} mice. If our hypothesis is correct, these animals will demonstrate decreased preferential NPC susceptibility following intracerebral alphavirus challenge.

In addition, we demonstrated increased basal expression of type I IFN pathway components and increased activation of the type I IFN pathway in mature neurons compared to NPCs. These phenotypes could additionally be examined *in vivo*. For

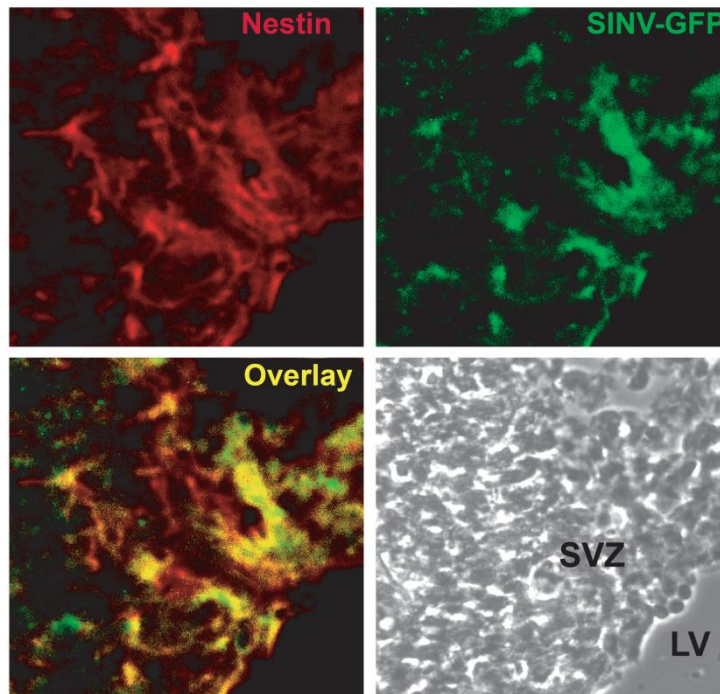


Figure IV-2. SINV replication co-localizes with NPC marker expression in the SVZ

Mice were intracerebrally inoculated with neuroadapted SINV expressing GFP. At day 6 post-inoculation, mice were sacrificed and brains were dissected, frozen, and transversely sectioned. GFP was co-localized with the NPC marker, nestin, using fluorescent immunohistochemistry (top and bottom left). Brightfield image depicts the subventricular zone (SVZ) adjacent to the lateral ventricle (LV) (bottom right).

example, we could observe relative basal type I IFN pathway component expression by immunohistochemistry in sections of rodent as well as human brain, as we have already obtained sections of adult human brain from the National Disease Research Interchange (NDRI). Comparative analysis of type I IFN pathway component expression in nestin-positive or Sox3-positive cells of the SVZ and SGZ to NF200-positive or NeuN-positive cells in surrounding regions of the CNS, perhaps regions demonstrating particular resistance to alphavirus infection, would be of interest. Finally, we could use *in vivo* mouse models to analyze differential response to type I IFN via examining induced expression of type I IFN-stimulated genes in NPCs versus mature neurons. This analysis could be conducted following type I IFN treatment and/or alphavirus infection, and would greatly enhance our understanding of innate antiviral NPC potential *in vivo*. As a number of type I IFN-stimulated genes have been shown to be promiscuously induced during virus infection (295-297), analysis in response to alphavirus challenge would require examination of IFN-stimulated genes that are strictly upregulated by type I IFN (e.g. the Mx proteins in Mx-competent mice).

Therapeutic potential

IFN- α has been shown to exacerbate neurotropic viral pathogenesis in the CNS by directly inducing neuronal dysfunction in a glutamate signaling-dependent manner (113). Therefore, IFN- α is not a potential therapeutic during acute infection with a neurotropic arbovirus. Additionally, immature mice demonstrate enhanced production of type I IFN in response to alphavirus infection (63), and this hyper-inflammatory response has been

correlated with heightened CNS pathology and fatal outcome (298). Our research would suggest that increased response to type I IFN, rather than increased production of type I IFN, is the critical component to enhanced neuronal defense.

Three protein isoforms of IFNAR2 have been described: a, b, and c (156). IFNAR2c is the signaling competent transmembrane isoform that has been the focus of all preceding discussion. IFNAR2b is a transmembrane protein with a shortened intracellular domain that likely functions as a dominant negative regulator (158). IFNAR2a is, in contrast, a soluble protein that has been most prominently described in the mouse model. Murine IFNAR2a transcripts have been identified in most tissues, including the brain (159). Furthermore, murine IFNAR2a has been detected by immunoblot in mouse serum, urine, and peritoneal fluid, suggesting that the IFNAR2a protein is secreted into biologic fluids *in vivo* and stable in biologic fluids over time. Finally, murine IFNAR2a was shown to bind type I IFN, competitively inhibiting type I IFN pathway activity in wild-type thymocytes while complementing type I IFN pathway activity in IFNAR2 knockout thymocytes (159). Together these data suggest that IFNAR2a has therapeutic potential. As a soluble protein with high stability and physiologic activity in biologic fluids, IFNAR2a could be dosed locally to the serum or directly to the CNS. Furthermore, IFNAR2a may be able to complement type I IFN pathway signaling in NPC that demonstrate low basal expression of IFNAR2. However, as IFNAR2a was shown to competitively inhibit type I IFN pathway signaling in wild-type thymocytes, mature neurons may be at risk for antagonization. Accordingly,

detailed *in vitro* analyses are required to elucidate the potential role for human IFNAR2a in complementing NPC signaling.

MXA IS A FUNCTIONAL ANTIVIRAL EFFECTOR IN HUMAN NEURONAL CELLS

Implications

Type I IFN mediates its cellular antiviral activity by stimulating the transcriptional upregulation of numerous cell-autonomous antiviral inhibitors. Included in this list is the large GTPase, MxA. We previously observed increased type I IFN-stimulated MxA expression in mature versus immature human neuronal cells. Therefore, we sought to directly assess whether overexpression of MxA in immature human neuronal cells was sufficient to confer decreased susceptibility to neurotropic arboviruses. Prior to our investigation, MxA had previously been shown to inhibit the replication of bunyaviruses (e.g. La Crosse virus (LACV) (232, 236, 237)) and Old World alphaviruses (e.g. Semliki Forest virus (SFV) (232, 238)). However, the antiviral function of MxA can be cell type-specific (233), and MxA function had not been assessed in human neurons. Additionally, antiviral MxA activity had not been characterized for the New World alphaviruses, including WEEV and VEEV.

Here we enhance understanding of MxA activity by demonstrating that MxA is a functional antiviral inhibitor in human neuronal cells. In response to CNS infection *in vivo*, a robust upregulation of neuronal Mx expression has been described (97, 100). Our results suggest that this upregulation has direct functional significance on neurotropic

arbovirus inhibition. Furthermore, we demonstrate that MxA has preferential activity against the bunyaviruses LACV and California encephalitis virus (CEV) compared to the New World alphaviruses WEEV and VEEV. This finding is consistent with previous work in a transgenic mouse model of MxA overexpression, where increased inhibition of LACV versus SFV was observed (232). Together these data suggest that MxA upregulation within CNS neurons confers enhanced protection against bunyavirus replication. However, as bunyaviruses are not known to be grossly more pathogenic than alphaviruses in the CNS, it follows that alternative type I IFN-induced antiviral inhibitors exist, which demonstrate particular effectiveness against the alphaviruses. Accordingly, type I IFN-dependent, yet Mx-independent, inhibition of SINV has been described (277), and alternative anti-alphavirus effectors have begun to emerge. For example, IFN-stimulated gene 20 (ISG20) and zinc finger antiviral protein (ZAP) were recently identified as potent alphavirus inhibitors (299). *In vitro*, ISG20 and ZAP robustly and additively inhibited SINV replication. Furthermore, ISG20 or ZAP overexpression was sufficient for decreased SINV-mediated neonatal mortality *in vivo*. Further investigation is required to test the competency of ISG20 and ZAP against the New World alphaviruses and in human neurons.

Limitations

We attempted to dissect the mechanism of MxA-mediated inhibition of neurotropic arboviruses in human neuronal cells. However, our reagents were not sufficient to address the question. First, we had access to goat antiserum raised against VEEV nucleocapsid that demonstrated non-specific cross-reactivity to other viral

proteins by immunoblot and non-specific cross-reactivity to host cellular proteins by immunocytochemistry. Additionally, we had access to mouse antiserum raised against whole CEV, and thus we were unable to observe nucleocapsid-specific relocalization in response to MxA overexpression. Accordingly, superior antibodies with limited non-specific cross-reactivity will be required to more definitively examine the effects of MxA overexpression on viral nucleocapsid subcellular localization. Second, we had a limited supply of MxA antibody and were unable to probe the counter-question: is MxA relocalized upon virus infection from diffuse cytoplasmic puncta to perinuclear puncta as has been described previously for the bunyaviruses (273)? Tagging MxA would be a superior future approach, allowing us to definitively localize MxA in the neuronal cell. Finally, our analyses were conducted using transient MxA overexpression. MxA proved slightly toxic to the Be(2)-c cells, which limited our potential to develop a stable overexpression cell line. However, stable conditional overexpression of MxA in Be(2)-c cells could be achieved, as a stable Tet-ON Be(2)-c cell line has already been established. This would be a superior future approach in terms of standardizing MxA overexpression throughout the human neuroblastoma cell culture while limiting MxA-mediated cellular toxicity.

Future directions

The mechanism of differential MxA-mediated inhibition of bunyavirus versus alphavirus replication has yet to be dissected. MxA is known to inhibit bunyavirus replication through sequestration of the viral nucleocapsid protein (273). This sequestration has been hypothesized to inhibit virus replication by: 1) directly depleting

nucleocapsid during virus assembly and 2) indirectly inhibiting genome amplification via loss of the nucleocapsid-RNA polymerase interaction (273). Furthermore, sequestration of viral ribonucleoproteins has been hypothesized to be a conserved mechanism of action for MxA across viral species (222). However, it remains to be determined whether MxA can bind and sequester the alphavirus homolog. Within bunyaviruses, the nucleocapsid sequence was determined to be highly conserved. However, the bunyavirus sequence overall was found to be highly divergent from that of the Old and New World alphavirus sequence. Accordingly, the alphavirus nucleocapsid may go unrecognized by MxA during infection. In support of this hypothesis, structural protein-independent MxA inhibition has been described for SFV using a viral replicon system (238), suggesting that alternative MxA targets may exist for the alphaviruses. Subsequent experiments should therefore focus on: 1) comparatively analyzing MxA binding to the bunyavirus versus alphavirus nucleocapsid and 2) identifying novel targets of MxA inhibition within the Old and New World alphaviruses. We specifically observed decreased levels of WEEV nonstructural protein 1 in response to MxA overexpression. Therefore, the alphavirus nonstructural proteins are potential targets of MxA inhibition.

Finally, we could comparatively analyze inhibition of Old versus New World alphaviruses by MxA. While exceptions exist, the Old World alphaviruses typically cause acute encephalitic disease in mice and an arthralgia-like syndrome in humans. In contrast, the New World alphaviruses are capable of causing acute encephalitic disease in humans. Therefore, it would be interesting to investigate whether MxA inhibits the replication of Old World alphaviruses to a greater extent than New World alphaviruses

within human neuronal cells. Enhanced MxA-mediated inhibition of Old World alphaviruses may suggest a role for neuronal MxA in the species-specific barrier to CNS infection.

Therapeutic potential

MxA upregulation within neurons confers enhanced protection against neurotropic arboviruses, and MxA induction is tightly regulated by a strict promoter requirement for type I (IFN- α and - β) and type III (IFN- λ) IFN (234). While excessive exposure to IFN- α has been shown to exacerbate neurotropic viral pathogenesis in the CNS (63, 113), it is yet unclear whether IFN- β and/or IFN- λ have therapeutic potential during acute viral encephalitis. Specific to IFN- β , treatment of MS patients with subtype 1b is sufficient for increased Mx expression in blood leukocytes (300), suggesting that IFN- α is not required for MxA upregulation *in vivo*. Furthermore, IFN- β -specific knockout mice demonstrate decreased Mx upregulation (301) and increased susceptibility to influenza A and coxsackievirus 3B (CV3B) (301, 302), suggesting an antiviral role for IFN- β that cannot be compensated for by IFN- α . Specific to IFN- λ , production of subtypes 1-3 has been described in human neuronal cells in response to activation of TLR3 (303). Furthermore, treatment of human neurons with IFN- λ is sufficient to inhibit replication of a neurotropic virus - herpes simplex virus - *in vitro* (304).

Therefore, IFN- β and - λ may be useful antiviral therapeutics during the acute phase of neurotropic arbovirus infection. Both IFN- β and - λ can upregulate antiviral inhibitors, including MxA, and can induce a potent antiviral response in tissue culture and *in vivo* mouse models. Finally, IFN- β has been used in the treatment of MS for over

a decade (102) and is known to be a safe therapeutic in the CNS. However, a direct investigation of IFN- β and/or - λ -mediated inhibition of neurotropic arboviruses within the CNS has yet to be conducted. Additionally, *in vivo* toxicology studies are required for IFN- β and IFN- λ at relevant antiviral treatment doses.

CONCLUSION

The work presented in this thesis has helped to define the neuronal innate immune response to a neurotropic arbovirus, with particular emphasis on how neuronal innate immune function changes with maturation. Specifically, our work identifies a novel maturation-dependent upregulation of neuronal type I IFN pathway function and demonstrates that this upregulation confers enhanced resistance to neurotropic arbovirus pathogenesis. Our careful dissection of maturation-dependent changes in the neuronal type I IFN signaling pathway *in vitro* paves the way for future investigations, which will elucidate the impact of neuron-specific innate immune function on global host defense against neurotropic arboviruses *in vivo*. Finally, as a result of the work presented in this thesis, we are one step closer to achieving the ultimate goal: the development of superior antiviral therapeutics that can be used safely in the treatment of neurotropic arbovirus encephalitis to reduce morbidity and mortality, particularly within the pediatric population.

APPENDIX:

STEM CELL CULTURE METHODS

In establishing a protocol for the routine derivation of neural progenitor cells (NPCs) and mature neurons from human embryonic stem cells (hESCs), various culture methods were explored. This appendix will summarize how we optimized our protocol for production of hESC-derived neurons.

MEDIA CONDITIONS

Optimizing NPC Culture Conditions

Zhang et al. developed NPCs from hESC lines H1 and H9 in DMEM/F12 media supplemented with N-2 ingredients (slightly modified concentrations), heparin, and fibroblast growth factor 2 (FGF-2) (*130*). In contrast, Reubinoff et al. developed NPCs from hESCs in DMEM/F12 media supplemented with B-27, glutamine, epidermal growth factor (EGF), and basic FGF (bFGF) (*131*). As Zhang et al. did not observe an increase in BrdU incorporation with combined EGF and FGF supplementation (*130*), we did not pursue the use of EGF further. However, we did comparatively analyze the effects of N-2 versus B-27 on NPC production in glutamine enriched DMEM/F12 media (**Appendix Table 1**). We qualitatively observed increased NPC viability with the use of B-27 versus

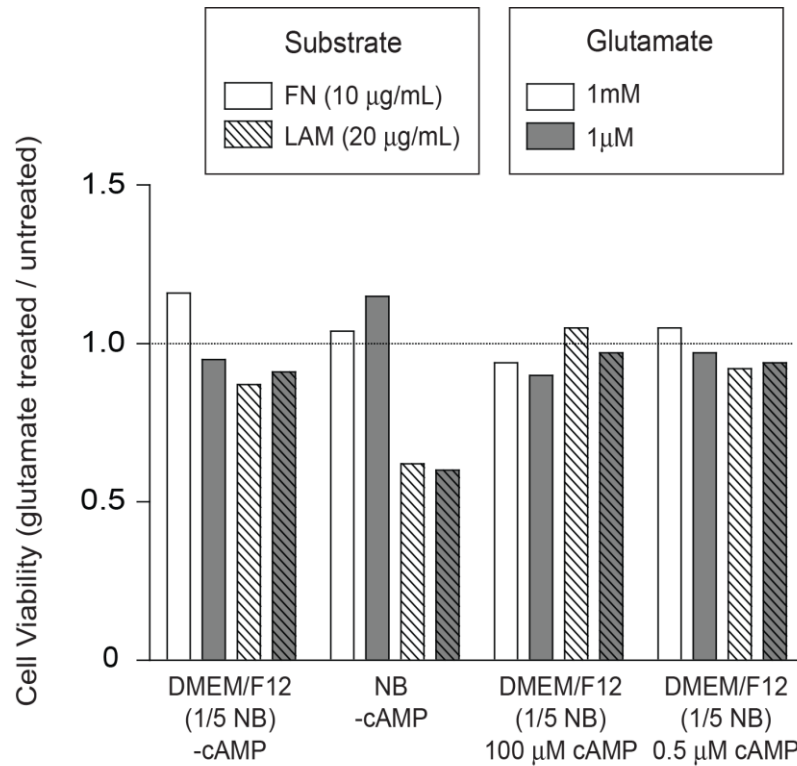
Appendix Table 1. Reagents for deriving NPCs and mature neurons from hESCs

Reagent	Product #	Special Instruction	Final []	Application
DMEM/F12	Invitrogen 11330032		1X	NPC
B-27 (minus A)	Invitrogen 12587010		2X	NPC
bFGF	Invitrogen 13256029	Dilute in 0.1% BSA to 2 µg/mL, aliquot, store at -20°C or 4°C for ≤ 1 week	20 ng/mL	NPC
noggin	R&D 1967-NG	Dilute 25µg in 250µL 0.1% BSA, store at 4°C for 1 month	500 ng/mL	NPC
Poly-D-lysine	Sigma P6407		50 µg/mL	NPC/mature
Laminin	Sigma L2020		20 µg/mL	NPC/mature
Neurobasal™	Invitrogen 21103049	store in dark	1X	mature
B-27	Invitrogen 17504044		2X	mature
N-2	Invitrogen 17502048		1X	mature
NEAA	Invitrogen 11140050		0.1 mM	mature
BDNF	ProSpec MGC34632	Dilute 10µg in no greater than 100µL H ₂ O, aliquot, store at -20°C or 4°C for ≤ 1 week	10 ng/mL	mature

N-2. However, we also observed increased NPC differentiation in response to B-27 supplementation. Therefore, we optimized the protocol by supplementing with B-27 minus vitamin A (**Appendix Table 1**), which was previously described to reduce neuronal differentiation of NPCs, as compared to the original B-27 formula (246). Accordingly, our final protocol for culturing NPCs from hESCs was as follows: DMEM/F12 media supplemented with B-27 (minus vitamin A) at 2X and bFGF at 20 ng/mL.

Optimizing Mature Neuronal Culture Conditions

Zhang et al. further developed NPCs into mature neurons in DMEM/F12 media supplemented with N-2, cAMP, and brain-derived neurotrophic factor (BDNF), in the absence of FGF-2 (130). However, subsequent protocols for deriving mature neurons have alternatively used NeurobasalTM media (247). Additionally, Reubinoff et al. continued to culture maturing neurons in B-27 supplemented media (131). Thus we chose to comparatively analyze various culture conditions for the derivation of mature neurons from NPCs. We examined sensitivity to glutamate as a maker of neuronal maturation, as glutamate-mediated cytotoxicity has previously been described in primary cultures of mature cortical neurons *in vitro* (305). We observed enhanced neuronal maturation using pure NeurobasalTM media supplemented with 1% N-2, 2% B-27, 0.1 mM non-essential amino acids, and 10 ng/mL human BDNF (**Appendix Figure 1**). Dibutyryl cyclic AMP was not found to be essential for derivation of mature neurons and was subsequently removed from the protocol. Qualitatively, hESC-derived NPCs that were cultured in pure NeurobasalTM media acquired cellular processes and tapered



Appendix Figure 1. The effects of culture conditions on neuronal maturation, as measured by glutamate-mediated cytotoxicity

Neuronal maturation is enhanced on laminin substrate in pure NeurobasalTM media; cAMP is not essential for derivation of mature neurons. hESC-derived NPCs were cultured on laminin (LAM) or fibronectin (FN) coated plates for two weeks in DMEM/F12 media, supplemented with NeurobasalTM, or pure NeurobasalTM media as indicated. Base media was further supplemented with 1% N-2, 2% B-27, 0.1 mM non-essential amino acids, 10 ng/mL human BDNF, and dibutyryl cyclic AMP (cAMP) as indicated. Cell viability was assessed by MTT assay at 24 hours post-treatment with glutamate.

replication at earlier times post-differentiation. This resulted in appreciably less dense mature neuronal cultures at the end of the two week protocol.

Finally, we attempted to enhance derivation of mature neurons through the use of mitotic inhibitors. Specifically, we tested cytosine arabinoside (Ara-C) at 1 μ M, uridine at 10 μ M, and 5-fluoro-2-deoxyuridine at 10 μ M. Although the mitotic inhibitors acutely promoted neuronal differentiation, the treatment became toxic to the cells at approximately one week post-differentiation, resulting in low yields of fibroblast-like cells by the end of the two week protocol. Accordingly, we refrained from using mitotic inhibitors in subsequent differentiations.

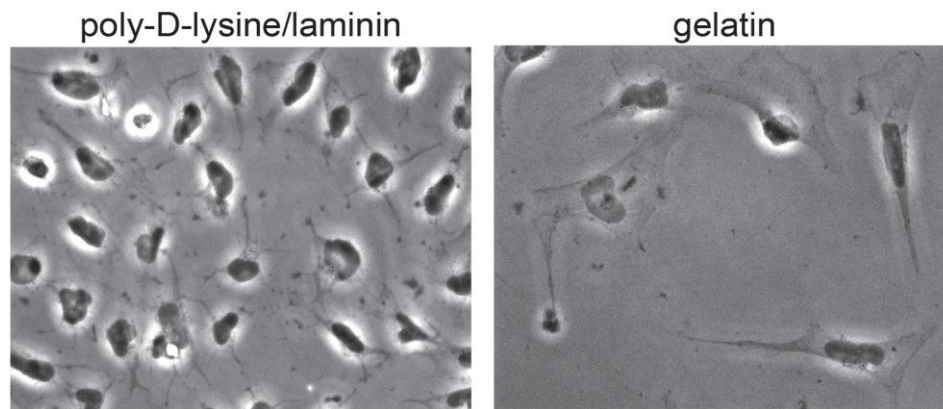
PLATING CONDITIONS

Plating Density

Various methods have been described for dissociating neuroectoderm including mechanical trituration by pipette (130) in addition to enzymatic digestion (247). In our hands, trypsin-EDTA treatment resulted in low NPC yields post-plating. Accordingly, we triturated our neurospheres for at least one minute prior to plating. This step of the protocol was extremely sensitive to technical error. Failure to sufficiently triturate neurospheres resulted in the laying down of large cellular clumps that were difficult to differentiate into mature neurons. In contrast, overly aggressive pipetting was highly toxic to the NPCs and resulted in low yields. Accordingly, plating the NPCs as clusters of 10-100 cells, at approximately 10 clusters per 35mm dish, is optimal for NPC survival and subsequent differentiation.

Extracellular Matrices

Various extracellular matrices have been described to support neuronal differentiation of hESCs including combinations of poly-D-lysine, ornithine, laminin, collagen, gelatin, fibronectin, and more recently, MatrigelTM (248). We comparatively analyzed NPC differentiation on the following substrates: gelatin (1x), ornithine (15 µg/mL)/fibronectin (10 µg/mL), poly-D-lysine (10-50 µg/mL)/laminin (20 µg/mL), and MatrigelTM (1/15 volume dilution in media, 1mL per 35mm dish). Post-neurosphere plating, we observed distinct, substrate-dependent cellular morphologies. Specifically, cells plated on either poly-D-lysine/laminin or MatrigelTM were observed to have contracted perikaryon and a number of small neurites, consistent with what has been previously described for NPCs (248). In contrast, cells plated on gelatin were observed to have large cell bodies with fibroblast-like extensions (**Appendix Figure 2**). Despite these divergent morphologies, all cells expressed the NPC marker, nestin. Accordingly, we chose to further examine substrate support of NPC differentiation to mature neurons. Over the course of the two week protocol, MatrigelTM, poly-D-lysine/laminin, and ornithine/fibronectin supported enhanced morphological differentiation to mature neurons. Specific to the fibronectin cultures, the cells were observed to organize into large multi-cellular tracts. However, immunocytochemistry analysis demonstrated decreased expression of mature neuronal markers in the fibronectin-supported cultures compared to the laminin-supported cultures. Moreover, laminin supported enhanced mature neuronal differentiation as measured by sensitivity to glutamate-mediated cytotoxicity (**Appendix Figure 1**). Accordingly, a poly-D-lysine/laminin substrate was



Appendix Figure 2. Extracellular matrices impact NPC morphology post-plating

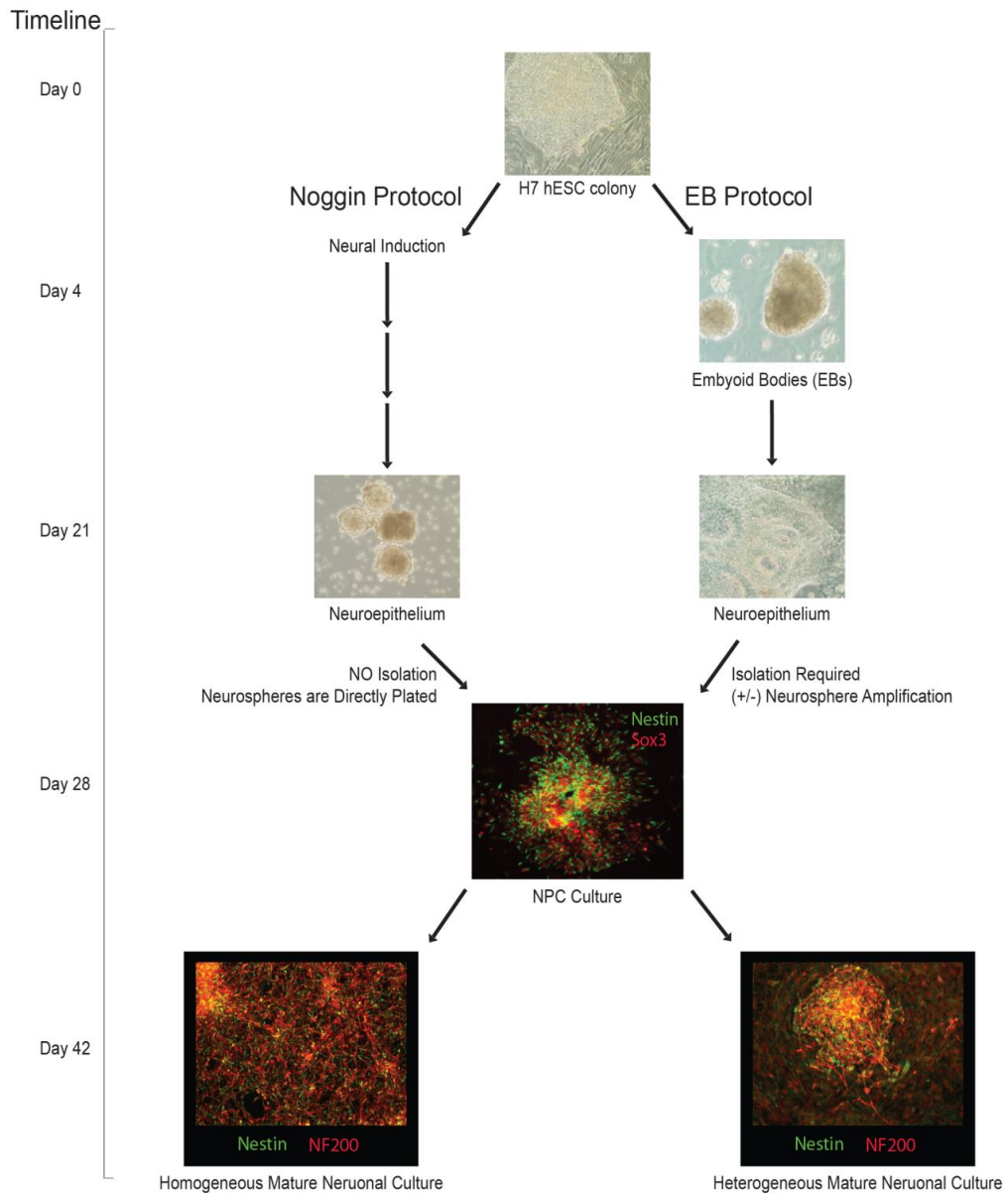
Brightfield images were taken two days following neurosphere plating. Cells plated on poly-D-lysine/laminin were observed to have contracted cell bodies and a number of small neurites. In contrast, cells plated on gelatin were observed to have large cell bodies and fibroblast-like extensions.

used in subsequent protocols. While MatrigelTM also robustly supported neuronal differentiation of hESCs, the reagent cost was prohibitive.

METHODS FOR NPC DERIVATION

Formation of Embryoid Bodies

Initially we attempted to derive NPCs from hESCs through the formation of embryoid bodies (EBs) as previously described (130) (**Appendix Figure 3**). In brief, H7 colonies were mechanically isolated from the mouse embryonic fibroblast (MEF) feeder layer and differentiated to EBs over the course of four days in low attachment plates and media lacking bFGF. Following this free-form differentiation period, EBs were collected, triturated, and plated on 1x gelatin in media supplemented with bFGF. The plated EBs were differentiated for a minimum of 21 days, at which point neuroepithelial rosettes could be clearly visualized. The neuroepithelium was subsequently mechanically dissected away from surrounding mesoderm, endoderm, and ectoderm, and was either: 1) directly replated or 2) further expanded as a neurosphere suspension culture prior to plating. In both adaptations of the EB protocol, we detected Sox3-positive and nestin-positive cells by immunocytochemistry post-plating. While nestin expression was rather homogeneous throughout the cultures, Sox3 expression was very heterogeneous, with a minority of cells (around 20%) staining robustly positive for Sox3. These data indicated to us that we were not producing pure populations of NPCs. Additionally, suspension culturing the neurospheres prior to plating did result in a more homogeneous Sox3 signal. However, it also resulted in a substantial degree of neuronal



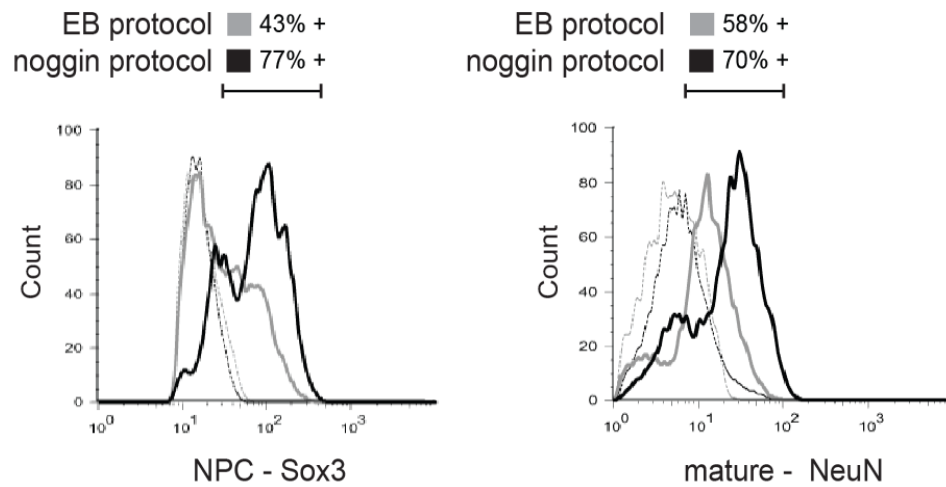
Appendix Figure 3. NPC derivation protocols, comparison by immunocytochemistry

The noggin protocol is superior for deriving pure neuronal populations from hESCs. hESCs were free-form differentiated into embryoid bodies (EB protocol) or induced to directly from neuroepithelium via noggin treatment (noggin protocol). Technical differences are indicated. Protocol outcomes were analyzed by immunocytochemistry, probing for expression of the indicated NPC or mature neuronal markers. Representative overlay images are shown.

differentiation in the NPC cultures (higher NF200 signal). Based on these data, we decided to explore alternative methods for deriving NPCs from hESCs.

Directed Neural Induction with Noggin

Derivation of NPCs from hESCs via directed neural induction with 500 ng/mL of noggin, a bone morphogenic protein (BMP) antagonist, has also been described (246) (**Appendix Figure 3**). In this protocol, hESCs are not allowed to freely differentiate into EBs, rather formation of neuroepithelium is directly induced. This adaptation eliminates the need to preferentially dissect neuroepithelial rosettes at later stages of differentiation and is therefore less technically advanced. In brief, H7 colonies were mechanically isolated from the MEF feeder layer (this is in contrast to the published protocol, which uses collagenase to dissociate the hESC colonies (246)). Subsequent to isolation, the hESCs were differentiated into neuroepithelium over the course of three weeks in low attachment plates and noggin-supplemented NPC media. After three weeks in suspension culture, neurospheres were clearly visualized. The neurospheres were collected, triturated to smaller aggregates, plated on poly-D-lysine/laminin, and allowed to expand as single cell NPC cultures for an additional one week prior to analysis with daily changes of noggin-free NPC media. The timing of our neurosphere plating is in contrast to the published protocol, which holds the neuroepithelium for an additional week in suspension culture, though with a similar change to noggin-free NPC media (246). Our adaptation was optimized to limit mature neuronal differentiation in the NPC cultures. However, NPC viability was qualitatively observed to be lower post-plating at three



Appendix Figure 4. NPC derivation protocols, comparison by flow cytometry

The noggin protocol is superior for deriving pure neuronal populations from hESCs. hESCs were free-form differentiated into embryoid bodies (EB protocol) or induced to directly from neuroepithelium via noggin treatment (noggin protocol). Protocol outcomes were analyzed by flow cytometry, probing for intracellular expression of Sox3 or NeuN in the NPC and mature neuronal cultures, respectively. Representative histograms are shown.

versus four weeks of suspension culture growth. Therefore, if NPC purity is not a concern, four weeks of suspension culture growth is optimal.

NPC cultures derived from hESCs via noggin induction were appreciably less diverse as qualitatively assessed by immunocytochemistry, staining for Sox3 and nestin, and quantitatively assessed by flow cytometry, staining for Sox3 (**Appendix Figure 4, left panel**). Additionally, NPC cultures derived using noggin induction produced less diverse populations of mature neurons, as quantitatively assessed by flow cytometry, staining for NeuN (**Appendix Figure 4, right panel**). Therefore, noggin induction was determined to be the superior method for deriving enriched populations of NPCs and mature neurons from H7 hESC colonies.

REFERENCES

1. Romero, J. R. (2008) Arboviruses, In *The Neurological Manifestations of Pediatric Infectious Diseases and Immunodeficiency Syndromes* (Barton, L. L., and Friedman, N. R., Eds.), pp 83-104, Humana Press, Totowa, New Jersey.
2. Hanson, R. P. (1957) An epizootic of equine encephalomyelitis that occurred in Massachusetts in 1831, *Am J Trop Med Hyg* 6, 858-862.
3. Meyer, K. F., Haring, C. M., and Howitt, B. (1931) The Etiology of Epizootic Encephalomyelitis of Horses in the San Joaquin Valley, 1930, *Science* 74, 227-228.
4. Lowry, P. W. (1997) Arbovirus encephalitis in the United States and Asia, *J Lab Clin Med* 129, 405-411.
5. Kuno, G., and Chang, G. J. (2005) Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends, *Clin Microbiol Rev* 18, 608-637.
6. Khoo, C. C., Piper, J., Sanchez-Vargas, I., Olson, K. E., and Franz, A. W. (2010) The RNA interference pathway affects midgut infection- and escape barriers for Sindbis virus in *Aedes aegypti*, *BMC Microbiol* 10, 130.
7. Wang, Z., Zhang, X., Li, C., Zhang, Y., Xin, D., and Zhao, T. (2010) Dissemination of western equine encephalomyelitis virus in the potential vector, *Culex pipiens pallens*, *J Vector Ecol* 35, 313-317.
8. Siler, J. F., Hall, M. W., and Hitchens, A. P. (1925) Transmission of dengue fever by mosquitoes, *P Soc Exp Biol Med* 23, 197-201.
9. Siler, J. F., Hall, M. W., and Hitchens, A. P. (1925) Results obtained in the transmission of dengue fever, *J Amer Med Assoc* 84, 1163-1172.

10. Griffin, D. E. (2001) Alphaviruses, In *Fields Virology, 4th Edition* (Knipe, D. M., and Howley, P. M., Eds.) 4 ed., pp 917-962, Lippincott-Raven, Philadelphia, PA.
11. Tsetsarkin, K. A., Vanlandingham, D. L., McGee, C. E., and Higgs, S. (2007) A single mutation in chikungunya virus affects vector specificity and epidemic potential, *PLoS Pathog* 3, e201.
12. (2005) Information of Arboviral Encephalitides, *Center for Disease Control and Prevention* <http://www.cdc.gov/ncidod/dvbid/arbor/arbdet.htm>.
13. Roos, K. L. (1999) Encephalitis, *Neurol Clin* 17, 813-833.
14. Grimley, P. M., and Friedman, R. M. (1970) Arboviral infection of voluntary striated muscles, *J Infect Dis* 122, 45-52.
15. Johnson, R. T. (1965) Virus Invasion of the Central Nervous System: A Study of Sindbis Virus Infection in the Mouse Using Fluorescent Antibody, *Am J Pathol* 46, 929-943.
16. Liu, C., Voth, D. W., Rodina, P., Shauf, L. R., and Gonzalez, G. (1970) A comparative study of the pathogenesis of western equine and eastern equine encephalomyelitis viral infections in mice by intracerebral and subcutaneous inoculations, *J Infect Dis* 122, 53-63.
17. Johnston, L. J., Halliday, G. M., and King, N. J. (2000) Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus, *J Invest Dermatol* 114, 560-568.
18. Dropulic, B., and Masters, C. L. (1990) Entry of neurotropic arboviruses into the central nervous system: an in vitro study using mouse brain endothelium, *J Infect Dis* 161, 685-691.
19. Cook, S. H., and Griffin, D. E. (2003) Luciferase imaging of a neurotropic viral infection in intact animals, *J Virol* 77, 5333-5338.
20. Monath, T. P., Cropp, C. B., and Harrison, A. K. (1983) Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy, *Lab Invest* 48, 399-410.

21. Charles, P. C., Walters, E., Margolis, F., and Johnston, R. E. (1995) Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse, *Virology* 208, 662-671.
22. Ehrenguber, M. U., Lundstrom, K., Schweitzer, C., Heuss, C., Schlesinger, S., and Gahwiler, B. H. (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures, *Proc Natl Acad Sci U S A* 96, 7041-7046.
23. Oliver, K. R., and Fazakerley, J. K. (1998) Transneuronal spread of Semliki Forest virus in the developing mouse olfactory system is determined by neuronal maturity, *Neuroscience* 82, 867-877.
24. German, A. C., Myint, K. S., Mai, N. T., Pomeroy, I., Phu, N. H., Tzartos, J., Winter, P., Collett, J., Farrar, J., Barrett, A., Kipar, A., Esiri, M. M., and Solomon, T. (2006) A preliminary neuropathological study of Japanese encephalitis in humans and a mouse model, *Trans R Soc Trop Med Hyg* 100, 1135-1145.
25. Vogel, P., Kell, W. M., Fritz, D. L., Parker, M. D., and Schoepp, R. J. (2005) Early events in the pathogenesis of eastern equine encephalitis virus in mice, *Am J Pathol* 166, 159-171.
26. Jackson, A. C., Moench, T. R., Griffin, D. E., and Johnson, R. T. (1987) The pathogenesis of spinal cord involvement in the encephalomyelitis of mice caused by neuroadapted Sindbis virus infection, *Lab Invest* 56, 418-423.
27. Pekosz, A., Phillips, J., Pleasure, D., Merry, D., and Gonzalez-Scarano, F. (1996) Induction of apoptosis by La Crosse virus infection and role of neuronal differentiation and human bcl-2 expression in its prevention, *J Virol* 70, 5329-5335.
28. Allsopp, T. E., Scallan, M. F., Williams, A., and Fazakerley, J. K. (1998) Virus infection induces neuronal apoptosis: A comparison with trophic factor withdrawal, *Cell Death Differ* 5, 50-59.
29. Mussgay, M., Enzmann, P. J., and Horst, J. (1970) Influence of an arbovirus infection (Sindbis virus) on the protein and ribonucleic acid synthesis of cultivated chick embryo cells, *Arch Gesamte Virusforsch* 31, 81-92.
30. Ubol, S., Park, S., Budihardjo, I., Desnoyers, S., Montrose, M. H., Poirier, G. G., Kaufmann, S. H., and Griffin, D. E. (1996) Temporal changes in chromatin,

intracellular calcium, and poly(ADP-ribose) polymerase during Sindbis virus-induced apoptosis of neuroblastoma cells, *J Virol* 70, 2215-2220.

31. Garry, R. F., Bishop, J. M., Parker, S., Westbrook, K., Lewis, G., and Waite, M. R. (1979) Na⁺ and K⁺ concentrations and the regulation of protein synthesis in Sindbis virus-infected chick cells, *Virology* 96, 108-120.
32. Lewis, J., Wesselingh, S. L., Griffin, D. E., and Hardwick, J. M. (1996) Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence, *J Virol* 70, 1828-1835.
33. Bourne, N., Scholle, F., Silva, M. C., Rossi, S. L., Dewsbury, N., Judy, B., De Aguiar, J. B., Leon, M. A., Estes, D. M., Fayzulin, R., and Mason, P. W. (2007) Early production of type I interferon during West Nile virus infection: role for lymphoid tissues in IRF3-independent interferon production, *J Virol* 81, 9100-9108.
34. Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989) Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins, *J Virol* 63, 3142-3150.
35. Marcus, P. I., and Fuller, F. J. (1979) Interferon induction by viruses. II. Sindbis virus: interferon induction requires one-quarter of the genome--genes G and A, *J Gen Virol* 44, 169-177.
36. Wahlberg, J. M., Bron, R., Wilschut, J., and Garoff, H. (1992) Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein, *J Virol* 66, 7309-7318.
37. Ross, T. M., Martinez, P. M., Renner, J. C., Thorne, R. G., Hanson, L. R., and Frey, W. H., 2nd. (2004) Intranasal administration of interferon beta bypasses the blood-brain barrier to target the central nervous system and cervical lymph nodes: a non-invasive treatment strategy for multiple sclerosis, *J Neuroimmunol* 151, 66-77.
38. Wang, J., Campbell, I. L., and Zhang, H. (2008) Systemic interferon-alpha regulates interferon-stimulated genes in the central nervous system, *Mol Psychiatry* 13, 293-301.

39. Ryman, K. D., Klimstra, W. B., Nguyen, K. B., Biron, C. A., and Johnston, R. E. (2000) Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism, *J Virol* 74, 3366-3378.
40. Samuel, M. A., and Diamond, M. S. (2005) Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival, *J Virol* 79, 13350-13361.
41. White, L. J., Wang, J. G., Davis, N. L., and Johnston, R. E. (2001) Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region, *J Virol* 75, 3706-3718.
42. Lobigs, M., Mullbacher, A., Wang, Y., Pavy, M., and Lee, E. (2003) Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus, *J Gen Virol* 84, 567-572.
43. Fragkoudis, R., Breakwell, L., McKimmie, C., Boyd, A., Barry, G., Kohl, A., Merits, A., and Fazakerley, J. K. (2007) The type I interferon system protects mice from Semliki Forest virus by preventing widespread virus dissemination in extraneural tissues, but does not mediate the restricted replication of avirulent virus in central nervous system neurons, *J Gen Virol* 88, 3373-3384.
44. Couderc, T., Chretien, F., Schilte, C., Disson, O., Brigitte, M., Guivel-Benhassine, F., Touret, Y., Barau, G., Cayet, N., Schuffenecker, I., Despres, P., Arenzana-Seisdedos, F., Michault, A., Albert, M. L., and Lecuit, M. (2008) A Mouse Model for Chikungunya: Young Age and Inefficient Type-I Interferon Signaling Are Risk Factors for Severe Disease, *PLoS Pathog* 4, e29.
45. Griffin, D. E. (1976) Role of the immune response in age-dependent resistance of mice to encephalitis due to Sindbis virus, *J Infect Dis* 133, 456-464.
46. Jahrling, P. B., Hesse, R. A., Anderson, A. O., and Gangemi, J. D. (1983) Oponization of alphaviruses in hamsters, *J Med Virol* 12, 1-16.
47. Levine, B., Hardwick, J. M., Trapp, B. D., Crawford, T. O., Bollinger, R. C., and Griffin, D. E. (1991) Antibody-mediated clearance of alphavirus infection from neurons, *Science* 254, 856-860.
48. Despres, P., Griffin, J. W., and Griffin, D. E. (1995) Antiviral activity of alpha interferon in Sindbis virus-infected cells is restored by anti-E2 monoclonal antibody treatment, *J Virol* 69, 7345-7348.

49. Griffin, D., Levine, B., Tyor, W., Ubol, S., and Despres, P. (1997) The role of antibody in recovery from alphavirus encephalitis, *Immunol Rev* 159, 155-161.
50. Kimura, T., and Griffin, D. E. (2000) The role of CD8(+) T cells and major histocompatibility complex class I expression in the central nervous system of mice infected with neurovirulent Sindbis virus, *J Virol* 74, 6117-6125.
51. Binder, G. K., and Griffin, D. E. (2001) Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons, *Science* 293, 303-306.
52. Griffin, D. E. (2003) Immune responses to RNA-virus infections of the CNS, *Nat Rev Immunol* 3, 493-502.
53. Przelomski, M. M., O'Rourke, E., Grady, G. F., Berardi, V. P., and Markley, H. G. (1988) Eastern equine encephalitis in Massachusetts: a report of 16 cases, 1970-1984, *Neurology* 38, 736-739.
54. Earnest, M. P., Goolishian, H. A., Calverley, J. R., Hayes, R. O., and Hill, H. R. (1971) Neurologic, intellectual, and psychologic sequelae following western encephalitis. A follow-up study of 35 cases, *Neurology* 21, 969-974.
55. Longshore, W. A., Jr., Stevens, I. M., Hollister, A. C., Jr., Gittelsohn, A., and Lennette, E. H. (1956) Epidemiologic observations on acute infectious encephalitis in California, with special reference to the 1952 outbreak, *Am J Hyg* 63, 69-86.
56. Hurwitz, E. S., Schell, W., Nelson, D., Washburn, J., and LaVenture, M. (1983) Surveillance for California encephalitis group virus illness in Wisconsin and Minnesota, 1978, *Am J Trop Med Hyg* 32, 595-601.
57. Murray, K. O., Walker, C., and Gould, E. (2011) The virology, epidemiology, and clinical impact of West Nile virus: a decade of advancements in research since its introduction into the Western Hemisphere, *Epidemiol Infect*, 1-11.
58. Monath, T. P., and Tsai, T. F. (1987) St. Louis encephalitis: lessons from the last decade, *Am J Trop Med Hyg* 37, 40S-59S.
59. Taylor, R. M., Hurlbut, H. S., Work, T. H., Kingston, J. R., and Frothingham, T. E. (1955) Sindbis virus: a newly recognized arthropodtransmitted virus, *Am J Trop Med Hyg* 4, 844-862.

60. Griffin, D. E., Levine, B., Ubol, S., and Hardwick, J. M. (1994) The effects of alphavirus infection on neurons, *Ann Neurol 35 Suppl*, S23-27.
61. Johnson, R. T., McFarland, H. F., and Levy, S. E. (1972) Age-dependent resistance to viral encephalitis: studies of infections due to Sindbis virus in mice, *J Infect Dis 125*, 257-262.
62. Ogata, A., Nagashima, K., Hall, W. W., Ichikawa, M., Kimura-Kuroda, J., and Yasui, K. (1991) Japanese encephalitis virus neurotropism is dependent on the degree of neuronal maturity, *J Virol 65*, 880-886.
63. Reinartz, A. B., Broome, M. G., and Sagik, B. P. (1971) Age-dependent resistance of mice to sindbis virus infection: viral replication as a function of host age, *Infect Immun 3*, 268-273.
64. Oliver, K. R., Scallan, M. F., Dyson, H., and Fazakerley, J. K. (1997) Susceptibility to a neurotropic virus and its changing distribution in the developing brain is a function of CNS maturity, *J Neurovirol 3*, 38-48.
65. Fazakerley, J. K., Pathak, S., Scallan, M., Amor, S., and Dyson, H. (1993) Replication of the A7(74) strain of Semliki Forest virus is restricted in neurons, *Virology 195*, 627-637.
66. Pattyn, S. R., De Vleeschauwer, L., and van der Groen, G. (1975) Replication of arboviruses in mouse organ cultures. II. Multiplication of virulent and avirulent Semliki Forest and western equine encephalitis viruses in mouse organ cultures, *Arch Virol 49*, 33-37.
67. Burdeinick-Kerr, R., and Griffin, D. E. (2005) Gamma interferon-dependent, noncytolytic clearance of sindbis virus infection from neurons in vitro, *J Virol 79*, 5374-5385.
68. Castorena, K. M., Peltier, D. C., Peng, W., and Miller, D. J. (2008) Maturation-dependent responses of human neuronal cells to western equine encephalitis virus infection and type I interferons, *Virology 372*, 208-220.
69. Chakraborty, S., Nazmi, A., Dutta, K., and Basu, A. (2010) Neurons under viral attack: victims or warriors?, *Neurochem Int 56*, 727-735.

70. Carrithers, M. D., Visintin, I., Kang, S. J., and Janeway, C. A., Jr. (2000) Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment, *Brain* 123 (Pt 6), 1092-1101.
71. Irani, D. N., and Griffin, D. E. (1996) Regulation of lymphocyte homing into the brain during viral encephalitis at various stages of infection, *J Immunol* 156, 3850-3857.
72. Fabry, Z., Topham, D. J., Fee, D., Herlein, J., Carlino, J. A., Hart, M. N., and Sriram, S. (1995) TGF-beta 2 decreases migration of lymphocytes in vitro and homing of cells into the central nervous system in vivo, *J Immunol* 155, 325-332.
73. Wekerle, H., Linington, C., Lassmann, H., and Meyermann, R. (1986) Cellular Immune Reactivity within the Cns, *Trends Neurosci* 9, 271-277.
74. Hickey, W. F., Hsu, B. L., and Kimura, H. (1991) T-lymphocyte entry into the central nervous system, *J Neurosci Res* 28, 254-260.
75. Irani, D. N., Lin, K. I., and Griffin, D. E. (1996) Brain-derived gangliosides regulate the cytokine production and proliferation of activated T cells, *J Immunol* 157, 4333-4340.
76. Flugel, A., Schwaiger, F. W., Neumann, H., Medana, I., Willem, M., Wekerle, H., Kreutzberg, G. W., and Graeber, M. B. (2000) Neuronal FasL induces cell death of encephalitogenic T lymphocytes, *Brain Pathol* 10, 353-364.
77. Neumann, H., Misgeld, T., Matsumuro, K., and Wekerle, H. (1998) Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor, *Proc Natl Acad Sci U S A* 95, 5779-5784.
78. Cardona, A. E., Pioro, E. P., Sasse, M. E., Kostenko, V., Cardona, S. M., Dijkstra, I. M., Huang, D., Kidd, G., Dombrowski, S., Dutta, R., Lee, J. C., Cook, D. N., Jung, S., Lira, S. A., Littman, D. R., and Ransohoff, R. M. (2006) Control of microglial neurotoxicity by the fractalkine receptor, *Nat Neurosci* 9, 917-924.
79. Hoek, R. M., Ruuls, S. R., Murphy, C. A., Wright, G. J., Goddard, R., Zurawski, S. M., Blom, B., Homola, M. E., Streit, W. J., Brown, M. H., Barclay, A. N., and Sedgwick, J. D. (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200), *Science* 290, 1768-1771.

80. Massa, P. T. (1993) Specific suppression of major histocompatibility complex class I and class II genes in astrocytes by brain-enriched gangliosides, *J Exp Med* 178, 1357-1363.
81. Bergmann, C. C., Lane, T. E., and Stohlman, S. A. (2006) Coronavirus infection of the central nervous system: host-virus stand-off, *Nat Rev Microbiol* 4, 121-132.
82. Ghoshal, A., Das, S., Ghosh, S., Mishra, M. K., Sharma, V., Koli, P., Sen, E., and Basu, A. (2007) Proinflammatory mediators released by activated microglia induces neuronal death in Japanese encephalitis, *Glia* 55, 483-496.
83. Mishra, M. K., Koli, P., Bhowmick, S., and Basu, A. (2007) Neuroprotection conferred by astrocytes is insufficient to protect animals from succumbing to Japanese encephalitis, *Neurochem Int* 50, 764-773.
84. Pope, J. G., Vanderlugt, C. L., Rahbe, S. M., Lipton, H. L., and Miller, S. D. (1998) Characterization of and functional antigen presentation by central nervous system mononuclear cells from mice infected with Theiler's murine encephalomyelitis virus, *J Virol* 72, 7762-7771.
85. Schafer, A., Whitmore, A. C., Konopka, J. L., and Johnston, R. E. (2009) Replicon particles of Venezuelan equine encephalitis virus as a reductionist murine model for encephalitis, *J Virol* 83, 4275-4286.
86. Massa, P. T., Whitney, L. W., Wu, C., Ropka, S. L., and Jarosinski, K. W. (1999) A mechanism for selective induction of 2'-5' oligoadenylate synthetase, anti-viral state, but not MHC class I genes by interferon-beta in neurons, *J Neurovirol* 5, 161-171.
87. Jarosinski, K. W., Whitney, L. W., and Massa, P. T. (2001) Specific deficiency in nuclear factor-kappaB activation in neurons of the central nervous system, *Lab Invest* 81, 1275-1288.
88. Medana, I., Li, Z., Flugel, A., Tschopp, J., Wekerle, H., and Neumann, H. (2001) Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity, *J Immunol* 167, 674-681.
89. Minghetti, L., Ajmone-Cat, M. A., De Bernardinis, M. A., and De Simone, R. (2005) Microglial activation in chronic neurodegenerative diseases: roles of apoptotic neurons and chronic stimulation, *Brain Res Brain Res Rev* 48, 251-256.

90. Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007) Neuronal 'On' and 'Off' signals control microglia, *Trends Neurosci* 30, 596-602.
91. Levite, M. (2008) Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors, *Curr Opin Pharmacol* 8, 460-471.
92. Rassa, J. C., Meyers, J. L., Zhang, Y., Kudravalli, R., and Ross, S. R. (2002) Murine retroviruses activate B cells via interaction with toll-like receptor 4, *Proc Natl Acad Sci U S A* 99, 2281-2286.
93. Bieback, K., Lien, E., Klagge, I. M., Avota, E., Schneider-Schaulies, J., Duprex, W. P., Wagner, H., Kirschning, C. J., Ter Meulen, V., and Schneider-Schaulies, S. (2002) Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling, *J Virol* 76, 8729-8736.
94. Zhou, Y., Ye, L., Wan, Q., Zhou, L., Wang, X., Li, J., Hu, S., Zhou, D., and Ho, W. (2009) Activation of Toll-like receptors inhibits herpes simplex virus-1 infection of human neuronal cells, *J Neurosci Res* 87, 2916-2925.
95. Peltier, D. C., Simms, A., Farmer, J. R., and Miller, D. J. (2010) Human Neuronal Cells Possess Functional Cytoplasmic and TLR-Mediated Innate Immune Pathways Influenced by Phosphatidylinositol-3 Kinase Signaling, *Journal of Immunology* 184, 7010-7021.
96. Lafon, M., Megret, F., Lafage, M., and Prehaud, C. (2006) The innate immune facet of brain: human neurons express TLR-3 and sense viral dsRNA, *J Mol Neurosci* 29, 185-194.
97. Delhaye, S., Paul, S., Blakqori, G., Minet, M., Weber, F., Staeheli, P., and Michiels, T. (2006) Neurons produce type I interferon during viral encephalitis, *Proc Natl Acad Sci U S A* 103, 7835-7840.
98. Daffis, S., Samuel, M. A., Suthar, M. S., Gale, M., Jr., and Diamond, M. S. (2008) Toll-like receptor 3 has a protective role against West Nile virus infection, *J Virol* 82, 10349-10358.
99. Schijns, V. E., Van der Neut, R., Haagmans, B. L., Bar, D. R., Schellekens, H., and Horzinek, M. C. (1991) Tumour necrosis factor-alpha, interferon-gamma and interferon-beta exert antiviral activity in nervous tissue cells, *J Gen Virol* 72 (Pt 4), 809-815.

100. Wang, J., and Campbell, I. L. (2005) Innate STAT1-dependent genomic response of neurons to the antiviral cytokine alpha interferon, *J Virol* 79, 8295-8302.
101. Detje, C. N., Meyer, T., Schmidt, H., Kreuz, D., Rose, J. K., Bechmann, I., Prinz, M., and Kalinke, U. (2009) Local type I IFN receptor signaling protects against virus spread within the central nervous system, *J Immunol* 182, 2297-2304.
102. Paty, D. W., and Li, D. K. (1993) Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group, *Neurology* 43, 662-667.
103. Teige, I., Treschow, A., Teige, A., Mattsson, R., Navikas, V., Leanderson, T., Holmdahl, R., and Issazadeh-Navikas, S. (2003) IFN-beta gene deletion leads to augmented and chronic demyelinating experimental autoimmune encephalomyelitis, *J Immunol* 170, 4776-4784.
104. Furlan, R., Bergami, A., Lang, R., Brambilla, E., Franciotta, D., Martinelli, V., Comi, G., Panina, P., and Martino, G. (2000) Interferon-beta treatment in multiple sclerosis patients decreases the number of circulating T cells producing interferon-gamma and interleukin-4, *J Neuroimmunol* 111, 86-92.
105. Lou, J., Gasche, Y., Zheng, L., Giroud, C., Morel, P., Clements, J., Ythier, A., and Grau, G. E. (1999) Interferon-beta inhibits activated leukocyte migration through human brain microvascular endothelial cell monolayer, *Lab Invest* 79, 1015-1025.
106. Yong, V. W. (2002) Differential mechanisms of action of interferon-beta and glatiramer acetate in MS, *Neurology* 59, 802-808.
107. Hohlfeld, R., and Wekerle, H. (2001) Immunological update on multiple sclerosis, *Curr Opin Neurol* 14, 299-304.
108. Liu, Z., Pelfrey, C. M., Cotleur, A., Lee, J. C., and Rudick, R. A. (2001) Immunomodulatory effects of interferon beta-1a in multiple sclerosis, *J Neuroimmunol* 112, 153-162.
109. Wang, X., Chen, M., Wandinger, K. P., Williams, G., and Dhib-Jalbut, S. (2000) IFN-beta-1b inhibits IL-12 production in peripheral blood mononuclear cells in an IL-10-dependent mechanism: relevance to IFN-beta-1b therapeutic effects in multiple sclerosis, *J Immunol* 165, 548-557.

110. Merimsky, O., and Chaitchik, S. (1992) Neurotoxicity of interferon-alpha, *Anticancer Drugs* 3, 567-570.
111. Campbell, I. L., Krucker, T., Steffensen, S., Akwa, Y., Powell, H. C., Lane, T., Carr, D. J., Gold, L. H., Henriksen, S. J., and Siggins, G. R. (1999) Structural and functional neuropathology in transgenic mice with CNS expression of IFN-alpha, *Brain Res* 835, 46-61.
112. Wang, J., Schreiber, R. D., and Campbell, I. L. (2002) STAT1 deficiency unexpectedly and markedly exacerbates the pathophysiological actions of IFN-alpha in the central nervous system, *Proc Natl Acad Sci U S A* 99, 16209-16214.
113. Sas, A. R., Bimonte-Nelson, H., Smothers, C. T., Woodward, J., and Tyor, W. R. (2009) Interferon-alpha causes neuronal dysfunction in encephalitis, *J Neurosci* 29, 3948-3955.
114. Temple, S. (2001) The development of neural stem cells, *Nature* 414, 112-117.
115. Lederer, C. W., and Santama, N. (2008) Neural stem cells: mechanisms of fate specification and nuclear reprogramming in regenerative medicine, *Biotechnol J* 3, 1521-1538.
116. Temple, S. (1989) Division and differentiation of isolated CNS blast cells in microculture, *Nature* 340, 471-473.
117. Cattaneo, E., and McKay, R. (1990) Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor, *Nature* 347, 762-765.
118. Reynolds, B. A., Tetzlaff, W., and Weiss, S. (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes, *J Neurosci* 12, 4565-4574.
119. Kilpatrick, T. J., and Bartlett, P. F. (1993) Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation, *Neuron* 10, 255-265.
120. Reynolds, B. A., and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system, *Science* 255, 1707-1710.

121. Lois, C., and Alvarez-Buylla, A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia, *Proc Natl Acad Sci U S A* 90, 2074-2077.
122. Pencea, V., Bingaman, K. D., Freedman, L. J., and Luskin, M. B. (2001) Neurogenesis in the subventricular zone and rostral migratory stream of the neonatal and adult primate forebrain, *Exp Neurol* 172, 1-16.
123. Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., and Gage, F. H. (1998) Neurogenesis in the adult human hippocampus, *Nat Med* 4, 1313-1317.
124. Kukekov, V. G., Laywell, E. D., Suslov, O., Davies, K., Scheffler, B., Thomas, L. B., O'Brien, T. F., Kusakabe, M., and Steindler, D. A. (1999) Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain, *Exp Neurol* 156, 333-344.
125. Kazanis, I., Lathia, J., Moss, L., and French-Constant, C. (2008) The neural stem cell microenvironment.
126. Maslov, A. Y., Barone, T. A., Plunkett, R. J., and Pruitt, S. C. (2004) Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice, *Journal of Neuroscience* 24, 1726-1733.
127. Sarnat, H. B. (2006) Role of the Fetal Ependyma, In *Child Neurology Seventh Edition* (Menkes, J. H., Sarnat, H.B., and Maria, B.L., Ed.), pp 274-275, Lippincott Williams & Wilkins, Philadelphia, PA.
128. Rao, M. S., Hattiangady, B., and Shetty, A. K. (2006) The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocampus, *Aging Cell* 5, 545-558.
129. Aizawa, K., Ageyama, N., Terao, K., and Hisatsune, T. (2011) Primate-specific alterations in neural stem/progenitor cells in the aged hippocampus, *Neurobiol Aging* 32, 140-150.
130. Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O., and Thomson, J. A. (2001) In vitro differentiation of transplantable neural precursors from human embryonic stem cells, *Nat Biotechnol* 19, 1129-1133.

131. Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T. (2001) Neural progenitors from human embryonic stem cells, *Nat Biotechnol* 19, 1134-1140.
132. Dhara, S. K., and Stice, S. L. (2008) Neural differentiation of human embryonic stem cells, *J Cell Biochem* 105, 633-640.
133. Kosugi, I., Shinmura, Y., Kawasaki, H., Arai, Y., Li, R. Y., Baba, S., and Tsutsui, Y. (2000) Cytomegalovirus infection of the central nervous system stem cells from mouse embryo: a model for developmental brain disorders induced by cytomegalovirus, *Lab Invest* 80, 1373-1383.
134. Lawrence, D. M., Durham, L. C., Schwartz, L., Seth, P., Maric, D., and Major, E. O. (2004) Human immunodeficiency virus type 1 infection of human brain-derived progenitor cells, *J Virol* 78, 7319-7328.
135. Krathwohl, M. D., and Kaiser, J. L. (2004) HIV-1 promotes quiescence in human neural progenitor cells, *J Infect Dis* 190, 216-226.
136. Feuer, R., Mena, I., Pagarigan, R. R., Harkins, S., Hassett, D. E., and Whitton, J. L. (2003) Coxsackievirus B3 and the neonatal CNS: the roles of stem cells, developing neurons, and apoptosis in infection, viral dissemination, and disease, *Am J Pathol* 163, 1379-1393.
137. Das, S., and Basu, A. (2008) Japanese encephalitis virus infects neural progenitor cells and decreases their proliferation, *J Neurochem* 106, 1624-1636.
138. Schwartz, L., Civitello, L., Dunn-Pirio, A., Ryschkewitsch, S., Berry, E., Cavert, W., Kinzel, N., Lawrence, D. M., Hazra, R., and Major, E. O. (2007) Evidence of human immunodeficiency virus type 1 infection of nestin-positive neural progenitors in archival pediatric brain tissue, *J Neurovirol* 13, 274-283.
139. Feuer, R., Pagarigan, R. R., Harkins, S., Liu, F., Hunziker, I. P., and Whitton, J. L. (2005) Coxsackievirus targets proliferating neuronal progenitor cells in the neonatal CNS, *J Neurosci* 25, 2434-2444.
140. Okamoto, S., Kang, Y. J., Brechtel, C. W., Siviglia, E., Russo, R., Clemente, A., Harrop, A., McKercher, S., Kaul, M., and Lipton, S. A. (2007) HIV/gp120 decreases adult neural progenitor cell proliferation via checkpoint kinase-mediated cell-cycle withdrawal and G1 arrest, *Cell Stem Cell* 1, 230-236.

141. Lee, M. H., Wang, T., Jang, M. H., Steiner, J., Haughey, N., Ming, G. L., Song, H., Nath, A., and Venkatesan, A. (2011) Rescue of adult hippocampal neurogenesis in a mouse model of HIV neurologic disease, *Neurobiol Dis* 41, 678-687.
142. Tsutsui, Y., Kosugi, I., Kawasaki, H., Arai, Y., Han, G. P., Li, L., and Kaneta, M. (2008) Roles of neural stem progenitor cells in cytomegalovirus infection of the brain in mouse models, *Pathol Int* 58, 257-267.
143. Das, S., Chakraborty, S., and Basu, A. (2010) Critical role of lipid rafts in virus entry and activation of phosphoinositide 3' kinase/Akt signaling during early stages of Japanese encephalitis virus infection in neural stem/progenitor cells, *J Neurochem* 115, 537-549.
144. Das, S., Ghosh, D., and Basu, A. (2009) Japanese encephalitis virus induce immuno-competency in neural stem/progenitor cells, *PLoS One* 4, e8134.
145. Han, G. P., Li, L., Kosugi, I., Kawasaki, H., Tsuchida, T., Miura, K., and Tsutsui, Y. (2007) Enhancement of susceptibility of adult mouse brain to cytomegalovirus infection by infusion of epidermal growth factor, *J Neurosci Res* 85, 2981-2990.
146. Isaacs, A., and Lindenmann, J. (1957) Virus interference. I. The interferon, *Proc R Soc Lond B Biol Sci* 147, 258-267.
147. David, M. (2002) Signal transduction by type I interferons, *Biotechniques Suppl*, 58-65.
148. Bogdan, C., Mattner, J., and Schleicher, U. (2004) The role of type I interferons in non-viral infections, *Immunol Rev* 202, 33-48.
149. Trinchieri, G., and Sher, A. (2007) Cooperation of Toll-like receptor signals in innate immune defence, *Nat Rev Immunol* 7, 179-190.
150. Pestka, S. (2007) The interferons: 50 years after their discovery, there is much more to learn, *J Biol Chem* 282, 20047-20051.
151. Vilcek, J. (2006) Fifty years of interferon research: aiming at a moving target, *Immunity* 25, 343-348.

152. Paul, S., Ricour, C., Sommereyns, C., Sorgeloos, F., and Michiels, T. (2007) Type I interferon response in the central nervous system, *Biochimie* 89, 770-778.
153. Colamonici, O. R., and Domanski, P. (1993) Identification of a novel subunit of the type I interferon receptor localized to human chromosome 21, *J Biol Chem* 268, 10895-10899.
154. Lutfalla, G., Roeckel, N., Mogensen, K. E., Mattei, M. G., and Uze, G. (1990) Assignment of the human interferon-alpha receptor gene to chromosome 21q22.1 by in situ hybridization, *J Interferon Res* 10, 515-517.
155. de Weerd, N. A., Samarajiwa, S. A., and Hertzog, P. J. (2007) Type I interferon receptors: biochemistry and biological functions, *J Biol Chem* 282, 20053-20057.
156. Lutfalla, G., Holland, S. J., Cinato, E., Monneron, D., Reboul, J., Rogers, N. C., Smith, J. M., Stark, G. R., Gardiner, K., Mogensen, K. E., and et al. (1995) Mutant U5A cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster, *Embo J* 14, 5100-5108.
157. Cohen, B., Novick, D., Barak, S., and Rubinstein, M. (1995) Ligand-induced association of the type I interferon receptor components, *Mol Cell Biol* 15, 4208-4214.
158. Gazziola, C., Cordani, N., Carta, S., De Lorenzo, E., Colombatti, A., and Perris, R. (2005) The relative endogenous expression levels of the IFNAR2 isoforms influence the cytostatic and pro-apoptotic effect of IFNalpha on pleomorphic sarcoma cells, *Int J Oncol* 26, 129-140.
159. Hardy, M. P., Owczarek, C. M., Trajanovska, S., Liu, X., Kola, I., and Hertzog, P. J. (2001) The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties, *Blood* 97, 473-482.
160. Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J. J. (1990) tyk2, prototype of a novel class of non-receptor tyrosine kinase genes, *Oncogene* 5, 1329-1336.
161. Krolewski, J. J., Lee, R., Eddy, R., Shows, T. B., and Dalla-Favera, R. (1990) Identification and chromosomal mapping of new human tyrosine kinase genes, *Oncogene* 5, 277-282.

162. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992) A protein tyrosine kinase in the interferon alpha/beta signaling pathway, *Cell* 70, 313-322.
163. Muller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A. G., Barbieri, G., Witthuhn, B. A., Schindler, C., and et al. (1993) The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction, *Nature* 366, 129-135.
164. Colamonici, O., Yan, H., Domanski, P., Handa, R., Smalley, D., Mullersman, J., Witte, M., Krishnan, K., and Krolewski, J. (1994) Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase, *Mol Cell Biol* 14, 8133-8142.
165. Colamonici, O. R., Uyttendaele, H., Domanski, P., Yan, H., and Krolewski, J. J. (1994) p135tyk2, an interferon-alpha-activated tyrosine kinase, is physically associated with an interferon-alpha receptor, *J Biol Chem* 269, 3518-3522.
166. Usacheva, A., Kotenko, S., Witte, M. M., and Colamonici, O. R. (2002) Two distinct domains within the N-terminal region of Janus kinase 1 interact with cytokine receptors, *J Immunol* 169, 1302-1308.
167. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C. W. (2002) Signaling through the JAK/STAT pathway, recent advances and future challenges, *Gene* 285, 1-24.
168. Fu, X. Y., Kessler, D. S., Veals, S. A., Levy, D. E., and Darnell, J. E., Jr. (1990) ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains, *Proc Natl Acad Sci U S A* 87, 8555-8559.
169. Fu, X. Y., Schindler, C., Improta, T., Aebersold, R., and Darnell, J. E., Jr. (1992) The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction, *Proc Natl Acad Sci U S A* 89, 7840-7843.
170. Schindler, C., Fu, X. Y., Improta, T., Aebersold, R., and Darnell, J. E., Jr. (1992) Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha, *Proc Natl Acad Sci U S A* 89, 7836-7839.

171. Wesoly, J., Szweykowska-Kulinska, Z., and Bluysen, H. A. (2007) STAT activation and differential complex formation dictate selectivity of interferon responses, *Acta Biochim Pol* 54, 27-38.
172. Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improta, T., Darnell, J. E., Jr., Stark, G. R., and Kerr, I. M. (1993) Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways, *Embo J* 12, 4221-4228.
173. Leung, S., Qureshi, S. A., Kerr, I. M., Darnell, J. E., Jr., and Stark, G. R. (1995) Role of STAT2 in the alpha interferon signaling pathway, *Mol Cell Biol* 15, 1312-1317.
174. Park, C., Li, S., Cha, E., and Schindler, C. (2000) Immune response in Stat2 knockout mice, *Immunity* 13, 795-804.
175. Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway, *Cell* 84, 431-442.
176. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease, *Cell* 84, 443-450.
177. Qureshi, S. A., Salditt-Georgieff, M., and Darnell, J. E., Jr. (1995) Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3, *Proc Natl Acad Sci U S A* 92, 3829-3833.
178. John, J., McKendry, R., Pellegrini, S., Flavell, D., Kerr, I. M., and Stark, G. R. (1991) Isolation and characterization of a new mutant human cell line unresponsive to alpha and beta interferons, *Mol Cell Biol* 11, 4189-4195.
179. Kimura, T., Kadokawa, Y., Harada, H., Matsumoto, M., Sato, M., Kashiwazaki, Y., Tarutani, M., Tan, R. S., Takasugi, T., Matsuyama, T., Mak, T. W., Noguchi, S., and Taniguchi, T. (1996) Essential and non-redundant roles of p48 (ISGF3 gamma) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies, *Genes Cells* 1, 115-124.

180. Kraus, T. A., Lau, J. F., Parisien, J. P., and Horvath, C. M. (2003) A hybrid IRF9-STAT2 protein recapitulates interferon-stimulated gene expression and antiviral response, *J Biol Chem* 278, 13033-13038.
181. Veals, S. A., Maria, T. S., and Levy, D. E. (1993) 2 Domains of Isgf3-Gamma That Mediate Protein-DNA and Protein-Protein Interactions during Transcription Factor Assembly Contribute to DNA-Binding Specificity, *Molecular and Cellular Biology* 13, 196-206.
182. Horvath, C. M., Stark, G. R., Kerr, I. M., and Darnell, J. E., Jr. (1996) Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex, *Mol Cell Biol* 16, 6957-6964.
183. Lau, J. F., Parisien, J. P., and Horvath, C. M. (2000) Interferon regulatory factor subcellular localization is determined by a bipartite nuclear localization signal in the DNA-binding domain and interaction with cytoplasmic retention factors, *Proc Natl Acad Sci U S A* 97, 7278-7283.
184. Hardy, M. P., Sanij, E. P., Hertzog, P. J., and Owczarek, C. M. (2003) Characterization and transcriptional analysis of the mouse Chromosome 16 cytokine receptor gene cluster, *Mamm Genome* 14, 105-118.
185. Hardy, M. P., Hertzog, P. J., and Owczarek, C. M. (2002) Multiple regions within the promoter of the murine Ifnar-2 gene confer basal and inducible expression, *Biochem J* 365, 355-367.
186. Marijanovic, Z., Ragimbeau, J., van der Heyden, J., Uze, G., and Pellegrini, S. (2007) Comparable potency of IFNalpha2 and IFNbeta on immediate JAK/STAT activation but differential down-regulation of IFNAR2, *Biochem J* 407, 141-151.
187. Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G., and Pellegrini, S. (2003) The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression, *Embo J* 22, 537-547.
188. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) SCF(HOS) ubiquitin ligase mediates the ligand-induced down-regulation of the interferon-alpha receptor, *Embo J* 22, 5480-5490.
189. Mizukoshi, E., Kaneko, S., Yanagi, M., Ohno, H., Matsushita, E., and Kobayashi, K. (1999) Upregulation of type I interferon receptor by IFN-gamma, *J Interferon Cytokine Res* 19, 1019-1023.

190. Fantuzzi, L., Eid, P., Malorni, W., Rainaldi, G., Gauzzi, M. C., Pellegrini, S., Belardelli, F., and Gessani, S. (1997) Post-translational up-regulation of the cell surface-associated alpha component of the human type I interferon receptor during differentiation of peripheral blood monocytes: role in the biological response to type I interferon, *Eur J Immunol* 27, 1075-1081.
191. Tan, Y. H., Schneider, E. L., Tischfield, J., Epstein, C. J., and Ruddle, F. H. (1974) Human chromosome 21 dosage: effect on the expression of the interferon induced antiviral state, *Science* 186, 61-63.
192. Wu, W. Z., Sun, H. C., Gao, Y. Q., Li, Y., Wang, L., Zhou, K., Liu, K. D., Iliakis, G., and Tang, Z. Y. (2004) Reduction in p48-ISGFgamma levels confers resistance to interferon-alpha2a in MHCC97 cells, *Oncology* 67, 428-440.
193. Wong, L. H., Krauer, K. G., Hatzinisiriou, I., Estcourt, M. J., Hersey, P., Tam, N. D., Edmondson, S., Devenish, R. J., and Ralph, S. J. (1997) Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma, *J Biol Chem* 272, 28779-28785.
194. Wong, L. H., Hatzinisiriou, I., Devenish, R. J., and Ralph, S. J. (1998) IFN-gamma priming up-regulates IFN-stimulated gene factor 3 (ISGF3) components, augmenting responsiveness of IFN-resistant melanoma cells to type I IFNs, *J Immunol* 160, 5475-5484.
195. Pattyn, E., Van Ostade, X., Schauvliege, L., Verhee, A., Kalai, M., Vandekerckhove, J., and Tavernier, J. (1999) Dimerization of the interferon type I receptor IFNAR2-2 is sufficient for induction of interferon effector genes but not for full antiviral activity, *J Biol Chem* 274, 34838-34845.
196. Jaks, E., Gavutis, M., Uze, G., Martal, J., and Piehler, J. (2007) Differential receptor subunit affinities of type I interferons govern differential signal activation, *J Mol Biol* 366, 525-539.
197. Kalie, E., Jaitin, D. A., Podoplelova, Y., Piehler, J., and Schreiber, G. (2008) The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities, *J Biol Chem* 283, 32925-32936.
198. Brideau-Andersen, A. D., Huang, X. J., Sun, S. C. C., Chen, T. T., Stark, D., Sas, I. J., Zadik, L., Dawes, G. N., Guptill, D. R., McCord, R., Govindarajan, S., Roy, A., Yang, S. M., Gao, J., Chen, Y. H., Skartved, N. J. O., Pedersen, A. K., Lin, D.,

- Locher, C. P., Rebbapragada, I., Jensen, A. D., Bass, S. H., Nissen, T. L. S., Viswanathan, S., Foster, G. R., Smons, J. A., and Patten, P. A. (2007) Directed evolution of gene-shuffled IFN-alpha molecules with activity profiles tailored for treatment of chronic viral diseases, *P Natl Acad Sci USA* 104, 8269-8274.
199. Constantinescu, S. N., Croze, E., Wang, C., Murti, A., Basu, L., Mullersman, J. E., and Pfeffer, L. M. (1994) Role of interferon alpha/beta receptor chain 1 in the structure and transmembrane signaling of the interferon alpha/beta receptor complex, *Proc Natl Acad Sci U S A* 91, 9602-9606.
200. Abramovich, C., Shulman, L. M., Ratovitski, E., Harroch, S., Tovey, M., Eid, P., and Revel, M. (1994) Differential tyrosine phosphorylation of the IFNAR chain of the type I interferon receptor and of an associated surface protein in response to IFN-alpha and IFN-beta, *Embo J* 13, 5871-5877.
201. Plataniias, L. C., and Colamonici, O. R. (1992) Interferon alpha induces rapid tyrosine phosphorylation of the alpha subunit of its receptor, *J Biol Chem* 267, 24053-24057.
202. Plataniias, L. C., Uddin, S., and Colamonici, O. R. (1994) Tyrosine phosphorylation of the alpha and beta subunits of the type I interferon receptor. Interferon-beta selectively induces tyrosine phosphorylation of an alpha subunit-associated protein, *J Biol Chem* 269, 17761-17764.
203. Yan, H., Krishnan, K., Greenlund, A. C., Gupta, S., Lim, J. T., Schreiber, R. D., Schindler, C. W., and Krolewski, J. J. (1996) Phosphorylated interferon-alpha receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein, *Embo J* 15, 1064-1074.
204. Wagner, T. C., Velichko, S., Vogel, D., Rani, M. R., Leung, S., Ransohoff, R. M., Stark, G. R., Perez, H. D., and Croze, E. (2002) Interferon signaling is dependent on specific tyrosines located within the intracellular domain of IFNAR2c. Expression of IFNAR2c tyrosine mutants in U5A cells, *J Biol Chem* 277, 1493-1499.
205. Russell-Harde, D., Wagner, T. C., Rani, M. R., Vogel, D., Colamonici, O., Ransohoff, R. M., Majchrzak, B., Fish, E., Perez, H. D., and Croze, E. (2000) Role of the intracellular domain of the human type I interferon receptor 2 chain (IFNAR2c) in interferon signaling. Expression of IFNAR2c truncation mutants in U5A cells, *J Biol Chem* 275, 23981-23985.

206. Li, X. X., Leung, S., Kerr, I. M., and Stark, G. R. (1997) Functional subdomains of STAT2 required for preassociation with the alpha interferon receptor and for signaling, *Molecular and Cellular Biology* 17, 2048-2056.
207. Nguyen, V. P., Saleh, A. Z., Arch, A. E., Yan, H., Piazza, F., Kim, J., and Krolewski, J. J. (2002) Stat2 binding to the interferon-alpha receptor 2 subunit is not required for interferon-alpha signaling, *J Biol Chem* 277, 9713-9721.
208. Saleh, A. Z., Fang, A. T., Arch, A. E., Neupane, D., El Fiky, A., and Krolewski, J. J. (2004) Regulated proteolysis of the IFN α 2 subunit of the interferon-alpha receptor, *Oncogene* 23, 7076-7086.
209. El Fiky, A., Pioli, P., Azam, A., Yoo, K., Nastiuk, K. L., and Krolewski, J. J. (2008) Nuclear transit of the intracellular domain of the interferon receptor subunit IFN α 2 requires Stat2 and Irf9, *Cell Signal* 20, 1400-1408.
210. Melen, K., Fagerlund, R., Franke, J., Kohler, M., Kinnunen, L., and Julkunen, I. (2003) Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein, *J Biol Chem* 278, 28193-28200.
211. Fagerlund, R., Melen, K., Kinnunen, L., and Julkunen, I. (2002) Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin alpha 5, *J Biol Chem* 277, 30072-30078.
212. Martinez-Moczygemba, M., Gutch, M. J., French, D. L., and Reich, N. C. (1997) Distinct STAT structure promotes interaction of STAT2 with the p48 subunit of the interferon-alpha-stimulated transcription factor ISGF3, *J Biol Chem* 272, 20070-20076.
213. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E., Jr. (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro, *Genes Dev* 3, 1362-1371.
214. Veals, S. A., Kessler, D. S., Josiah, S., Leonard, D. G., and Levy, D. E. (1991) Signal transduction pathway activating interferon-alpha-stimulated gene expression, *Br J Haematol* 79 Suppl 1, 9-13.
215. Bluysen, H. A. R., and Levy, D. E. (1997) Stat2 is a transcriptional activator that requires sequence-specific contacts provided by Stat1 and p48 for stable interaction with DNA, *Journal of Biological Chemistry* 272, 4600-4605.

216. Levy, D. E., Kessler, D. S., Pine, R., Reich, N., and Darnell, J. E., Jr. (1988) Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control, *Genes Dev* 2, 383-393.
217. Pellegrini, S., and Schindler, C. (1993) Early events in signalling by interferons, *Trends Biochem Sci* 18, 338-342.
218. de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., Silverman, R. H., and Williams, B. R. (2001) Functional classification of interferon-stimulated genes identified using microarrays, *J Leukoc Biol* 69, 912-920.
219. Der, S. D., Zhou, A., Williams, B. R., and Silverman, R. H. (1998) Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays, *Proc Natl Acad Sci U S A* 95, 15623-15628.
220. Gao, S., von der Malsburg, A., Paeschke, S., Behlke, J., Haller, O., Kochs, G., and Daumke, O. (2010) Structural basis of oligomerization in the stalk region of dynamin-like MxA, *Nature* 465, 502-506.
221. Janzen, C., Kochs, G., and Haller, O. (2000) A monomeric GTPase-negative MxA mutant with antiviral activity, *J Virol* 74, 8202-8206.
222. Haller, O., and Kochs, G. (2011) Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity, *J Interferon Cytokine Res* 31, 79-87.
223. Lindenmann, J. (1964) Inheritance of Resistance to Influenza Virus in Mice, *Proc Soc Exp Biol Med* 116, 506-509.
224. Staeheli, P., and Sutcliffe, J. G. (1988) Identification of a second interferon-regulated murine Mx gene, *Mol Cell Biol* 8, 4524-4528.
225. Haller, O., Stertz, S., and Kochs, G. (2007) The Mx GTPase family of interferon-induced antiviral proteins, *Microbes Infect* 9, 1636-1643.
226. Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G., and Haller, O. (1988) Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation, *Mol Cell Biol* 8, 4518-4523.

227. Staeheli, P., and Haller, O. (1985) Interferon-induced human protein with homology to protein Mx of influenza virus-resistant mice, *Mol Cell Biol* 5, 2150-2153.
228. Aebi, M., Fah, J., Hurt, N., Samuel, C. E., Thomis, D., Bazzigher, L., Pavlovic, J., Haller, O., and Staeheli, P. (1989) cDNA structures and regulation of two interferon-induced human Mx proteins, *Mol Cell Biol* 9, 5062-5072.
229. Pavlovic, J., Zurcher, T., Haller, O., and Staeheli, P. (1990) Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein, *J Virol* 64, 3370-3375.
230. Stertz, S., Reichelt, M., Krijnse-Locker, J., Mackenzie, J., Simpson, J. C., Haller, O., and Kochs, G. (2006) Interferon-induced, antiviral human MxA protein localizes to a distinct subcompartment of the smooth endoplasmic reticulum, *J Interferon Cytokine Res* 26, 650-660.
231. King, M. C., Raposo, G., and Lemmon, M. A. (2004) Inhibition of nuclear import and cell-cycle progression by mutated forms of the dynamin-like GTPase MxB, *Proc Natl Acad Sci U S A* 101, 8957-8962.
232. Hefti, H. P., Frese, M., Landis, H., Di Paolo, C., Aguzzi, A., Haller, O., and Pavlovic, J. (1999) Human MxA protein protects mice lacking a functional alpha/beta interferon system against La crosse virus and other lethal viral infections, *J Virol* 73, 6984-6991.
233. Schneider-Schaulies, S., Schneider-Schaulies, J., Schuster, A., Bayer, M., Pavlovic, J., and ter Meulen, V. (1994) Cell type-specific MxA-mediated inhibition of measles virus transcription in human brain cells, *J Virol* 68, 6910-6917.
234. Holzinger, D., Jorns, C., Stertz, S., Boisson-Dupuis, S., Thimme, R., Weidmann, M., Casanova, J. L., Haller, O., and Kochs, G. (2007) Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling, *J Virol* 81, 7776-7785.
235. Ronni, T., Matikainen, S., Lehtonen, A., Palvimo, J., Dellis, J., Van Eylen, F., Goetschy, J. F., Horisberger, M., Content, J., and Julkunen, I. (1998) The proximal interferon-stimulated response elements are essential for interferon responsiveness: a promoter analysis of the antiviral MxA gene, *J Interferon Cytokine Res* 18, 773-781.

236. Frese, M., Kochs, G., Feldmann, H., Hertkorn, C., and Haller, O. (1996) Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein, *J Virol* 70, 915-923.
237. Miura, T. A., Carlson, J. O., Beaty, B. J., Bowen, R. A., and Olson, K. E. (2001) Expression of human MxA protein in mosquito cells interferes with LaCrosse virus replication, *J Virol* 75, 3001-3003.
238. Landis, H., Simon-Jodicke, A., Kloti, A., Di Paolo, C., Schnorr, J. J., Schneider-Schaulies, S., Hefti, H. P., and Pavlovic, J. (1998) Human MxA protein confers resistance to Semliki Forest virus and inhibits the amplification of a Semliki Forest virus-based replicon in the absence of viral structural proteins, *J Virol* 72, 1516-1522.
239. Burdeinick-Kerr, R., Wind, J., and Griffin, D. E. (2007) Synergistic roles of antibody and interferon in noncytolytic clearance of Sindbis virus from different regions of the central nervous system, *J Virol* 81, 5628-5636.
240. Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007) Neuronal 'On' and 'Off' signals control microglia, *Trends Neurosci* 30, 596-602.
241. Massa, P. T., Whitney, L. W., Wu, C., Ropka, S. L., and Jarosinski, K. W. (1999) A mechanism for selective induction of 2'-5' oligoadenylate synthetase, anti-viral state, but not MHC class I genes by interferon-beta in neurons, *J Neurovirol* 5, 161-171.
242. Yin, J., Gardner, C. L., Burke, C. W., Ryman, K. D., and Klimstra, W. B. (2009) Similarities and differences in antagonism of neuron alpha/beta interferon responses by Venezuelan equine encephalitis and Sindbis alphaviruses, *J Virol* 83, 10036-10047.
243. Flohr, F., Schneider-Schaulies, S., Haller, O., and Kochs, G. (1999) The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures, *FEBS Lett* 463, 24-28.
244. Berge, T. O., Tigertt, W. D., and Banks, I. S. (1961) Attenuation of Venezuelan Equine Encephalomyelitis Virus by in Vitro Cultivation in Guinea-Pig Heart Cells, *Am J Hyg* 73, 209-&.

245. Carpenter, M. K., Inokuma, M. S., Denham, J., Mujtaba, T., Chiu, C. P., and Rao, M. S. (2001) Enrichment of neurons and neural precursors from human embryonic stem cells, *Exp Neurol* 172, 383-397.
246. Cohen, M. A., Itsykson, P., and Reubinoff, B. E. (2007) Neural differentiation of human ES cells, *Curr Protoc Cell Biol Chapter 23*, Unit 23 27.
247. Pankratz, M. T., Li, X. J., Lavaute, T. M., Lyons, E. A., Chen, X., and Zhang, S. C. (2007) Directed neural differentiation of human embryonic stem cells via an obligated primitive anterior stage, *Stem Cells* 25, 1511-1520.
248. Ma, W., Tavakoli, T., Derby, E., Serebryakova, Y., Rao, M. S., and Mattson, M. P. (2008) Cell-extracellular matrix interactions regulate neural differentiation of human embryonic stem cells, *BMC Dev Biol* 8, 90.
249. Levine, B., Huang, Q., Isaacs, J. T., Reed, J. C., Griffin, D. E., and Hardwick, J. M. (1993) Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene, *Nature* 361, 739-742.
250. Sammin, D. J., Butler, D., Atkins, G. J., and Sheahan, B. J. (1999) Cell death mechanisms in the olfactory bulb of rats infected intranasally with Semliki forest virus, *Neuropathol Appl Neurobiol* 25, 236-243.
251. Rehberg, E., Kelder, B., Hoal, E. G., and Pestka, S. (1982) Specific molecular activities of recombinant and hybrid leukocyte interferons, *J Biol Chem* 257, 11497-11502.
252. Peltier, D. C., Simms, A., Farmer, J. R., and Miller, D. J. (2010) Human neuronal cells possess functional cytoplasmic and TLR-mediated innate immune pathways influenced by phosphatidylinositol-3 kinase signaling, *J Immunol* 184, 7010-7021.
253. van Boxel-Dezaire, A. H., Rani, M. R., and Stark, G. R. (2006) Complex modulation of cell type-specific signaling in response to type I interferons, *Immunity* 25, 361-372.
254. Bianchi, T. I., Aviles, G., Monath, T. P., and Sabattini, M. S. (1993) Western equine encephalomyelitis: virulence markers and their epidemiologic significance, *Am J Trop Med Hyg* 49, 322-328.

255. Bianchi, T. I., Aviles, G., and Sabattini, M. S. (1997) Biological characteristics of an enzootic subtype of western equine encephalomyelitis virus from Argentina, *Acta Virol* 41, 13-20.
256. Schoepp, R. J., Smith, J. F., and Parker, M. D. (2002) Recombinant chimeric western and eastern equine encephalitis viruses as potential vaccine candidates, *Virology* 302, 299-309.
257. Reed, D. S., Larsen, T., Sullivan, L. J., Lind, C. M., Lackemeyer, M. G., Pratt, W. D., and Parker, M. D. (2005) Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques, *J Infect Dis* 192, 1173-1182.
258. Spotts, D. R., Reich, R. M., Kalkhan, M. A., Kinney, R. M., and Roehrig, J. T. (1998) Resistance to alpha/beta interferons correlates with the epizootic and virulence potential of Venezuelan equine encephalitis viruses and is determined by the 5' noncoding region and glycoproteins, *J Virol* 72, 10286-10291.
259. Hirsch, M., Knight, J., Tobita, M., Soltys, J., Panitch, H., and Mao-Draayer, Y. (2009) The effect of interferon-beta on mouse neural progenitor cell survival and differentiation, *Biochem Biophys Res Commun* 388, 181-186.
260. Wellen, J., Walter, J., Jangouk, P., Hartung, H. P., and Dihne, M. (2009) Neural precursor cells as a novel target for interferon-beta, *Neuropharmacology* 56, 386-398.
261. Makela, J., Koivuniemi, R., Korhonen, L., and Lindholm, D. (2010) Interferon-gamma produced by microglia and the neuropeptide PACAP have opposite effects on the viability of neural progenitor cells, *PLoS One* 5, e11091.
262. Peng, H., Whitney, N., Wu, Y., Tian, C., Dou, H., Zhou, Y., and Zheng, J. (2008) HIV-1-infected and/or immune-activated macrophage-secreted TNF-alpha affects human fetal cortical neural progenitor cell proliferation and differentiation, *Glia* 56, 903-916.
263. Eantuzzi, L., Eid, P., Malorni, W., Rainaldi, G., Gauzzi, M. C., Pellegrini, S., Belardelli, F., and Gessani, S. (1997) Post-translational up-regulation of the cell surface-associated alpha component of the human type I interferon receptor during differentiation of peripheral blood monocytes: role in the biological response to type I interferon, *Eur J Immunol* 27, 1075-1081.

264. Zurney, J., Howard, K. E., and Sherry, B. (2007) Basal expression levels of IFNAR and Jak-STAT components are determinants of cell-type-specific differences in cardiac antiviral responses, *J Virol* 81, 13668-13680.
265. Weaver, S. C., and Reisen, W. K. (2010) Present and future arboviral threats, *Antiviral Res* 85, 328-345.
266. Schnorr, J. J., Schneider-Schaulies, S., Simon-Jodicke, A., Pavlovic, J., Horisberger, M. A., and ter Meulen, V. (1993) MxA-dependent inhibition of measles virus glycoprotein synthesis in a stably transfected human monocytic cell line, *J Virol* 67, 4760-4768.
267. Staeheli, P., and Pavlovic, J. (1991) Inhibition of vesicular stomatitis virus mRNA synthesis by human MxA protein, *J Virol* 65, 4498-4501.
268. Pavlovic, J., Haller, O., and Staeheli, P. (1992) Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle, *J Virol* 66, 2564-2569.
269. Hammon, W. M., Reeves, W. C., and Sather, G. (1952) California encephalitis virus, a newly described agent. II. Isolations and attempts to identify and characterize the agent, *J Immunol* 69, 493-510.
270. Bennett, R. S., Ton, D. R., Hanson, C. T., Murphy, B. R., and Whitehead, S. S. (2007) Genome sequence analysis of La Crosse virus and in vitro and in vivo phenotypes, *Virol J* 4, 41.
271. Calisher, C. H., Monath, T. P., Muth, D. J., Lazuick, J. S., Trent, D. W., Franc, D. B., Kemp, G. E., and Chandler, F. W. (1980) Characterization of Fort Morgan virus, an alphavirus of the western equine encephalitis virus complex in an unusual ecosystem, *Am J Trop Med Hyg* 29, 1428-1440.
272. Peng, W., Peltier, D. C., Larsen, M. J., Kirchhoff, P. D., Larsen, S. D., Neubig, R. R., and Miller, D. J. (2009) Identification of thieno[3,2-b]pyrrole derivatives as novel small molecule inhibitors of neurotropic alphaviruses, *J Infect Dis* 199, 950-957.
273. Kochs, G., Janzen, C., Hohenberg, H., and Haller, O. (2002) Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes, *Proc Natl Acad Sci U S A* 99, 3153-3158.

274. Turan, K., Mibayashi, M., Sugiyama, K., Saito, S., Numajiri, A., and Nagata, K. (2004) Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome, *Nucleic Acids Res* 32, 643-652.
275. Kochs, G., and Haller, O. (1999) GTP-bound human MxA protein interacts with the nucleocapsids of Thogoto virus (Orthomyxoviridae), *J Biol Chem* 274, 4370-4376.
276. Dittmann, J., Stertz, S., Grimm, D., Steel, J., Garcia-Sastre, A., Haller, O., and Kochs, G. (2008) Influenza A virus strains differ in sensitivity to the antiviral action of Mx-GTPase, *J Virol* 82, 3624-3631.
277. Ryman, K. D., Meier, K. C., Nangle, E. M., Ragsdale, S. L., Korneeva, N. L., Rhoads, R. E., MacDonald, M. R., and Klimstra, W. B. (2005) Sindbis virus translation is inhibited by a PKR/RNase L-independent effector induced by alpha/beta interferon priming of dendritic cells, *J Virol* 79, 1487-1499.
278. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., and Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist, *Nucleic Acids Res* 36, W465-469.
279. Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoepfner, D. J., Ravin, R., Poser, S. W., Rueger, M. A., Bae, S. K., Kittappa, R., and McKay, R. D. (2006) Notch signalling regulates stem cell numbers in vitro and in vivo, *Nature* 442, 823-826.
280. Sabin, A. B. (1947) Epidemic encephalitis in military personnel; isolation of Japanese B virus on Okinawa in 1945, serologic diagnosis, clinical manifestations, epidemiologic aspects and use of mouse brain vaccine, *J Am Med Assoc* 133, 281-293.
281. Dickerson, R. B., Newton, J. R., and Hansen, J. E. (1952) Diagnosis and immediate prognosis of Japanese B encephalitis; observations based on more than 200 patients with detailed analysis of 65 serologically confirmed cases, *Am J Med* 12, 277-288.
282. Smorodintsev, A. A. (1958) Tick-borne spring-summer encephalitis, *Prog Med Virol* 1, 210-247.

283. Grascenkov, N. I. (1964) Tick-Borne Encephalitis in the Ussr, *Bull World Health Organ* 30, 187-196.
284. Perry, A. K., Chen, G., Zheng, D., Tang, H., and Cheng, G. (2005) The host type I interferon response to viral and bacterial infections, *Cell Res* 15, 407-422.
285. Gaspard, N., and Vanderhaeghen, P. (2011) From stem cells to neural networks: recent advances and perspectives for neurodevelopmental disorders, *Dev Med Child Neurol* 53, 13-17.
286. Gaillard, A., Prestoz, L., Dumartin, B., Cantereau, A., Morel, F., Roger, M., and Jaber, M. (2007) Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons, *Nat Neurosci* 10, 1294-1299.
287. Gaspard, N., Bouschet, T., Hourez, R., Dimidschstein, J., Naeije, G., van den Aemele, J., Espuny-Camacho, I., Herpoel, A., Passante, L., Schiffmann, S. N., Gaillard, A., and Vanderhaeghen, P. (2008) An intrinsic mechanism of corticogenesis from embryonic stem cells, *Nature* 455, 351-357.
288. Takahashi, K., and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126, 663-676.
289. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131, 861-872.
290. Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O., and Jaenisch, R. (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease, *Proc Natl Acad Sci U S A* 105, 5856-5861.
291. Betts, D. H., and Kalionis, B. (2010) Viable iPSC mice: a step closer to therapeutic applications in humans?, *Mol Hum Reprod* 16, 57-62.
292. Hamner, S., Skoglosa, Y., and Lindholm, D. (1999) Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat nervous system, *Neuroscience* 91, 673-684.

293. Labrada, L., Liang, X. H., Zheng, W., Johnston, C., and Levine, B. (2002) Age-dependent resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse ISG12, *J Virol* 76, 11688-11703.
294. Byrnes, A. P., Durbin, J. E., and Griffin, D. E. (2000) Control of Sindbis virus infection by antibody in interferon-deficient mice, *J Virol* 74, 3905-3908.
295. Geiss, G., Jin, G., Guo, J., Bumgarner, R., Katze, M. G., and Sen, G. C. (2001) A comprehensive view of regulation of gene expression by double-stranded RNA-mediated cell signaling, *J Biol Chem* 276, 30178-30182.
296. Espert, L., Rey, C., Gonzalez, L., Degols, G., Chelbi-Alix, M. K., Mechti, N., and Gongora, C. (2004) The exonuclease ISG20 is directly induced by synthetic dsRNA via NF-kappaB and IRF1 activation, *Oncogene* 23, 4636-4640.
297. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3, *Nature* 413, 732-738.
298. Trgovcich, J., Aronson, J. F., Eldridge, J. C., and Johnston, R. E. (1999) TNFalpha, interferon, and stress response induction as a function of age-related susceptibility to fatal Sindbis virus infection of mice, *Virology* 263, 339-348.
299. Zhang, Y., Burke, C. W., Ryman, K. D., and Klimstra, W. B. (2007) Identification and characterization of interferon-induced proteins that inhibit alphavirus replication, *J Virol* 81, 11246-11255.
300. Kracke, A., von Wussow, P., Al-Masri, A. N., Dalley, G., Windhagen, A., and Heidenreich, F. (2000) Mx proteins in blood leukocytes for monitoring interferon beta-1b therapy in patients with MS, *Neurology* 54, 193-199.
301. Deonarain, R., Cerullo, D., Fuse, K., Liu, P. P., and Fish, E. N. (2004) Protective role for interferon-beta in coxsackievirus B3 infection, *Circulation* 110, 3540-3543.
302. Koerner, I., Kochs, G., Kalinke, U., Weiss, S., and Staeheli, P. (2007) Protective role of beta interferon in host defense against influenza A virus, *J Virol* 81, 2025-2030.

303. Zhou, L., Wang, X., Wang, Y. J., Zhou, Y., Hu, S., Ye, L., Hou, W., Li, H., and Ho, W. Z. (2009) Activation of toll-like receptor-3 induces interferon-lambda expression in human neuronal cells, *Neuroscience* 159, 629-637.
304. Li, J., Hu, S., Zhou, L., Ye, L., Wang, X., Ho, J., and Ho, W. (2011) Interferon lambda inhibits herpes simplex virus type I infection of human astrocytes and neurons, *Glia* 59, 58-67.
305. Zhang, Y., and Bhavnani, B. R. (2006) Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens, *BMC Neurosci* 7, 49.