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Role of Interferon-α in HIV-associated Neurocognitive Disorders and Neuroinflammation

By

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Role of Interferon-α in HIV-associated Neurocognitive Disorders and Neuroinflammation

By

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B.S., University of South Carolina - Aiken, 2007

Advisor: William R. Tyor, Ph.D.

An abstract of
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Abstract

Role of Interferon-α in HIV-associated Neurocognitive Disorders and Neuroinflammation

By: Cari Fritz Kessing

Elevated levels of interferon-alpha (IFNα) in the central nervous system (CNS) are linked to cognitive dysfunction in patients with inflammatory CNS diseases, most prominently in HIV-associated neurocognitive disorders (HAND). Nearly half of the roughly 34 million HIV-infected patients worldwide suffer from HAND and the prevalence of HAND continues to rise. Antiretroviral therapies are not very effective in treating HAND and people develop HAND even while on antiretroviral therapy.

This research focuses on investigating the signaling mechanisms involved in IFNα neurotoxicity and testing potential therapeutics because evidence strongly suggests that IFNα is prominently involved in HAND pathogenesis. We hypothesize that better treatments can be developed for HAND by blocking IFNα or associated downstream signaling.

In order to test this hypothesis, we utilized an HIVE/SCID mouse model to assess the efficacy of a novel IFNα blocker, B18R. We found that treating HIVE/SCID mice with B18R blocked the neurotoxic effects of IFNα on neurons and subsequently prevented pathology associated with HAND. To compliment this *in vivo* work, we also used a primary rat neuronal culture system to assess alternate therapeutic targets involved in IFNα signaling. We found that IFNα causes decreased dendritic length and branching when exposed to neurons within 24 hours. We determined that the IFNα receptor was only partially responsible for IFNα induced neurotoxicity, but that blocking the NR2A subunit alone also partially prevents neurotoxicity after exposure to IFNα based on resultant effects on dendrites.

The results of these studies provide better insight into the mechanism(s) of IFNα induced neurotoxicity and provide potential therapeutic targets for HAND and possibly other inflammatory central nervous system diseases.

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Dedication

This work is dedicated to my parents, George Fritz and Marti French, for their unwavering support and faith in me. I could not have succeeded without them.

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List of Abbreviations

Ab: antibody
AIDS: acquired immune deficiency syndrome
ALS: amyotrophic lateral sclerosis
ANI: asymptomatic neurocognitive impairment
AP5: 2R-amino-5-phosphonovaleric acid
BBB: blood brain barrier
cART: combined antiretroviral therapy
CNS: central nervous system
CSF: cerebrospinal fluid
DNA: deoxyribonucleic acid
ELISA: enzyme-linked immunosorbent assay
EPSP: excitatory post synaptic potential
GAS: IFN-gamma activation site
gp120: envelope glycoprotein 120
HAD: HIV associated dementia
HAND: HIV associate neurocognitive disorders
HIV: human immunodeficiency virus
HIVE: HIV encephalitis
IC: intracranial
IHC: immunohistochemistry
IFNAR: type I interferon receptor
IFNα: interferon alpha
IFNβ: interferon beta
IFNγ: interferon gamma
IL: interleukin
IP: intraperitoneal
IRF: interferon regulatory factor
ISG: interferon stimulated gene
ISGF: interferon-stimulated growth factor
ISRE: interferon-stimulated response element
IV: intravenous
JAK: Janus kinase
LTP: long-term potentiation
MAP2: microtubule associated protein 2
MCP-1/CCL2: monocyte chemoattractant protein-1
MDM: monocyte-derived macrophages
MK801: antagonists dizocilpine maleate
MND: mild neurocognitive disorder
MRI: magnetic resonance imaging
MS: multiple sclerosis
NAb: neutralizing antibody
NMDA: N-methyl-D-aspartate
OAS: 2'–5' oligoadenylate synthetase
PBS: Phosphate buffered saline

PCR: polymerase chain reaction
PKR: dsRNA-dependent protein kinase
RNA: ribonucleic acid
ROS: reactive oxygen species
SCID: severe combined immunodeficient
SIV: simian immunodeficiency virus
SLE: systemic lupus erythematosus
STAT: signal transducer and activator of transcription
SV: sindbis virus
Tat: trans-activator of transcription
TCN201: 3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazino]carbonyl] benzyl] benzenesulphonamide
TLR: toll-like receptor
TNFα: tumor necrosis factor-α

Chapter 1:

Introduction to IFNα Neurotoxicity

Chapter adapted from:

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1. Introduction

Interferons are a group of cytokines that are synthesized and secreted during inflammation by a wide variety of cells including macrophages, monocytes, T lymphocytes, glial cells, and even neurons (Dafny 1998). Interferons are characterized by their ability to interfere with virus replication and profoundly alter the immune response to bacterial and viral infections. There are three main classes of interferons (IFN): type I IFN, type II IFN, and the less studied type III IFN. All interferons share the common feature of being upregulated during viral infections and are immunomodulatory. Type I interferons, including IFNα and IFNβ, are particularly potent antiviral agents.

The IFNβ protein is encoded by one gene, whereas there are thirteen IFNα genes encoding twelve proteins that have been identified in humans. They share homology across multiple species (Pestka 1997, Pestka, Krause et al. 2004). The various functions of all of the IFNα subtypes have yet to be completely determined (van Pesch, Lanaya et al. 2004, Moll, Maier et al. 2011). Particularly in the CNS, it is unknown if there are specific IFNα subtypes that are important. Despite binding to the same receptor, IFNα and IFNβ have somewhat different functions. These differences are most evident in the central nervous system (CNS) where IFNα has been shown to be neurotoxic, whereas IFNβ only rarely has side effects such as depression (Mattson, Niiranen et al. 1983, Meyers, Scheibel et al. 1991, Rho, Wesselingh et al. 1995, Valentine, Meyers et al. 1998, Mayr, Zeitlhofer et al. 1999, Schaefer, Engelbrecht et al. 2002, Dafny and Yang 2005, Paul, Ricour et al. 2007, Sas, Bimonte-Nelson et al. 2009). Some studies suggest the differences between the type I interferon functions, such as CNS

toxicity, are due to the variations in the affinity of type I interferons for the type I interferon receptor (IFNAR) subunits. These differing receptor affinities between IFNα and IFNβ can result in alterations in their use of specific JAK/STAT signaling pathways (MacDonald, Kuhl et al. 1990, Platanias, Uddin et al. 1996, Cutrone and Langer 1997, Marijanovic, Ragimbeau et al. 2007).

The IFNAR consists of two subunits, IFNAR1 and IFNAR2. As stated above, various subtypes of IFNα differ from IFNβ in their binding affinities to IFNAR1 and IFNAR2 (Marijanovic, Ragimbeau et al. 2007). Generally, subunit IFNAR1 associates with tyrosine kinase 2 (TYK2) and IFNAR2 subunit associates with JAK1 (Figure 1.1). Upon receptor engagement and subsequent dimerization of the IFNR, these associated proteins become phosphorylated and activated (Darnell, Kerr et al. 1994, Platanias 2005). Autophosphorylation of the JAK kinases then recruit STAT1 and STAT2 to the cell membrane where they are also phosphorylated. They then dimerize and bind to IFN-regulatory factor 9 (IRF9). This STAT1-STAT2-IRF9 interaction constitutes the IFN-stimulated gene factor 3 (ISGF3) complex that subsequently translocates to the nucleus. The ISGF3 complex then binds IFN-stimulated response elements (ISREs) on DNA to initiate gene transcription (Platanias 2005). Other downstream cascades of the JAK/STAT pathway may involve STAT1 homodimerization with GAS (IFN-gamma activation site) elements to initiate transcription of other interferon stimulating genes (ISGs). Stimulation of the JAK/STAT pathway results in production of many viral replication interfering proteins such as 2'–5' oligoadenylate synthetase (OAS), dsRNA-dependent protein kinase (PKR), MxA, and more IFNα (Ransonoff 1998). PKR phosphorylates the translation initiation

factor 2a (eIF2a) that inhibits the initiation of protein synthesis and therefore helps prevent production of new viral particles (Meurs, Chong et al. 1990, Elde, Child et al. 2009). OAS activates a cellular endoribonuclease RNase L that degrades viral and cellular RNAs, additionally inhibiting viral particle formation (Silverman 2007). Proteins upregulated by IFNα block viral replication through multiple mechanisms, activate immune cells, and assist uninfected cells in establishing an anti-viral state. IFNα has multiple immunomodulatory functions, which are beyond the scope of this commentary (Platanias 2005).

2. Clinical Implications

2.1. IFNα therapy side effects

IFNα has both antiviral and antitumor effects and is therefore used to successfully treat a variety of diseases, such as hepatitis B and lymphoma. Despite the benefits of IFNα therapy, neurological and psychiatric side effects are common with high-dose therapy and less severe neuropsychiatric side effects have been observed during low-dose therapy in a smaller portion of patients (Table 1) (Renault and Hoofnagle 1989, Haria and Benfield 1995, Okanoue, Sakamoto et al. 1996, Valentine, Meyers et al. 1998, Kirkwood, Bender et al. 2002, Schaefer, Engelbrecht et al. 2002). IFNα has been used as a therapy in a variety of viral infections, such as hepatitis B and C and herpes (Valentine, Meyers et al. 1998, Lieb, Engelbrecht et al. 2006, Drozdz, Borkowska et al. 2007, Pawelczyk, Pawelczyk et al. 2008). Some studies involving IFNα treated hepatitis

patients have not found cognitive impairments (Fontana, Bieliauskas et al. 2007, Fontana, Bieliauskas et al. 2010), whereas other studies using pegylated IFNα with or without ribavirin found impaired cognitive functioning. This included impaired executive and working memory, which is related to defects in hippocampal and prefrontal cortex functions (Reichenberg, Gorman et al. 2005, Lieb, Engelbrecht et al. 2006, Pawelczyk, Pawelczyk et al. 2008).

IFNα therapy has been used in amyotrophic lateral sclerosis (ALS). High-dose IFNα in these patients resulted in side effects similar to severe acute encephalitis, such as cognitive dysfunction and disorientation. This was reversible when treatment was terminated (Farkkila, Iivanainen et al. 1984, Iivanainen, Laaksonen et al. 1985).

IFNα has also been used as a treatment for cancer (Pavol, Meyers et al. 1995). Studies found that IFNα appeared to reduce tumor growth in patients diagnosed with various cancers such as myeloma, hairy-cell leukemia, breast cancer, Kaposi's sarcoma, and Non-Hodgkin's lymphoma. To try to improve results subsequent studies increased the amount of IFNα. However, it was found that long term and/or high dose IFNα therapy resulted in cognitive dysfunction including cognitive slowing, disorientation, memory deficits, and impaired motor and executive function (Smedley, Katrak et al. 1983, Adams, Quesada et al. 1984, Meyers, Scheibel et al. 1991, Pavol, Meyers et al. 1995, Mayr, Zeitlhofer et al. 1999).

There are important features of cognitive dysfunction associated with IFNα treatment, which have often been characterized as subcortical. Cognitive

dysfunction usually subsides shortly after treatment is terminated or the dose is reduced, implying a reversible effect of IFNα.

2.2. CNS Inflammatory diseases

In addition to adverse neurological effects of IFNα given therapeutically, increased IFNα levels brought on by various inflammatory conditions have been linked to diseases with adverse CNS manifestations. These include HIV-associated neurocognitive disorders (HAND), systemic lupus erythematosus (SLE), multiple sclerosis (MS), rabies, and others (Table 1). Patients with these conditions can develop signs of cognitive dysfunction similar to those treated with IFNα. When carefully characterized, the cognitive dysfunction is usually attributed to frontal-subcortical areas, just as they are in IFNα treated patients. Interestingly, increased levels of IFNα in the cerebrospinal fluid (CSF) were found in the majority of 200 newborns and children with various diseases such as herpes encephalitis, mumps encephalitis, viral meningitis, SLE, and others (Dussaix, Lebon et al. 1985). IFNα has also been reported to be elevated in the CSF of patients with meningoencephalitis, a disease which is almost always associated with cognitive dysfunction (i.e., delirium) (Bocket, Delforge et al. 1992). Again, like cognitive dysfunction related to treatment with IFNα, delirium associated with elevated IFNα in conditions such as meningoencephalitis is also typically reversible.

IFNα was found in all active MS lesions in several case studies (Traugott and Lebon 1988). It is important to note that nearly 70% of MS patients suffer

from cognitive dysfunction, which is often characterized as subcortical. High levels of IFNα were found in the CSF of most patients with Aicardi-Goutieres syndrome, a rare genetic disease associated with progressive encephalopathy in infants (Goutieres, Aicardi et al. 1998). IFNα levels have been correlated with severity of disease in SLE patients (Pascual, Banchereau et al. 2003). Induction of dendritic cells by IFNα may drive the autoimmune response in SLE (Blanco, Palucka et al. 2001, Pascual, Banchereau et al. 2003). IFNα is capable of initiating the differentiation of precursor cells into dendritic cells resulting in the continuous activation and differentiation of dendritic cells that is observed in SLE (Paquette, Hsu et al. 1998, Santini, Lapenta et al. 2000). High levels of IFNα were found in patients with neuropsychiatric systemic lupus erythematosus (Jonsen, Bengtsson et al. 2003). In addition, Shiozawa et al. found that elevated levels of IFNα in the CSF of SLE patients were associated with symptoms resembling autism and schizophrenia (Shiozawa, Kuroki et al. 1992). Similarly, IFNα levels were elevated in the CSF of SLE patients with lupus psychosis, the most common CNS manifestation in SLE patients. Patients diagnosed with lupus psychosis manifest symptoms including schizophrenia, depression, and autism. These psychiatric symptoms subsided when the patients were given steroids to reduce serum IFNα (Hooks, Jordan et al. 1982, Ytterberg and Schnitzer 1982, Shiozawa, Kuroki et al. 1992).

The clinical evidence associating elevated CNS IFNα levels in a wide variety of diseases associated with cognitive dysfunction is compelling. It is important to emphasize that the cognitive impairments associated with IFNα therapy are different from the side effects seen with IFNβ therapy, which is only

rarely associated with depression (Fragoso, Frota et al. 2010). These side effect differences between two very similar [type I] interferons are probably due to the IFNAR signaling differences mentioned above. Understanding the molecular mechanisms of IFNα in neuronal functions leading to cognitive dysfunction could offer insights into new treatments for the conditions discussed above.

3. IFNα Role in HIV-associated Neurocognitive Disorders

3.1. HIV-associated neurocognitive disorders

HIV primarily infects and destroys CD4+ T lymphocytes and is the cause of acquired immune deficiency syndrome (AIDS). Over 33 million people worldwide are infected. This number has continued to rise due to spreading of the infection, population growth and, more recently, the life-prolonging effects of combined antiretroviral therapy (cART). HIV-associated dementia (HAD), which develops in approximately 10% of HIV-infected people, is the most severe form of HAND and is typified by significant impairment in activities of daily living and largely a subcortical dementia (Valcour, Sithinamsuwan et al. 2010). Since the introduction of cART in 1996, there has been a decline in death rate and incidence of HAD in infected patients. However the prevalence of HAND has been increasing due to an aging population of HIV-infected people who are more susceptible to cognitive dysfunction because of age and cardiovascular risks (Valcour, Shikuma et al. 2004, Ellis, Rosario et al. 2010, McArthur, Steiner et al. 2010). In addition, recently there has been a greater appreciation of the milder

forms of HAND. These milder forms of HAND include asymptomatic neurocognitive impairment (ANI; only diagnosed with neuropsychological testing) and mild neurocognitive disorder (MND). Despite the advent of cART, HAND develops in approximately 40 to 50% of people and can even occur in patients while they are taking cART and with reduced systemic viral load (Liner, Ro et al. 2010). Since as many as 15 to 20 million people worldwide may have some form of HAND, it is important to discern its pathogenesis so that alternative treatments can be developed.

HIV encephalitis (HIVE) is a common manifestation of AIDS and for the purposes of this review HIVE will refer to the histopathological expression of HIV CNS infection (Price, Navia et al. 1986). HIV enters the CNS through a leaky blood brain barrier (BBB) via infected mononuclear phagocytes. Virus enters the CNS early after systemic infection where it resides in the CNS throughout the lifespan of the infected individual (Gray, Scaravilli et al. 1996). It is also possible that HIV invades the CNS by crossing the BBB as free virus or by means of HIV-infected CD4+T cells that cross the BBB. HIV-infected macrophages in the CNS spread the infection to other resident mononuclear phagocytes such as perivascular macrophages and microglia. Typically, HIV has been found in most abundance in the basal ganglia, specifically the caudate nucleus. HIV-infected cells have also been found in the hippocampus, an area known for memory formation (Wiley, Soontornniyomkij et al. 1998, Petito, Adkins et al. 2003, Kumar, Borodowsky et al. 2007).

Associated pathology of HIVE includes HIV-infected mononuclear phagocytes (i.e., macrophages and microglia), multinucleated giant cells,

microglial nodules, diffuse myelin pallor, gliosis, and neuronal abnormalities (Navia, Cho et al. 1986, Petito, Cho et al. 1986, Wiley and Achim 1994, Masliah, Roberts et al. 2004). Gliosis refers to the increased activation of astrocytes and microglia, resident CNS cells that have complex functions including reaction to inflammatory conditions by secretion of various cytokines. Neuronal abnormalities in HIVE include decreased neuronal cell counts, abnormal and decreased dendritic arborizations, and neuronal apoptosis (Petito, Cho et al. 1986, Masliah, Roberts et al. 2004).

Because HIV does not directly infect significant numbers of neurons, neuronal damage is believed to be indirectly caused by the effects of various putative neurotoxins produced by mononuclear phagocytes and astrocytes (Figure 1.2) (Tyor, Wesselingh et al. 1995, Zheng, Thylin et al. 2001, Ryan, Cotter et al. 2002, Cook, Dasgupta et al. 2005). A number of immune factors are increased in HIVE, including cytokines, such as tumor necrosis factor-α (TNFα) and IFNα, chemokines like monocyte chemoattractant protein-1 (MCP-1/CCL2) (Kelder, McArthur et al. 1998), and various metabolites such as arachidonic acid and quinolinic acid (Heyes, Brew et al. 1991, Genis, Jett et al. 1992). These substances have been shown to have direct or indirect toxic effects on neurons. Neuronal damage may also occur by injury from neurotoxic HIV proteins, such as gp120 and Tat, which directly bind to neuronal receptors (Dreyer, Kaiser et al. 1990). Quinolinic acid, Tat, and gp120 can interact through the NMDA receptor to produce neurotoxicity.

Other virological features of HIV such as cell tropism or specific sequence differences may also play a role, but the details of these implications are beyond

the scope of this review (Heyes, Brew et al. 1991, Lipton, Singel et al. 1994, Nath 2002, van Marle and Power 2005, Rao, Sas et al. 2008). Despite many studies implicating a wide variety of factors as potential neurotoxins or causes of HIVE and HAND, the exact factor(s) playing a prominent role in pathogenesis have not been conclusively determined.

The potential neurological effects of increased CNS IFNα have been studied in HAD. IFNα was measured by ELISA in the CSF of patients with HIV infection, with and without HAD (Rho, Wesselingh et al. 1995). Individuals with HAD had significantly elevated CSF levels of IFNα compared with HIV positive patients without neurocognitive impairment. Immunohistochemistry for IFNα in the brains of HIV infected individuals at autopsy revealed staining present on macrophages and astrocytes (Rho, Wesselingh et al. 1995), consistent with previously reported staining in multiple sclerosis brains (Traugott and Lebon 1988).

A subsequent study using gene array performed on postmortem brain tissue from HAD individuals showed upregulation of a number of IFNα associated genes compared to HIV-infected individuals who did not have HAD. In addition, genes associated with dendritic stability like microtuble associated protein 2 (MAP2) (discussed later) were downregulated (Masliah, Roberts et al. 2004). Reverse transcriptase polymerase chain reaction (RT-PCR) for IFNα mRNA also has indicated that IFNα is actively produced within the brains of individuals with HAD (Rho, Wesselingh et al. 1995, Perrella, Carreiri et al. 2001). Finally, another study revealed that IFNα levels in CSF correlated with cerebral atrophy measured by MRI (Perrella, Carreiri et al. 2001). Taken together, these

studies indicate that IFNα is elevated in the CNS along with downstream IFNα stimulated gene products in association with HAD.

3.2. HIVE animal models

In order to begin to determine whether specific, putative neurotoxins are causing HAND, a SCID mouse model was developed (Tyor, Power et al. 1993, Persidsky, Limoges et al. 1996, Avgeropoulos, Kelley et al. 1998, Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2007). This model reflects similar behavioral and pathological signs seen in humans. The HIVE/SCID mouse model utilizes intracranial injections of HIV-infected human macrophages into the frontal cortex and deep nuclei (e.g., caudate). HIVE/SCID mice have demonstrated behavioral abnormalities in both the Morris water maze and water radial arm maze (WRAM) (Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2007). The mice portray pathological similarities to humans including multinucleated giant cells, increased microgliosis and astrogliosis, decreased dendritic arborization, and increased production of inflammatory cytokines and chemokines. Recent data using this HIVE/SCID mouse model implies that neuronal apoptosis is not an important initial event in HIV-associated cognitive dysfunction, but that physiological dysfunction of neurons and disruption of dendritic arborization is an important early pathogenic event (Cook, Dasgupta et al. 2005). Studies using the HIVE /SCID mouse model indicate that increased IFNα levels in the brains of these mice correlate with worsening of HIVE and behavioral abnormalities during WRAM testing (Sas,

Bimonte-Nelson et al. 2007). Systemic treatment with neutralizing antibodies to IFNα, which cross the BBB, significantly decreased microgliosis, prevented loss of dendritic arborization and improved behavioral abnormalities in HIVE/SCID mice (Sas, Bimonte-Nelson et al. 2009). This HIVE/SCID mouse data compliments the clinical studies of patients treated with IFNα summarized above, which demonstrated improved cognitive function once treatment was ceased. In addition, using the SIV pigtailed macaque model of HIVE, IFNα was not present less than 6 days post-infection (Alammar, Gama et al. 2011). However interestingly, IFNα increases 45 days post-infection, which may correspond with later time points during SIV encephalitis that eventually lead to the development of cognitive dysfunction.

The pathogenesis of HIVE leading to cognitive dysfunction and ultimately HAD most likely begins with reversible neuronal physiological dysfunction perhaps when relatively few putative neurotoxins (e.g. IFNα) are playing a prominent role (Figure 1.2). Frank dementia (HAD) is probably indicative of some amount of neuronal death, seen in later stages of more severe encephalitis during a time when multiple neurotoxins are in play. We hypothesize that IFNα is most important during HAND in earlier phases, when cognitive dysfunction is still reversible. Therefore, it is possible that treatment of IFNα toxicity early in HAND patients, who may or may not be also on cART, could ameliorate cognitive dysfunction in the majority of these people.

4. Additional Studies Supporting the Role of IFNα in Neuronal Dysfunction and Possible Mechanisms

4.1. IFNα toxicity mouse models

Aside from the mouse studies of HIVE above, other *in vivo* data also support the theory that IFNα is involved in cognitive dysfunction. Transgenic mice designed to overexpress IFNα in astrocytes develop severe encephalitis and cognitive abnormalities (Campbell, Krucker et al. 1999). The mice displayed signs of neurological abnormalities including decreased appetite due to effects on hypothalamus, deficits in spatial learning, and seizures related to elecrophysiological hyperexcitability. Histopathological signs included neurodegeneration, such as swelling and deterioration of neuronal profiles and axons, gliosis, and calcifications. Decreased motor activity has been seen in mice treated with IFNα (Segall and Crnic 1990, Dunn and Crnic 1993, Makino, Kitano et al. 2000). One study attempted to simulate typical interferon treatment given to patients by giving repeated intraperitoneal injections of IFNα to mice over a 5 day period. This resulted in decreased motor activity (Dunn and Crnic 1993). Similarly, a single systemic intravenous injection of IFNα results in decreased motor activity in mice (Makino, Kitano et al. 2000).

4.2. In vitro studies of IFNα neurotoxicity

4.2.1. IFNAR activation

The mechanism of IFNα neurotoxicity is not well established (Figure 1.3). IFNα may have both direct and indirect negative effects on neurons due to the

diverse signaling cascades associated with the IFNAR. Sas et al. showed a dose dependent effect of IFNα resulting in simplification of dendritic arborization in primary rat neuronal cultures (Sas, Bimonte-Nelson et al. 2009). In neurons, IFNα has also been shown to activate the JAK/STAT pathway through phosphorylation of STAT1 leading to activation of IFNα induced genes (Wang and Campbell 2005).

Interferon-stimulated gene 15 (ISG15) is highly upregulated after IFNα receptor engagement (Wang et. al. 2007). ISG15 is a ubitquitin-like molecule known to be secreted by monocytes and lymphocytes during an infection and plays a role in certain neurological diseases. During a viral response when ISG15, like other antiviral cytokines, are upregulated to initiate an antiviral response, ISG15 targets viral proteins for breakdown by binding to them and preventing efficient viral assembly (Lenschow et. al. 2007). Additionally, ISG15 has been shown to prevent release of HIV virions and is upregulated in HAD patients (Lenschow et. al. 2007, Masliah et. al. 2005). ISG15 is also upregulated in the HIVE/SCID model and a key marker for IFNAR activation through our studies.

STAT1 activation, upstream of ISG15 upregulation, has been linked to dendritic retraction in sympathetic and hippocampal neurons when treated with the type II interferon, IFNγ (Kim, Beck et al. 2002). IFNγ is known to activate both STAT1 and STAT3 after receptor engagement. Kim et.al found that STAT3 did not affect dendritic arborization, but STAT1 activation was required for the dendritic retraction seen in IFNγ treated cultures. As mentioned previously, STAT1 is activated by IFNα engaging the IFNAR and STAT1 is then

phosphorylated. Therefore, IFNAR engagement is likely required for IFNα neurotoxicity.

4.2.2. NMDAR and MAP2

IFNα has an indirect effect on neurons by affecting glutamate levels and signaling resulting in changes in excitability (D'Arcangelo, Grassi et al. 1991, Dafny 1998, Campbell, Krucker et al. 1999). Glutamate is a major excitatory transmitter in the CNS and binds to N-methyl-D-aspartic acid receptor (NMDAR), among others. NMDAR is an ionotropic glutamate receptor composed of 2 NR1 subunits and two NR2 or NR3 subunits (Cull-Candy, Brickley et al. 2001) and controls synaptic plasticity and memory function. NMDAR is both voltage-dependent and ligand-gated and it requires co-activations with glutamate and glycine (or d-serine) (Wolosker 2007). The NR2 unit binds glutamate and the NR1 subunit is required for binding glycine, whereas NR3 subunits have an inhibitory effect. There are 8 splice variants of the NR1 subunit and 4 isoforms of the NR2 subunit (N2A, N2B, N2C, N2D). Unlike NR1 subunit, NR2 subunit is critical for controlling electrophysiological activities and is expressed differently depending on the cell type. More interestingly, immature neurons predominantly express NR2B and as the neurons mature, NR2A subunit expression begins to outnumber the NR2B subunits (Paoletti and Neyton 2007). There are several drugs known to specifically inhibit the NMDAR subunits. Ifenprodil binds NR2B and TCN201 selectively binds NR2A. These inhibitors will be described in more detail later.

NMDAR activation is highly regulated in the CNS glutamate transporters maintain extracelluar levels of glutamate at low levels (Hardingham, Glazewski et al. 2003). Macrophages and microglia can damage neurons during CNS inflammation due to infection by causing excessive activations of receptors like NMDAR. NMDAR is known to be vulnerable to HIV infection in the CNS (Petito, Cho et al. 1986, Masliah, Roberts et al. 2004). Previous *in vitro* studies showed that IFNα toxicity is partially mediated by NMDA receptors (Sas, Bimonte-Nelson et al. 2009). Rat neuronal cultures containing 5% glial elements (mostly astrocytes) were treated with NMDA antagonists dizocilpine maleate (MK801) or 2R-amino-5-phosphonovaleric acid (AP5) after exposure to IFNα. MK801 is a non-competitive antagonist of the NMDAR and AP5 is a competitive NMDAR antagonist. Treatment with either of these NMDA antagonists prevented IFNα-associated loss of dendritic length, but did not prevent the loss of dendritic branching (Sas, Bimonte-Nelson et al. 2009). Since treating neuronal cultures with neutralizing antibodies to IFNα prevents both dendritic length and branching abnormalities, it is likely that neuronal toxicity is due to IFNAR engagement by IFNα, but further studies are needed to establish this relationship. Interestingly, glutamate-mediated neurotoxicity in this setting is specific to dendritic length, not branching, suggesting that IFNα has other direct and/or indirect effects on neuronal branching. The exact association of IFNα with NMDA neurotoxic effects is also unclear. It may be that IFNα stimulates glutamate release from neurons, but further studies are required to elucidate the relationship between NMDA neurotoxicity and IFNα (Sas, Bimonte-Nelson et al. 2009).

NMDAR activation has also been shown to play a role in MAP2 breakdown in rat hippocampal neurons after oxygen-glucose deprivation (Buddle, Eberhardt et al. 2003). This study showed that MAP2 breakdown was not related to free radical production, but occurred through NMDAR activation and calcium influx. Taken together, these data suggest a possible link between increased IFNα levels in the CNS and NMDAR activation with an end result being MAP2 breakdown, decreased dendritic arborization, and cognitive dysfunction. MAP2 is a critical cytoskeletal protein involved in maintaining the stability of neuronal dendrites as well as facilitating dendritic formation. It is downregulated in patients with HAD (see above). Decreased MAP2 expression has also been shown in the HIVE/SCID mouse model (Cook-Easterwood, Middaugh et al. 2007). More research is needed to evaluate the mechanism of IFNAR and NMDA signaling and MAP2 breakdown or deactivation resulting in decreased dendritic maintenance and/or growth.

4.2.3. Signal transmission effects

Synaptic transmission and membrane potential is also affected by excessive levels of IFNα. *In vitro* studies using rat hippocampal neurons showed that IFNα inhibits long-term potentiation (LTP) and reduces excitatory postsynaptic potentials (EPSP) (Dafny 1998, Mendoza-Fernandez, Andrew et al. 2000). LTP involves the signal transmission between neurons required for memory and learning function and EPSP reflects the stability of neuron firing and overall health.

4.2.4. Inflammatory influence

The neurotoxic actions of IFNα may also be related to a multiplicative effect with other inflammatory cytokines that are known to be upregulated by IFNα signaling including IL-1, IL-6, and TNFα, as well as the generation of free radicals (Hansson, Romero et al. 2004). IFNα acts on surrounding mononuclear phagocytes influencing hydrogen peroxide, superoxide, and nitric oxide production, which may have neurotoxic effects (Hansson, Romero et al. 2004). IFNα actions may influence upregulation of additional inflammatory cytokines resulting in more severe neurodegeneration and ultimately cell death.

5. Conclusions and future perspectives

Clinical studies and *in vivo* and *in vitro* data demonstrate that IFNα is neurotoxic. Patients treated with high doses of IFNα show neurological symptoms that largely resolve after treatment is removed. The effects of high levels of IFNα in the CNS and the resulting neurotoxicity have been associated with patients suffering from HAND. Behavioral deficits and associated histopathological effects have also been shown in a variety of *in vivo* studies including transgenic mice, IFNα treated mice, and HIVE/SCID mice. *In vitro* studies have been used to delineate the specific effects of IFNα and have been effective in showing a dose dependent effect of IFNα on dendritic simplification. IFNα is known to increase production of inflammatory cytokines, activate critical immune cells, increase cell stress, increase production of ROS and metabolites through multiple signaling cascades initiated through activation of the IFNAR.

Inhibiting IFNα through treatment in CNS inflammatory diseases may improve cognitive function in patients by preventing the neurotoxic effects of IFNα. It is reasonable to believe it is possible to specifically inhibit IFNα and still maintain important immunomodulatory functions of interferons. This is because the other type I interferon, IFNβ, and the type II interferon, IFNγ, also inhibit viral replication and share many immunomodulatory pathways with IFNα. Determining the mechanism(s) of how IFNα acts as a neurotoxin is a critical step to find effective and specific targets downstream of IFNAR engagement that may have therapeutic implications in a variety of neurological disorders involving inflammation and cognitive dysfunction.

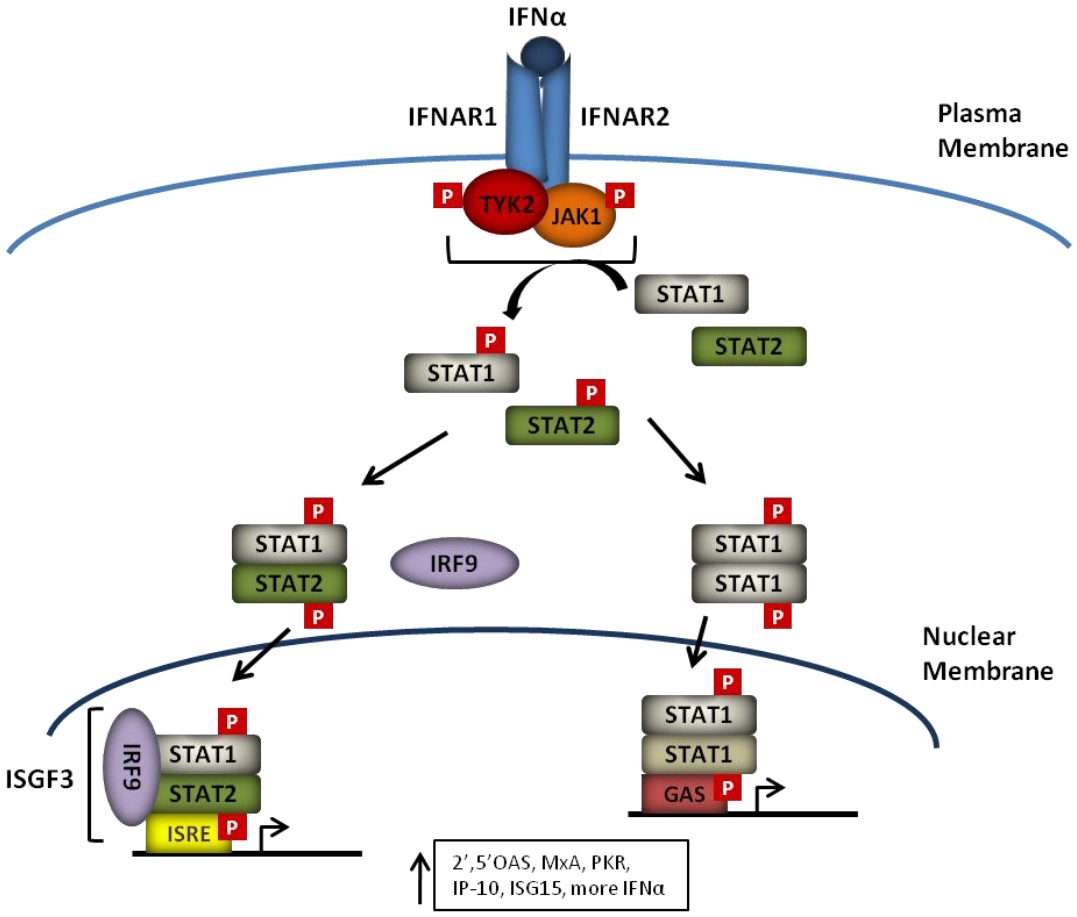


Figure 1.1. IFNα binds to its receptor, IFNAR, recruiting kinases to the cellular membrane and activating JAK/STAT pathway resulting in upregulation of ISGs such as OAS, MxA, PKR, and more type 1 interferons.

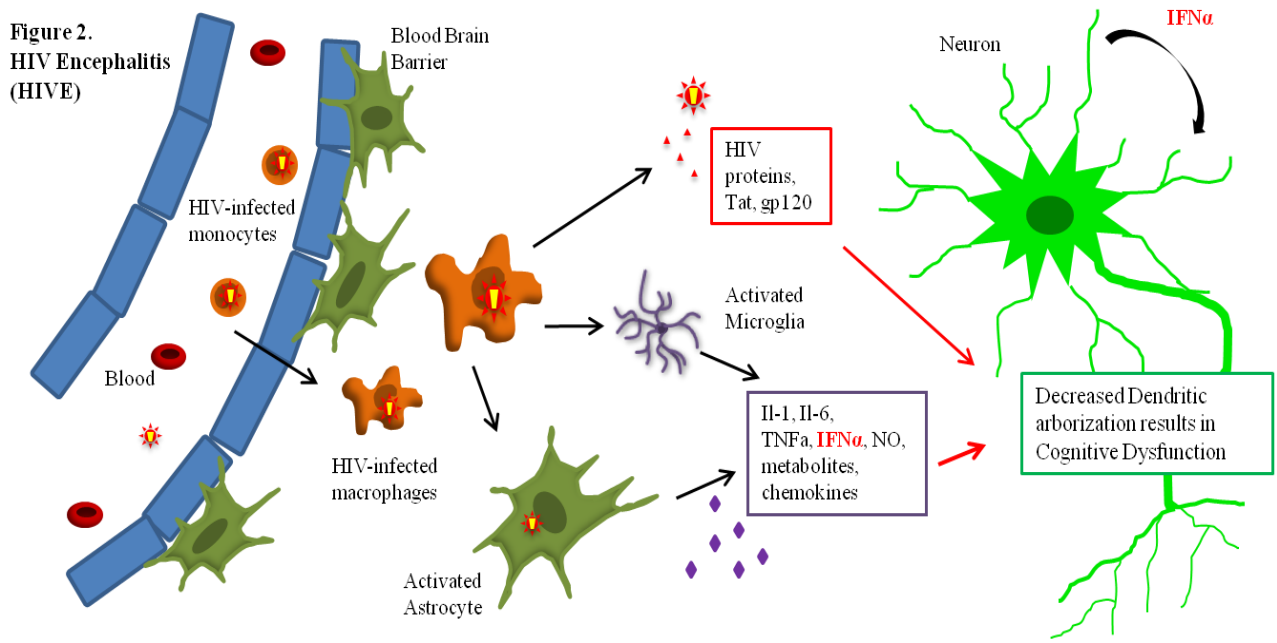


Figure 1.2. HIV enters the CNS early in infection and infects and activates local cells such as microglia and astrocytes. These cells secrete neurotoxins and viral proteins resulting in increased gliosis, abnormalities to dendritic arborization, and eventually neuronal loss.

Table 1. Clinical implications of IFNα neurotoxicity

Influence of IFNα in inflammatory neurodegenerative diseases	
Disease	**References**
HIV-associated neurocognitive disorders (HAND)	12, 55-68
Systemic lupus erythematosus (SLE)	14, 46, 47, 50-53
Multiple sclerosis (MS)	44
Various encephalitides	42, 43
Aicardi-Goutieres syndrome	45
Cognitive dysfunction linked to IFNα therapy for various diseases	
Hepatitis B and C	8, 14, 26, 28, 30-32, 36, 37
Herpes	8, 42
Cancer	6, 7, 9, 10, 14, 27, 30, 41
Amyotrophic lateral sclerosis (ALS)	37, 38

Table 1.1. IFNα neurotoxicity has multiple clinical applications in both neurodegenerative diseases and as a therapeutic treatment. Listed above are various diseases involved in excess IFNα in the CNS that result in cognitive dysfunction.

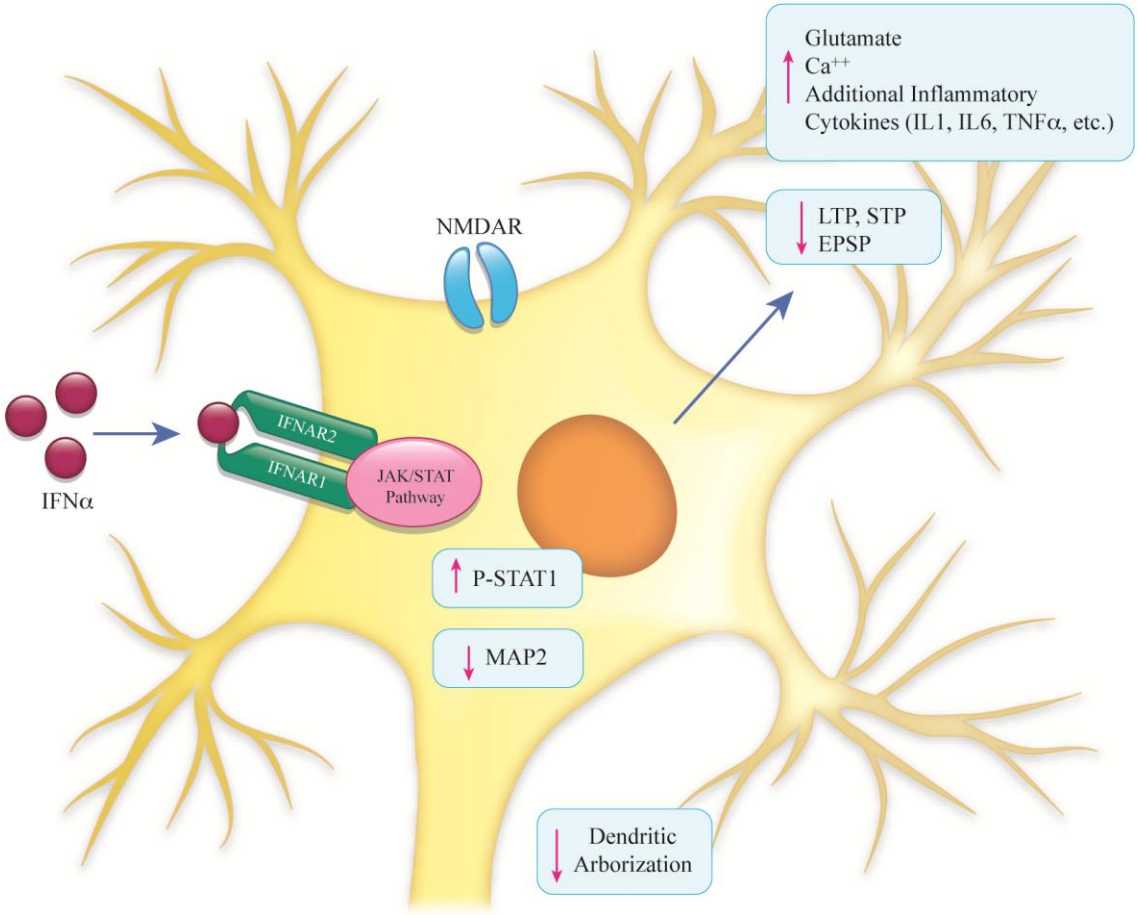


Figure 1.3. Excess IFNα in the CNS results in decreased dendritic arborization and cognitive function. Upregulation of IFNα results in increased glutamate levels, Ca^{+}, and additional inflammatory cytokines that all most likely play roles in neuronal degradation.

Chapter 2:

B18R crosses the BBB and decreases ISG signaling in SV infected mice

Introduction

Early in the HIV epidemic, over 15% of HIV-infected individuals with advanced disease developed dementia prior to death. Today, with the advent of combination antiretroviral therapy (cART), the incidence of HIV-associated dementia (HAD) has been reduced. However, the prevalence of HAD has increased due to the prolonged life of HIV-infected individuals using ART treatment. In addition, around 40-50% of HIV-infected patients still develop HIV associated neurocognitive disorders (HAND) meaning cART is only partially effective in treating HAND and more effective treatments are needed.

During HIV-infection, infected monocytes cross the blood brain barrier (BBB), activating a variety of nucleated cells to secrete chemokines and cytokines leading to inflammation in the brain and HIV encephalitis (HIVE) pathology. Interferon alpha (IFNα) is a pleomorphic cytokine secreted by a variety of nucleated cells in the brain, including neurons, during viral infection. Increased levels of IFNα have been seen in the CNS of HIV-infected patients with dementia compared to HIV-infected patients without dementia. IFNα has also been shown *in vitro* to have toxic effects on neurons causing decreased dendritic arborization that was restored with neutralizing antibodies to IFNα. This data showing the reversible effect of IFNα is consistent with human studies where patients treated with high doses of IFNα showed cognitive problems that dissipated once treatment was stopped. In addition, previous studies in our lab showed that treating HIVE/SCID mice with intraperitoneally (IP) injected IFNα neutralizing antibody led to improved cognitive function compared to the untreated group.

In this study, we are interested in determining the efficacy of a novel interferon-alpha (IFNα) blocker, B18R. B18R is a vaccinia virus-encoded receptor that binds and neutralizes IFNα. B18R hinds to all IFNα subtypes and with greater affinity than the IFNα receptor, the type I interferon receptor. Our preliminary study included assessing if B18R crossed the BBB, which was not previously known. The BBB consists of eight junctions between endothelial cells that filters molecules and substances from entering the central nervous system (CNS) making the CNS an immunological privileged organ. Previous studies have found that cART drugs are not efficient at crossing the BBB (Cook-Easterwood, Middaugh et al. 2007) and therefore identifying a HAND drug that can cross the BBB efficiently is necessary.

To accomplish this, a sindbis virus (SV) mouse model of encephalitis was utilized (Sas, Jones et al. 2008). The SV encephalitis mouse model involves inoculating normal mice intracranially with sindbis virus. After 3-5 days, the SV mice develop a robust encephalitis evident throughout the brain. This model was previously used to show that neutralizing antibodies to IFNα effectively crossed the BBB and significantly decreased levels of IFNα in the brain. We therefore wanted to use the SV model to determine if B18R protein would cross the BBB and effectively inhibit IFNα prior to using B18R in an HIVE mouse model (see Chapter 3). The SV model is advantageous to the HIVE/SCID mouse model because the SV model is a quicker, more affordable, and robust encephalitis mouse model than the latter. Additionally this study also determined the half-life of B18R *in vivo*.

We treated SV infected mice with a single dose of B18R and found that B18R did cross the BBB based on immunohistochemistry staining. Additionally, we found that interferon stimulated gene 15 (ISG15) was downregulated, reflecting efficient blockage of IFNα. ISG15 was downregulated within 1 hour after a single B18R dose. After a single dose of B18R, ISG15 levels were back to control levels within 6 hours.

Materials and Methods

SV Mice

Four week old B6.CB17-*Prkdc*^{scid}/SzJ male mice were obtained from Jackson Laboratory. Mice were acclimated in single housed cages for 1 week prior to infection. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Administration Medical Center and were in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Infection and B18R treatment

Mice were injected intracranially (IC) with sindbis virus (SV) titered at 1000PFU/20 μl in the right frontal lobe (n=2 per group, n=10 total). After 5 days, mice were separated into 5 groups (untreated controls, 1hr B18R treated, 3hr B18R treated, 6hr B18R treated, and 18R B18R treated) and administered either saline or B18R (vaccinia virus-encoded neutralizing type I interferon receptor; type I IFN inhibitor) at 30 μg/300 μl intraperitoneal (IP). Mice were sacrificed 1hr, 3hr, 6hr, 12hr post-IP B18 administration. Brains were extracted and snap

frozen in tissue freezing media for immunocytochemistry and real-time PCR analysis.

Toxicity Study

In order to insure that the B18R protein preparation was non-toxic to mice, a toxicity study was carried out. Four-week old B6.CB17-*Prkdc*^{scid}/SzJ male mice obtained from Jackson Laboratory were given intraperitoneal (IP) injections of 300μl of B18R or saline twice daily for 8 days. The mice were then monitored for physical and behavioral changes which are indicative of toxicity. Physical perimeters monitored include weight, appearance of fur and ear positioning. To monitor behavior, the mice are given fresh bedding daily to ensure that they are shredding, also their level of lethargy is recorded. Food and water intake is also recorded daily.

Immunohistochemistry and anti-B18R antibody development

Brains were sectioned and stained using an immunoperoxidase method previously described (Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2007, Sas, Bimonte-Nelson et al. 2009). Tissues were stained with anti-B18R (1:500, Meiogen). To stain for B18R, antibody (Ab) was produced in rabbits by injection of resuspended inclusion body pellets (Cocalico Biologicals, Stevens, PA). Serum was run through Pierce Protein A columns according to the manufacturer's protocol (Pierce Biotechnology, IL). Sections were stained with B18R (1:500). Sections (5μm) were blocked in 2% normal goat serum (NGS) in PBS for 20 min. Sections were then incubated in primary mAb (1:500 diluted in

2% NGS) for 45 min. Slides were then incubated in biotinylated goat-anti-rabbit secondary antibody diluted (1:100) in 2% NGS (Vector, Burlingame, CA) for 30 min. Endogenous peroxidase was blocked with 0.1% H_2O_2 in methanol for 30 min and slides were incubated in avidin DH-biotin complex (ABC complex) (Vector) for 30 min. For color development, sections were incubated in 0.7 mg/ml Diaminobenzidine (Sigma) for 20 min. Sections were washed, dehydrated and finally cover-slipped. To demonstrate the capability of our IHC staining to detect B18R, mice were IC inoculated with B18R. Brains were stained using anti-B18R antibody which acted as positive controls.

To determine specificity of the anti-B18R antibody, B18R and brain lysate samples were assessed on a PAGE gel. Protein was harvested from SCID mouse brain using RIPA buffer with 10 µL/ml protease inhibitors (Thermo Scientific). Total protein concentration was determined by a Pierce® BCA Protein Assay Kit. B18R alone, B18R with brain lysate, and brain lysate samples were then loaded on a 7.5% polyacrylamide gel. Protein was run at 200V for 40 minutes and then transferred to nitrocellulose membrane using the Trans-blot Turbo transfer system (Biorad). The anti-B18R antibody was used as primary antibody at 1:2500. The membrane was then washed and incubated with goat anti-rabbit IgG HRP-linked secondary antibody at 1:10000 (Cell Signaling). The membrane was then exposed to X-ray film using Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Detection of a single band representing B18R at 37.9 kD was observed in the brain lysate samples containing B18R. No bands were evident in brain lysate alone when probed with anti-B18R antibody (Figure 2.1).

Real-time PCR

Levels of mouse interferon-induced gene15 (*ISG15*) and interferon-alpha 4 gene (*IFNA4*) were analyzed using real-time PCR with a Biorad C1000 Thermocycler. Levels were normalized to the levels of Glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) which was used as the endogenous control for all experiments. *ISG15*, *IFNA4*, and *GAPDH* primers were designed using Assays-by-Design (Applied Biosystems).

Results

anti-B18R specifically binds B18R

B18R in brain lysate was run on a PAGE gel and probed with anti-B18R antibody (2.1). A single-band at 37 kD representing B18R was evident in the brain lysate. Lanes containing brain lysate samples with no evident bands demonstrates no non-specific binding of anti-B18R. Additionally, mice inoculated directly with B18R to determine staining efficacy in brain tissue were assessed. Brain tissue was probed with the same anti-B18R antibody and specific staining was seen along injection site (Figure 2.2).

B18R crosses the BBB

Immunostaining for B18R at 6 hours post-IP injection was roughly equivalent to the amount of staining observed at 1 and 3 hours after the final IP injection of B18R (data not shown). However, immunostaining for B18R at 12 hours was probably slightly less than at 6 hours. Figure 2.3 depicts B18R staining which likely represents B18R bound to IFNα on the surface of cells such as neurons and glia. Immunostaining with the rabbit Ab to B18R on SV infected brain that did not receive B18R treatment was completely negative (data not shown).

B18R reduces IFNα stimulated gene expression in brain tissue

To further examine IFNα inhibition in the brain, real-time PCR was performed on mRNA obtained from brain tissue of SV mice. Several genes were

analyzed by real-time PCR including IFNA4, to demonstrate IFNα mRNA expression in the mice, and ISG15, a downstream gene activated by IFNα. In mice treated with B18R, ISG 15 and IFNA4 gene levels were decreased (Figure 2.4). Levels of ISG15 seem to recover to levels found in untreated mice in a time dependant manner. Levels of ISG15 showed a time-dependant decrease, with the greatest effect at 1 hour post-B18R treatment and a less severe decrease at 3 hours. At 6 and 12 hours after treatment, the levels of ISG15 transcripts were equal to the levels in mice that were not treated with B18R.

Discussion

These preliminary studies in SV infected mice show that B18R protein efficiently crosses the blood brain barrier (BBB) as demonstrated by immunohistochemistry staining of B18R. Data showing decreased IFNα associated genes implies effective B18R inhibition of IFNα in the brains of mice infected with SV. ISG expression was decreased within an hour after a single treatment of B18R. ISG levels began to return to elevated levels by 3-6 hours post-injection, indicating a 4 hour half-life. This data indicates an efficient dose treatment for encephalitis in mice will likely require 3 injections of 30 μg/300μl per injection daily.

Future studies will include testing B18R in our HIVE/SCID mouse model over an extended period of time and assessing effects of B18R on HIVE pathology, as well as using real-time PCR measures that will reflect downstream events of IFNα as outcomes.

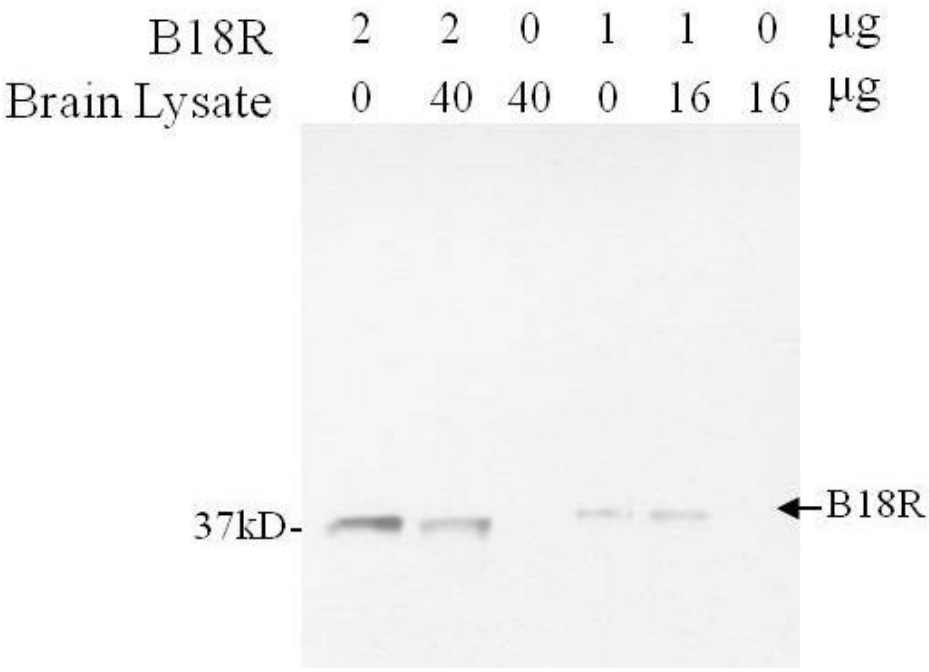


Figure 2.1: Anti-B18R antibody binds specifically to B18R in brain lysate. B18R protein alone, B18R and brain lysate, and brain lysate alone was run on a PAGE gel and probed with anti-B18R. Bands specific to B18R were observed at 37 kD.

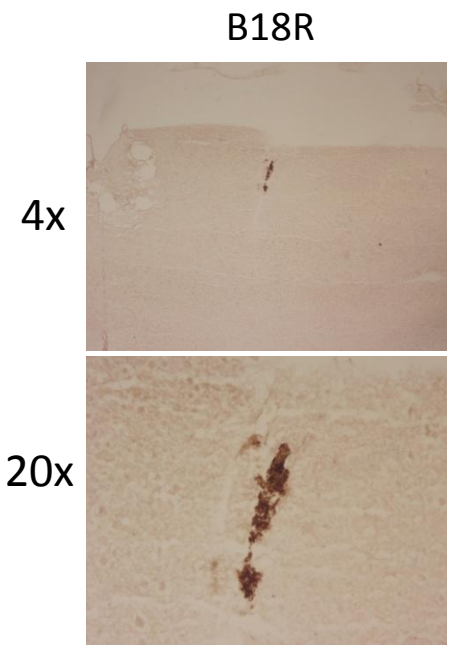


Figure 2.2: Immunocytochemistry staining of brain tissue probed with anti-B18R from mice directly inoculated IC with B18R at 4x and 20x magnification.

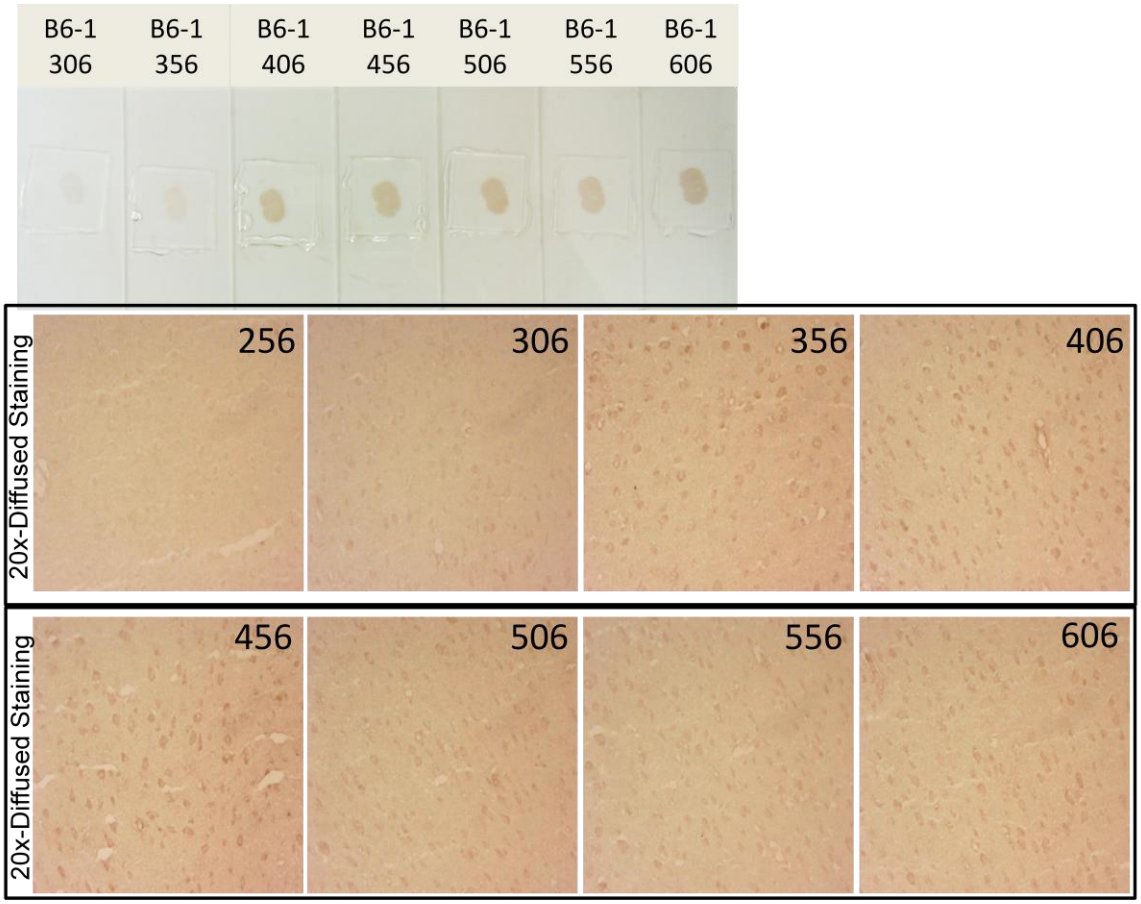


Figure 2.3: Immunohistochemistry staining of B18R in SV mice treated with B18R. The top panel shows a gross view of brain sections. The bottom panels show 20x photomicrographs of rabbit polyclonal immunostaining for B18R. Note the darkest amount of staining at about section #450, closest to the injection site of SV and the highest amount of perivascular inflammation. The stained cells include neurons and glia.

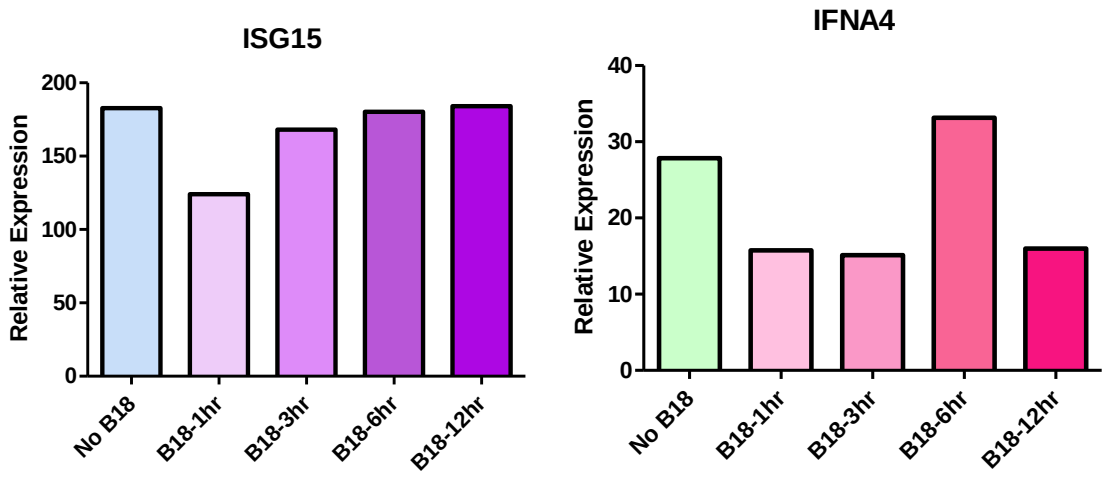


Figure 2.4: Real-time PCR on mouse brain tissue shows that B18R treated SV mice have decreased ISG15 and IFNA4 gene expression. Decreased ISG15 and IFNA4 gene expression demonstrates that B18R effectively inhibits IFNα in the brain of mice.

Chapter 3:

B18R neutralizes interferon alpha and alleviates histopathological complications in HIVE mice

Chapter adapted from:

Fritz-French C, Shawahna R, Ward JE, Maroun LE, Tyor WR. The recombinant vaccinia virus gene product B18R neutralizes interferon alpha and alleviates histopathological complications in HIV encephalitis mouse model. *Journal of Interferon and Cytokine Research*. In Press.

Abstract

Interferon-alpha (IFNα) has been identified as a neurotoxin that plays a prominent role in HIV-associated neurocognitive disorders (HAND) and HIV encephalitis (HIVE) pathology. IFNα is associated with cognitive dysfunction in other inflammatory diseases where IFNα is upregulated. Trials of monoclonal anti-IFNα antibodies have been generally disappointing possibly due to high specificity to limited IFNα subtypes and low affinity.

We investigated a novel IFNα inhibitor, B18R, in an HIV-encephalitis (HIVE)/Severe Combined Immunodeficiency (SCID) mouse model. Immunostaining for B18R in systemically treated HIVE/SCID mice suggested the ability of B18R to cross the blood brain barrier (BBB). Real-time PCR indicated that B18R treatment resulted in a decrease in gene expression associated with IFNα signaling in the brain. Mice treated with B18R were found to have decreased mouse mononuclear phagocytes and significant retention of neuronal arborization compared to untreated HIVE/SCID mice.

Increased mononuclear phagocytes and decreased neuronal arborization are key features of HIVE. These results suggest that B18R crosses the BBB, blocks IFNα signaling, and prevents key features of HIVE pathology. These data suggest that the high affinity and broad IFNα sub-type specificity of B18R make it a viable alternative to monoclonal antibodies for the inhibition of IFNα in the immune-suppressed environment.

Introduction

The advent of combined antiretroviral therapy (cART) has markedly reduced Acquired Immunodeficiency Syndrome (AIDS) related morbidity and mortality. Despite this reduction, HIV-associated neurocognitive disorders (HAND) are still reported in up to 50% of HIV seropositive patients (Liner, Ro et al. 2010). HAND remains among the most common causes of cognitive dysfunction in young adults and it is expected that more patients will develop the severe form known as HIV-associated dementia (HAD) due to an aging patient (Ellis, Rosario et al. 2010, McArthur, Steiner et al. 2010).

Reaching sufficient therapeutic concentrations in the central nervous system (CNS) is a prerequisite for any effective therapy for HAND. However, the ability of different ART agents to cross the blood-brain barrier (BBB) and reach effective concentrations in the brain varies widely from agent to agent (Cook, Dasgupta et al. 2005, Brew, Halman et al. 2007). Additionally, the prevalence of HAND continues to increase in patients despite cART, reinforcing the need for specific treatments targeted to HAND (Liner, Ro et al. 2010).

Delineating pathogenic mechanisms often leads to new therapeutic avenues. Recently, several agents targeting specific HAND pathogenic mechanisms were investigated in small clinical trials (Schifitto, Sacktor et al. 1999, Sacktor, Skolasky et al. 2000, Clifford, McArthur et al. 2002, Goodkin, Vitiello et al. 2006, Schifitto, Peterson et al. 2006, Nakasujja, Miyahara et al. 2013). Despite showing some trends, the results of these trials were largely disappointing since some agents may have failed to penetrate the BBB and/or did not improve cognitive function in HAND patients. Given the results of these

treatment trials and the apparent failure of cART to prevent HAND, there is a great need to develop therapies that reduce the significant, worldwide morbidity of HAND.

HIV invades the CNS shortly after exposure. With progression of disease, patients may develop HAND and its related histopathological entity, HIV-encephalitis (HIVE), manifested by the presence of HIV-infected macrophages and microglia, multinucleated giant cells, activated astrocytes (astrogliosis), and neuronal damage (Tyor, Glass et al. 1992, Wiley and Achim 1994). In response to HIV invasion, the immune system increases the production of cytokines like tumor necrosis factor-α, chemokines like monocyte chemoattractant protein-1, and various metabolites such as reactive oxygen species (ROS) (Genis, Jett et al. 1992, Kelder, McArthur et al. 1998). These immune factors produced by macrophages, microglia, and astrocytes, together with HIV proteins such as gp120 and tat, cause neuronal damage (Louboutin and Strayer 2012). Neuronal damage is associated with a decrease in the number of dendritic arborizations (Sas, Bimonte-Nelson et al. 2007), and later, the occurrence of neuronal apoptosis leading to dementia.

The neurotoxicity of interferon-α (IFNα) has been well established in CNS inflammatory diseases as well as in cases of patients receiving interferon therapy (Rho, Wesselingh et al. 1995, Valentine, Meyers et al. 1998, Sas, Bimonte-Nelson et al. 2009, Fritz-French and Tyor 2012). Earlier basic and clinical research suggested a significant role of IFNα in HIVE and pathogenesis of HAND (Rho, Wesselingh et al. 1995, Fritz-French and Tyor 2012). Neuronal defects and upregulated IFNα levels are critical in both HAND and HIVE. Clinical studies

showed that patients treated with IFNα suffer reversible cognitive impairment and complications resembling those seen in HAND (Scheibel, Valentine et al. 2004). Similarly, Rho et al. reported a correlation between the severity of dementia and the levels of IFNα in HIV seropositive patients.

Our previous studies used an HIVE/Severe combined immunodeficiency (SCID) mouse model to investigate the role of IFNα (Sas, Bimonte-Nelson et al. 2007, Sas, Bimonte-Nelson et al. 2009). The HIVE/SCID mouse model used shows similar histopathology and behavioral changes seen in HAND in humans (Tyor, Power et al. 1993, Avgeropoulos, Kelley et al. 1998, Cook, Dasgupta et al. 2005, Cook-Easterwood, Middaugh et al. 2007). Additionally, Sas et al. found a direct correlation between the amount of mouse IFNα expression in the brain and cognitive performance in the HIVE/SCID mice (Sas, Bimonte-Nelson et al. 2007). Systemically treating these HIVE/SCID mice with neutralizing antibodies (NAb) to IFNα resulted in significant amelioration of behavioral and pathological features. However, administering NAb to HAND patients is probably not practical for multiple reasons (Sas, Bimonte-Nelson et al. 2009). Our previous studies (Sas, Bimonte-Nelson et al. 2009) like those of others (Maroun, Heffernan et al. 2000), used polyclonal antibody preparations. Polyclonal antibodies have diverse epitope specificity and thus, inhibit multiple IFN-α subtypes, of which there are 12 functional subtypes in humans. However, administration of polyclonal antibodies is prohibitive due to serum sickness.

In addition, the use of monoclonal anti- IFNα antibodies in clinical trials has been generally disappointing probably primarily because monoclonal antibodies cannot provide the wide specificity needed to fully neutralize multiple

IFN-α subtypes (Petri, Wallace et al. 2013). B18R is a recombinant vaccinia virus gene product that based on its specificity, affinity, and immunogenicity is a more optimal IFNα inhibitor than neutralizing antibodies used previously (Higgs, Zhu et al. 2013). Complete data is not yet available, however B18R has been shown to inhibit all type I IFN-α sub-types tested thus far including IFN-β but does not inhibit type III (Huang, Smirnov et al. 2007).

In this study, we used an HIVE/SCID mouse model (Figure 3.1) to investigate the effects of B18R as a novel IFNα blocker. We report that intraperitoneal (IP) injection of this novel IFNα blocker alleviates histopathological complications in the HIVE/SCID mouse model.

Methods

Human MDMs and HIV infection

Human monocyte-derived macrophages (MDMs) were cultured and infected as described in our previous reports (Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2009). Briefly, primary human monocytes and HIV-1 ADA were obtained from Dr. Howard Gendelman, University of Nebraska Medical Center, Omaha, NE. Purified primary human macrophages ($1x10^8$) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% human serum, glutamine (Sigma-Aldrich), penicillin–streptomycin (Sigma-Aldrich), and monocyte-colony stimulating factor (Sigma-Aldrich) at 37 °C with 5% CO_2 in Teflon-coated culture flasks (CoStar, Cambridge, MA) for 1 week. Cells were counted and culture media was exchanged every 2-3 days. On day 7, cells were divided into two cultures and each was re-suspended in 9 mL of media (DMEM with 10% human serum, glutamine, and penicillin– streptomycin). 1 mL of HIV-1ADA (0.1 multiplicity of infection) was added to the first culture and 1 mL of media was added to the second culture. The first culture was labeled HIV infected and the second was labeled control. After hour incubation, cells were resuspended in media at the rate of $1x10^6$ cells/mL. After 2 weeks, MDMs were collected and re-suspended in phosphate buffered saline (PBS) for intracranial (IC) inoculation.

Animals and inoculation

Five week old B6.CB17-Prkdc^{scid}/SzJ male mice were obtained from Jackson Laboratory, Cold Harbor, ME. Mice were singly housed in micro-isolator

cages for 1 week to acclimate before the experiment. Mice were given free access to food and water. All cages, bedding, food and water supplied were sterilized by autoclaving prior to use. All procedures were approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Administration Medical Center and were in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Mice were IC inoculated either with 10^5 HIV-infected (*n=16*) or uninfected (control; *n=8*) human MDMs re-suspended in 30 μl PBS into the right frontal lobe under xylazine (5 mg/kg) and ketamine (95 mg/kg) anesthesia. IC inoculation was performed using a syringe fitted with a collar to control depth to 3 mm below the skull surface.

Production of B18R

B18R (Normferon™, gift from Meiogen Biotechnology Corp., DE) was produced using modified standard recombinant procedures (Symons, Alcami et al. 1995). Briefly, the B18R gene minus the transmembrane region was sub-cloned into plasmid pET29a and grown in *E. coli* strain BL-21. Following 1 mM IPTG induction, the cultures were pelleted and frozen. The pellets were digested with lysozyme and inclusion bodies pelleted at 15,000g for 30 min at 4°C. After unfolding in 6M Guanidium HCl, 0.1M Tris HCl (pH = 8.0), 1.5 mM EDTA, 6 mM dithiothreitol, the protein was refolded in refold buffer [(0.2M Guanidium HCl, 0.7M L-arginine, 1.5 mM EDTA, 50 mM Tris HCl, 10 mM NaCl (pH=8.5)] with reduced glutathione (0.615 g/L) and glutathione oxidized hydrate (0.245 g/L) added just before use.

The refolded and concentrated protein was stored at -20°C in 50% glycerin. The glycerin was diluted when the protein was concentrated and further purified by elution from a cation exchange resin (POROS HS50, Life Technologies**)** with 1M NaCl. The protein gave a single 37 kD band on PAGE gel analysis. . B18R has been extensively studied *in vitro* (Liptakova, Kontsekova et al. 1997, Alcami 2000). Here we study its potential for use *in vivo*.

For *in vivo* potency estimation, a test of B18R was run in non-immunocompromised animals. Six week old C57/BL6 mice were induced to high levels of IFNα by IP injection of poly I:C (300 microliters at 1 mg/ml/PBS). Three hours later, they were divided into two groups of 3 mice each and B18R (120µg/300µl) or vehicle injected. Serum was collected for IFNα ELISA analysis (PBL product number 42120) 45 minutes and 4.5 hours after these injections. At 45 minutes IFNα was reduced to non-detectable levels in the B18R injected group (vehicle: mean= 420.8925+/- 138.548 [SEM] pg/ml vs. B18R injected: mean =<37 pg/ml). At 4.5 hours, IFNα was reduced to 55% of the control level (mean = 330.91 +/- 87.81 [SEM] pg/ml (vehicle) vs. mean = 180.96 +/- 62.9 [SEM] pg/ml (B18R)). No adverse effects of B18R injection were seen either in these normal mice or in the SCID mice used in the experiments reported here.

B18R treatment

Immediately after IC inoculation with either infected or uninfected MDMs, control and infected mice received either saline or B18R treatment. Each mouse received 3 IP injections of saline or B18R per day over a period of 10 days. HIV infected mice were separated into 2 groups of 8 each. The first group (HIV-

Saline; *n=8*) and control mice (*n=8*) received saline. The second HIV infected mice group (HIV-B18R; *n=8*) received B18R. Each mouse of the HIV-B18R group received 50 μg of B18R per day suspended in 900 μL of PBS in divided doses (300μL x 3 times a day). Preliminary data indicated the half-life of B18R as measured by ISG15 gene expression in the brain at 1, 3, 6, and 12 hours after B18R injections to be 2-3 hours (see Chapter 2), therefore three times a day dosage was chosen. Mice were monitored for physical and behavioral changes and mice showed no signs of toxicity. Physical perimeters monitored included weight, appearance of fur and ear positioning, and appearance of injection area. To monitor behavior, the mice were given fresh bedding daily to ensure that they were shredding and their level of lethargy was also assessed. Food and water intake was assessed daily and found to be normal.

Immunohistochemistry (IHC)

At day 10 post IC inoculation, all mice were sacrificed under xylazine (5 mg/kg) and ketamine (95 mg/kg) anesthesia and brains were extracted, snap frozen in tissue-freezing medium and stored at -70^{o}C until cryo-sectioning. Snap frozen brain tissues were taken and 10 and 5μm-thick coronal sections were made from the frontal lobe through the temporal-parietal lobe from each brain (Leica CM1850 UV, Leica Microsystems Inc, Buffalo Grove, IL). In brief, slides were collected starting at section number 100. Approximately 9 sets of serial coronal sections were collected from each mouse. Each set consisted of two 10μm and three 5μm-thick sections and each subsequent set was cut at 225-μm intervals (i.e., 45 sections apart). Sections were stained using an immunoperoxidase

method as previously described (Avgeropoulos, Kelley et al. 1998). In brief, to stain for microglial CD45, rat anti-mouse monoclonal antibody (mAb) was used (AbD Serotec, UK). Sections (5μm) were blocked in 2% normal rabbit serum (NRS) in PBS for 20 min. Sections were then incubated in primary mAb (1:50 diluted in 2% NRS) for 45 min. Slides were then incubated in biotinylated goat-anti-rabbit secondary antibody diluted (1:100) in 2% NRS (Vector, Burlingame, CA) for 30 min. Endogenous peroxidase was blocked with 0.1% H_2O_2 in methanol for 30 min and slides were incubated in avidin DH-biotin complex (ABC complex) (Vector) for 30 min. For color development, sections were incubated in 0.7 mg/ml Diaminobenzidine (Sigma) for 20 min. Sections were washed, dehydrated and finally cover-slipped.

To stain for HIV p24 antigen, human macrophages EBM11, glial fibrillary acidic protein (GFAP) that identifies astrocytes and microtubule associated protein-2 (MAP2) that identifies neuronal dendrites, mouse-on-mouse (M.O.M.) monoclonal Abs were used. Briefly, sections (10μm for p24 and EBM11 and 5μm for GFAP and MAP2) were incubated in 0.1% H_2O_2 in methanol for 30 min to block endogenous peroxidase. Sections were then incubated in M.O.M. blocking reagent (Vector) diluted by 1:20 dilution in PBS overnight at 4 ^{o}C followed by 1h at room temperature. Primary mAbs were applied diluted in Protein Concentrate (1:12.5 in PBS) at the following dilutions: 1:50 for p24 (Dako, Denmark); 1:20 for EBM11 (DAKO, Denmark); 1:750 for GFAP (Chemicon, Temecula, CA); and 1:200 for MAP2 (Chemicon, Temecula, CA) for 30 min. Sections were then incubated in secondary biotinylated antibody diluted (1:250) in protein concentrate for 10 min. Slides were then incubated in ABC complex for 5 min. Similar to CD45

staining, Diaminobenzidine was applied for 20 min to develop color. Finally, slides were washed, dehydrated and cover-slipped. Slides were then reviewed under light microscope (Olympus microscope: Melville, NY).

To stain for B18R, sections were stained with anti-B18R (1:500, Meiogen) as described in Chapter 2.

Densitometry scoring

The optical density was obtained as follows: x4 (GFAP), 20x (CD45 and MAP2) and 40x (B18R) images were captured using a QImaging Retiga EXi digital camera attached to an Olympus BX51 microscope. Three sections for GFAP and B18R and two sections for CD45 in the injection site area were analyzed from each mouse. Images were then analyzed by ImageJ 1.45S software (NIH, Bethesda, MD). Microscope and image capture parameters were kept constant. Each slide was subjected to the ImageJ software by selecting the representative GFAP-positive area and adjustments were made to include all GFAP and CD45 positive staining. Artifactual signal such as empty ventricles or folded tissue was not measured. For B18R, positive and background staining was measured in the injection site area in all B18R and saline injected mice. A mean optical density value was generated from each slide which was then averaged for each mouse prior to statistical analysis.

For MAP2 staining, mean optical density in the un-injected hemisphere (left) was assigned as a control for the injected hemisphere (right). The intensity of signal was measured in both left and right hemispheres and the percent reduction in MAP2 staining was considered a surrogate for the percent reduction

in dendritic arborization. A mean value was calculated from each section (left and right) and then averaged by mouse from two sections before the statistical analysis.

Human MDMs and p24-positive cells

EBM11 (human MDMs) and p24 positively stained cells in three consequential sections in the injection site area were counted from each slide and then averaged for each mouse as previously described (Avgeropoulos, Kelley et al. 1998). We counted a p24 or EBM11 positively stained cell as a single cell regardless of whether it was a single human macrophage or a single multinucleated cell. Dark brown reaction indicated positive staining for EBM11 or p24. Researchers were blinded prior to counting cells on slides.

RNA extraction and cDNA synthesis

We collected the intervening sections (45 sections between each set; refer to heading Immunohistochemistry) which were not used for IHC as previously described. RNA was extracted from these sections using the RNeasy Mini Kit according to the manufacturer (Qiagen) protocol. For each brain we used approximately 1 μg of RNA for cDNA synthesis. The first-strand cDNA synthesis was performed using the iScript cDNA Synthesis Kit according to the manufacturer (Biorad) protocol.

Real-time PCR

Levels of mouse interferon-induced gene15 (*ISG15*), interferon-alpha 4 gene (*IFNA4*), and interferon-alpha response gene 15 (*Ifrg15*) were analyzed using real-time PCR with a Biorad C1000 Thermocycler. Levels were normalized to the levels of Glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*), a housekeeping gene, which was used as the endogenous control for all experiments. *ISG15*, *IFNA4*, *Ifrng15*, and *GAPDH* primers were designed using Assays-by-Design (Applied Biosystems). cDNA samples were added to TaqMan Assay mix (Applied Biosystems) and the primer/probe mix. Relative quantification of the ISG15 gene in unknown samples was done in comparison to the levels of GAPDH in each sample. The relative changes in gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistics

Analyses of densitometry vales and relative gene expression levels were performed using One-way ANOVA and an unpaired t test in GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, CA). Significance was set at $p<0.05$ for all analyses.

Results

HIV infected MDMs in mouse brain

We counted the number of EBM11 and p24 positively stained cells corresponding to the number of human MDMs and HIV-infected human MDMs respectively in the brains of mice IC inoculated with uninfected (control) human MDMs, mice IC inoculated with HIV-infected MDMs treated with saline (HIV-Saline) and mice IC inoculated with HIV-infected MDMs treated with B18R (HIV-B18R). There was no statistically significant difference between the numbers of EBM11 positively stained cells across the three groups (data not shown). These findings indicate that approximately the same number of HIV-infected and uninfected human MDMs were inoculated into the control and HIV-infected mouse brains. Staining showed human MDMs scattered around the injection site. These findings were consistent with our previously reported data (Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2009).

Similarly, there was no significant difference in the numbers of p24 positively stained cells in the brains of HIV-Saline and HIV-B18R treated mice, indicating the presence of the same number of HIV-infected human MDMs in both groups. As measured by p24positive human MDM, CNS HIV did not increase (see Supplemental Figure 3.1). This is consistent with previous studies using polyclonal anti-IFNα antiserum (Sas, Bimonte-Nelson et al. 2009).

B18R crossed the blood-brain barrier

We examined mouse brain using an antibody against B18R, which showed the presence of B18R in the brain parenchyma and suggested localization on glia and neurons (data not shown). B18R densitometry indicated that mice IC inoculated with HIV-infected human MDMs that received IP injections of B18R had significantly higher densitometric values compared to background staining detected in mice that received saline treatment (HIV-B18R vs. control; $p<0.001$) as shown in Figure 3.2.

B18R inhibited IFNα signaling in the brain

To assess the ability of the IP injected B18R to cross the mouse BBB and block IFNα signaling pathways in the brain, we used real-time PCR to measure mRNA levels for ISG15, *IFNA4,* and *Ifrng15,* genes that are activated after IFNα receptor engagement. The mRNA levels of ISGs in mice IC inoculated with HIV-infected human MDMs treated with saline (HIV-Saline) were significantly higher ($p<0.001$) than those observed in control and HIV-B18R as shown in Figure 3.3. Interestingly, the B18R treatment resulted in ISGs mRNA levels similar to those observed in the control mice group, suggesting that B18R completely inhibited IFNα signaling in brain.

Astrogliosis

GFAP densitometry showed that mice IC inoculated with HIV-infected human MDMs had significantly higher astrogliosis than mice IC inoculated with uninfected human MDMs (HIV-Saline and HIV-B18R vs. control; $p<0.001$) as shown in Figure 3.4A (See also Supplemental Figure 3.2). This finding is

consistent with our previously reported data (Avgeropoulos, Kelley et al. 1998, Sas, Bimonte-Nelson et al. 2009). Densitometry values for astrogliosis were slightly lower in B18R treated mice IC inoculated with HIV-infected human MDMs compared to the HIV-Saline treated mice, but the difference was not statistically significance.

Mouse macrophages and microglia

CD45 densitometry showed that mice IC inoculated with HIV-infected human MDMs had significantly higher presence of mouse macrophages and microglia, than in control mice (HIV-Saline vs. control; $p<0.001$) as shown in Figure 3.4B. Interestingly, B18R treatment significantly reduced (HIV-Saline vs. HIV-B18R; $p<0.05$) CD45 staining in mice IC inoculated with HIV-infected human MDMs. However, B18R treatment did not bring the level of mouse macrophages and microglia staining down to control levels. Visibly less CD45 staining is evident in the HIV-B18R treated mice versus the HIV-Saline treated mice (Supplemental Figure 3.2).

Neuronal dendritic arborization

In accordance with previously reported data (Cook-Easterwood, Middaugh et al. 2007, Sas, Bimonte-Nelson et al. 2007), our results indicate that mice IC inoculated with HIV-infected human MDMs had more dendritic loss, as measured by MAP2 staining ($p<0.05$) compared to control mice (Figure 3.4C). Treatment with B18R showed a significant improvement of MAP2 staining compared to saline injected mice. The area of the neuronal degeneration is visibly

larger in the HIV-Saline mice compared to the HIV-B18R treated mice as seen in Supplemental Figure 3.2.

Discussion

In this investigation B18R treatment alleviated histopathological complications seen in an HIVE/SCID mouse model. Our data indicate that the IP injected B18R was capable of crossing the BBB, as evident by the IHC staining and densitometry results. B18R treatment reduced the severity of histopathology and inhibited IFNα mediated signaling in mouse brains. Importantly, the treatment with B18R had no effect on HIV infection since approximately the same numbers of p24 positively stained MDMs were found in saline and B18R treated mice (Supplemental Figure 3.1).

Elevated IFNα levels in the CNS are associated with cognitive abnormalities (Fritz-French and Tyor 2012). The expression of type I IFN genes was shown to be elevated in the frontal cortex of HAD patients (Masliah, Roberts et al. 2004). Previous reports showed that the levels of IFNα were significantly higher in the CSF of HIV-infected patients with dementia compared with HIV patients without dementia or HIV-negative individuals (Rho, Wesselingh et al. 1995, Krivine, Force et al. 1999). Interestingly, IFNα levels were directly correlated with the severity of dementia. Our mouse model reflects these clinical studies showing elevated levels of IFNα, increased IFN-mediated signaling (Figure 3.3), and abnormal cognitive behavior.

Previous studies in our lab found that treating HIVE/SCID mice with IFNα NAbs alleviated cognitive dysfunction and improved HIVE in mice. B18R protein is a more ideal treatment than NAbs because it is a direct IFNα binding protein with higher affinity for IFNα than the type I interferon receptor and is a multi-subtype IFNα inhibitor, suggesting the possibility of very low dose therapy

with B18R. The binding constant of B18R surpasses other antibodies by more than an order of magnitude (Boder, Midelfort et al. 2000). Cytokine binding proteins with low affinity run the risk of agonist rather than antagonist activity by stabilizing normally short lived cytokines (Klein and Brailly 1995). Antibody affinity can be increased using phage display technology but, with humanized antibodies, these modifications generally increase the risk of human constant region auto-antibody induction (Boder, Midelfort et al. 2000).

In clinical practice, all injected proteins are immunogenic whether human or non-human (CHMP 2010). This suggests that NAbs maybe endogenously produced to counteract injected monoclonal antibodies resulting in treatment failure (Leroy, Baud et al. 1998, Hemmer, Stuve et al. 2005). For example, the anti- IFNα fully human IgG used in clinical trials induced an immune response in up to 20% of injected patients (CHMP 2010). In addition to being anti-idiotypic, these antibodies can often bind to human IgG constant regions with the associated risk of inducing immune complex-mediated disease (CHMP 2010). Viral immunomodulatory proteins are often modified human proteins but, in some cases, they have no amino acid sequence homology to any human protein (Lucas and McFadden 2004). B18R has minimal, if any, amino acid sequence homology to any human protein and thus provides the advantage that its expected immunogenicity in immune-competent individuals is not likely to induce autoimmunity.

In viral infections significant type I interferon, including IFNα, is produced, which plays a vital role in suppressing viral replication. Consequently, reducing type I interferons with B18R could result in a damaging effect on the

immune system's ability to mount an appropriate antiviral response leading to increased viral loads. However, previous studies showed that IFNβ (Sas, Bimonte-Nelson et al. 2009) and likely IFNγ and type III interferons are still present in HIVE/SCID mice, where IFNα has been blocked. In addition, B18R treatment is expected to improve the ability of patients to fight infection. This somewhat counter-intuitive result was demonstrated in clinical trials in late-stage HIV+ patients where improved blood lymphocyte counts and decreased viral load was observed in those HIV infected patients who were successfully immunized against their own IFN (Gringeri, Musicco et al. 1999). Despite these cautions regarding potential B18R treatment in humans, the present study strongly suggests that HIV loads were not affected by B18R treatment in the HIVE/SCID mice.

The vast majority of HIV infected cells in the CNS are macrophages and microglia (i.e., increased presence of mononuclear phagocytes) (Glass and Wesselingh 2001). The presence of increased numbers of macrophages and microglia, other than demonstrating HIV infection of brain, is arguably the hallmark of HIVE (Glass and Wesselingh 2001). These cells are probably the main source of HIV, HIV proteins like Tat, cellular metabolites that are potential neurotoxins, and IFNα (Tyor 2009). Therefore, reducing the number of reactive macrophages and microglia in HIVE may, in and of itself, alleviate neuronal abnormalities and thus cognitive dysfunction. Interestingly, our results show that the IP injected B18R in HIVE/SCID mice significantly reduced the presence of mouse macrophages and microglia (Figure 3.4B). Results of this study are consistent with our previously reported data in which IP injections of IFNα NAbs

significantly reduced the presence of mouse macrophages and microglia in HIVE/SCID mice.

In our study, B18R did not significantly reduce astrogliosis (Figure 3.4A). These results were consistent with those reported by Sas et al. in which the IP injected IFNα NAbs did not reduce astrogliosis. Therefore, astrogliosis might not be a critical feature of HIVE. Our previous studies using cART in this HIVE/SCID mouse model have also suggested that astrogliosis is not as important as increased numbers of macrophages and microglia in the pathogenesis of HAND (Cook, Dasgupta et al. 2005, Cook-Easterwood, Middaugh et al. 2007). While cART in HIVE/SCID mice reduced astrogliosis, it had no effect on MAP2 reductions (i.e., neuronal integrity) or behavioral dysfunction (Cook-Easterwood, Middaugh et al. 2007). Taken together these studies suggest that reducing the number of macrophages and microglia, but not astrocytes, in HIVE is associated with preservation of neuronal integrity.

Although HIV does not infect neurons, neurotoxicity in HAND is evident by neuronal and neurocognitive abnormalities (McArthur, Steiner et al. 2010). As stated above, infected macrophages and microglia in HIVE interact with other cells in the CNS to produce substances like TNFα and IFNα which cause neurotoxicity (Tyor 2009). In the present study, B18R preserved dendritic arborization as shown by MAP2 staining compared to saline injected mice (Figure 3.4C). The deleterious action of IFNα on neuronal function and anatomy was demonstrated in our previous *in vivo* and *in vitro* investigations. Although the exact mechanism has to be elucidated, our *in vitro* experiments suggest that part of this mechanism involves N-methyl-D-aspartate (NMDA) receptors. This is

interesting because other putative HIVE neurotoxins (e.g., gp120) have also been linked to NMDA toxicity (Potter, Figuera-Losada et al. 2013, Yang, Hu et al. 2013). Therefore this may represent a final common pathway for several neurotoxins implicated in HAND pathogenesis. Our results suggest that B18R would be an effective therapeutic agent to prevent or reduce IFNα neurotoxicity in HAND. Future studies will look at behavior in HIVE/SCID mice to see if B18R treatment not only prevents cognitive dysfunction, but reverses it, as seen in patients that discontinue IFNα therapy.

It is important to note that the pathogenesis of HAND likely involves multiple putative neurotoxins that are not limited to IFNα. The severe form of HAND (i.e., HAD) may result from synergistic neurotoxicity of viral proteins like Tat and gp120 in addition to IFNα and other neurotoxins, such as free radicals (Tyor 2009). Nevertheless, it seems likely that IFNα is a major source of neurotoxicity in HAND, especially early in the course of the disease. Here, we have shown that B18R treatment resulted in improvement in HIVE/SCID mice, suggesting that B18R is likely to be an effective therapy in humans. Future studies will investigate the effects of B18R on improving behavioral memory tasks in cognitively impaired HIVE/SCID mice as was previously investigated with IFNα NAb and in combination with cART. If these studies are successful, B18R may be moved forward into Phase I trials in humans with HAND.

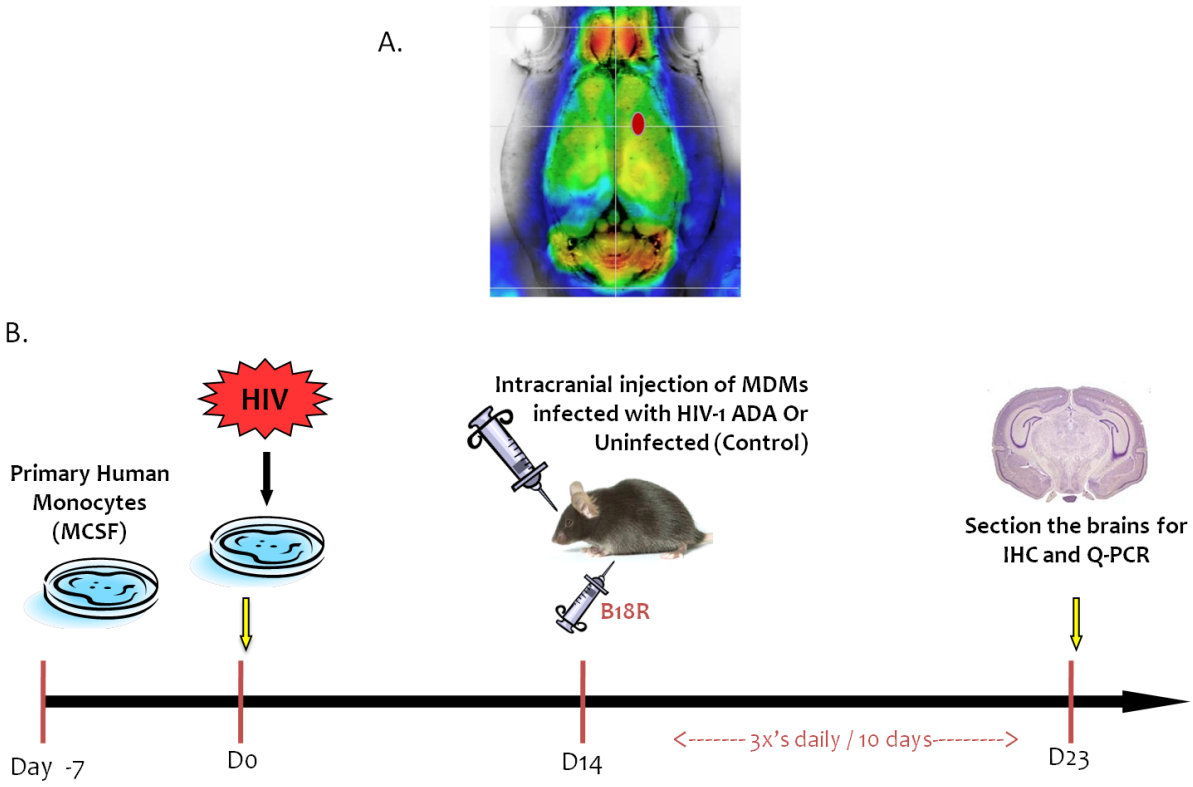


Figure 3.1. HIVE/SCID mouse model experimental design involves inoculating SCID mice in the frontal right cortex with human MDMs (A). Time course of cell infection and mouse inoculation and B18R treatment is shown in B.

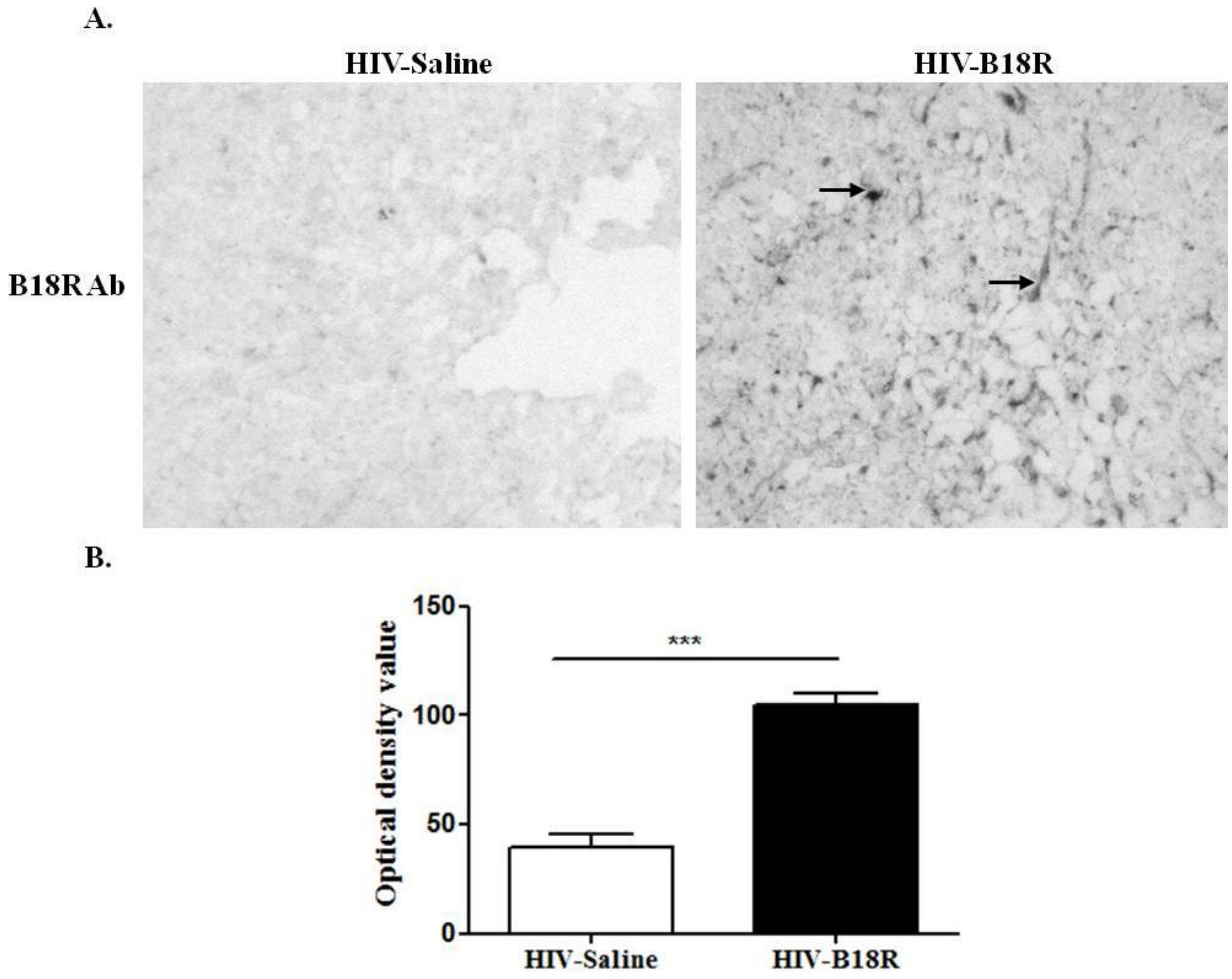


Figure 3.2: Immunohistochemistry staining for B18R in mouse HIV-infected brain tissue in B18R treated and untreated groups (A). Arrows indicate positive B18R staining on cells within the brain. Densitometry values (B) of B18R in brain sections of mice inoculated with HIV-infected human MDMs and treated with saline (HIV-Saline; *n=8*) or B18R (HIV-B18R; *n=8*) (***p <0.001).

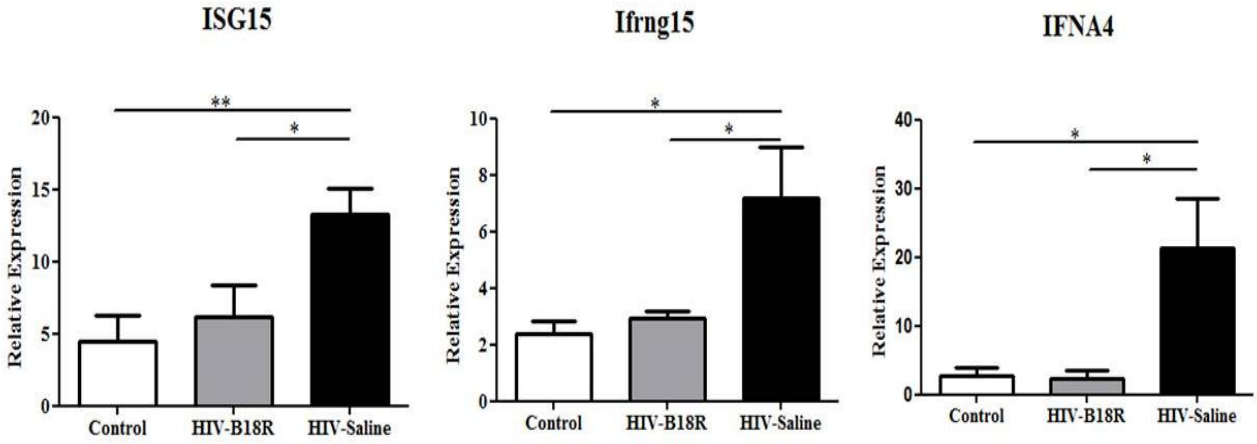


Figure 3.3: Real-time PCR analysis of interferon stimulated genes (ISGs) (*ISG15, Ifrg15,* and *IFNA4*) mRNA in control (mice inoculated with uninfected human MDMs and treated with saline; *n=8*), HIV-Saline (mice inoculated with HIV-infected human MDMs and treated with saline; *n=8*) and HIV-B18R (mice inoculated with HIV-infected human MDMs and treated with B18R; *n=8*) (*p <0.05, **p<0.01).

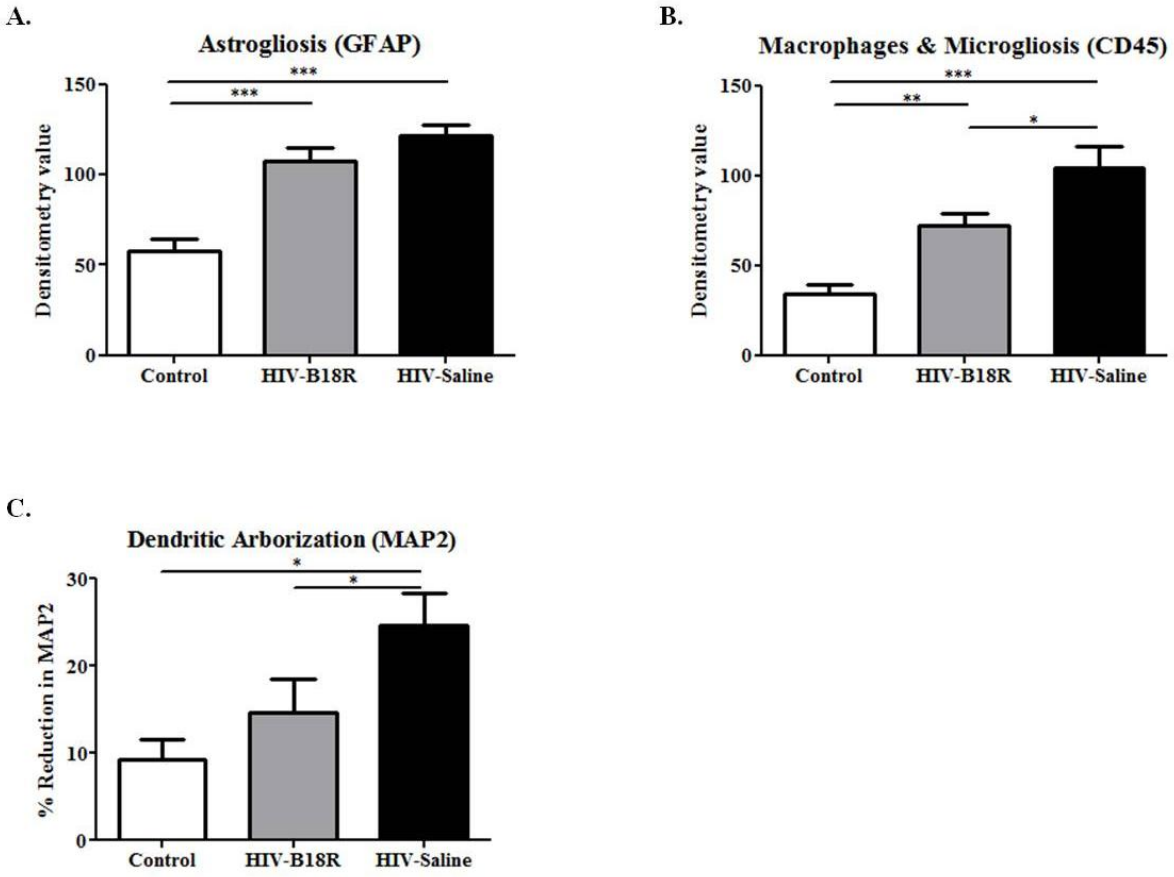
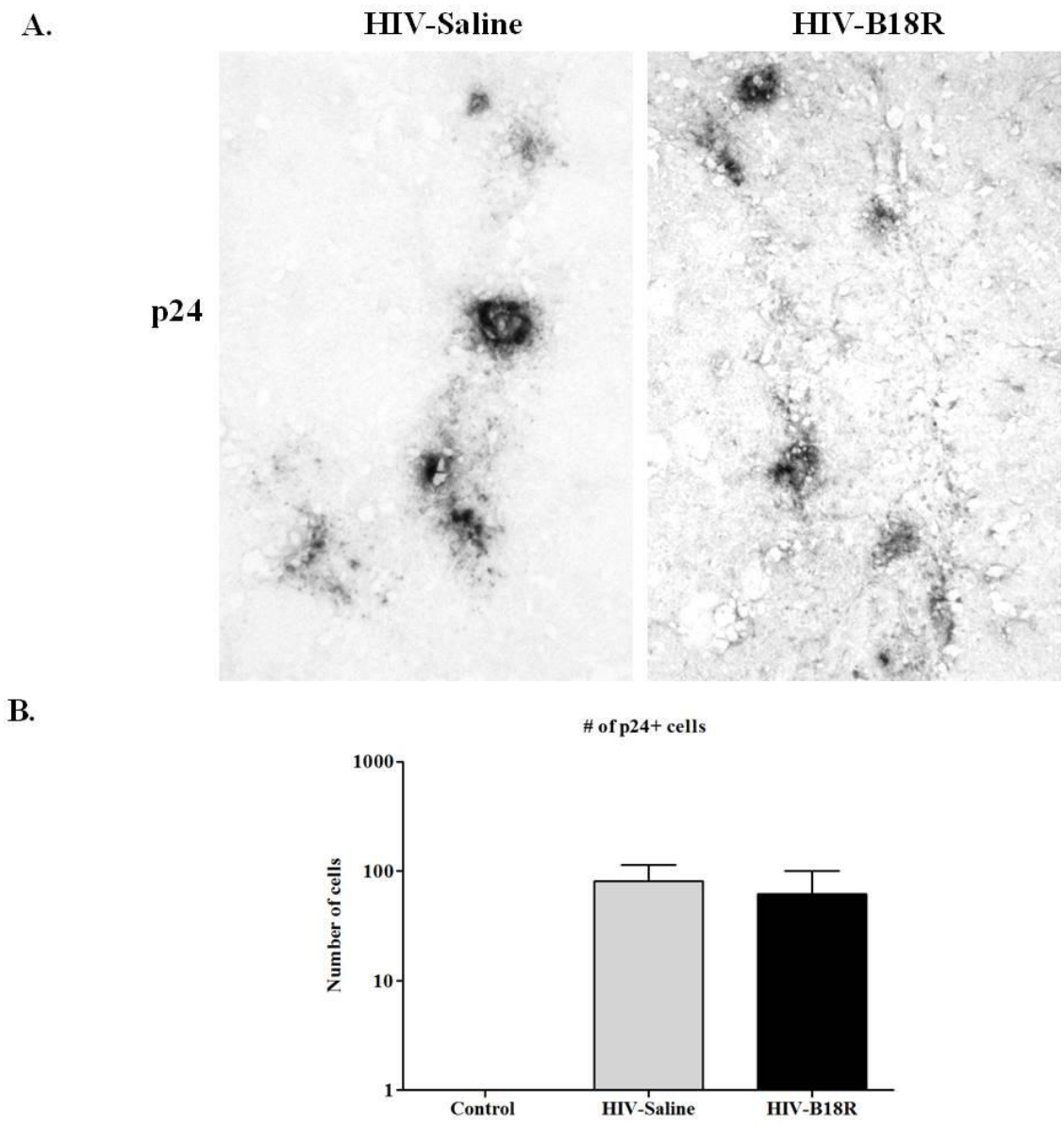
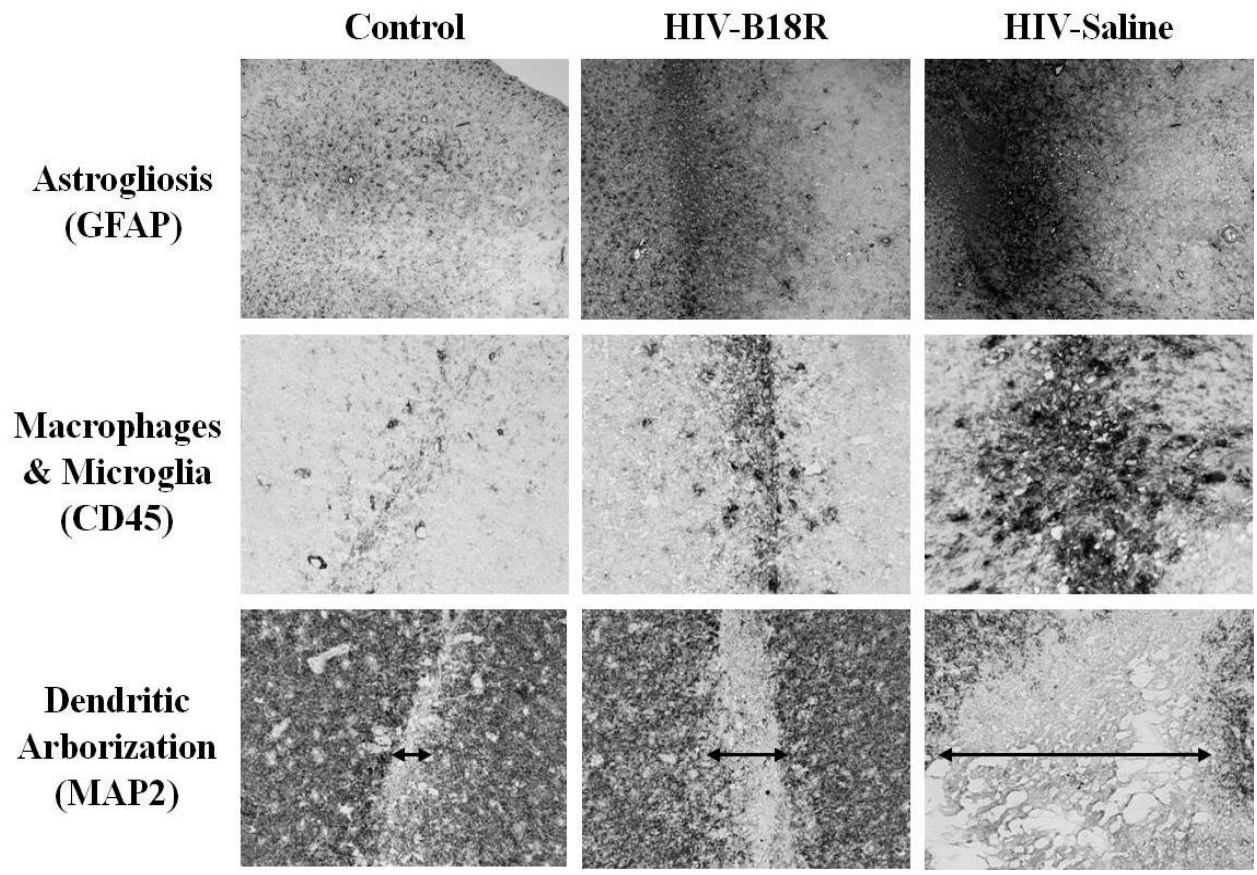


Figure 3.4: Densitometry values of astrogliosis, microgliosis, and dendritic arborization in all mice. Astrocytes (mouse GFAP) expression was slightly decreased in HIV-B18R mice compared to HIV (A) (***p<0.001). Mice treated with B18R had significantly less staining for mouse macrophages and microglia (mouse CD45) compared with HIV mice (B) (*p<0.05, **p<0.01, ***p<0.001). B18R treated mice were significantly protected against reduction in dendritic arborization (mouse MAP2) compared to HIV mice (C) (*p<0.05).



Supplemental Figure 3.1 Immunohistochemistry staining for p24 positive human MDMs for saline and B18R treated HIVE/SCID mice (A). Number of p24 positively stained human MDMs counted in brain sections of control (*n=8*), HIV-Saline (*n=8*) and HIV-B18R (*n=8*) (B). Expectedly, there was no positive staining for p24 in all control mice. There was no significant difference between the counts of positive cells across the treated and untreated groups.



Supplemental Figure 3.2: Immunohistochemistry stain representations for astrogliosis (mouse GFAP), macrophages and microglia (mouse CD45), and dendritic arborization (mouse MAP2) in mouse brain tissue for control, HIV-B18R, and HIV-saline groups. Arrows shown for dendritic arborization illustrate the increase in reduction of MAP2 staining that is seen during severe inflammation in HIV-saline mice brains. Densitometry analysis and statistics are presented in Figure 3.4.

Chapter 4:

Interferon-α induces neurotoxicity through activation of the type I receptor and the NR2A subunit of the NMDA receptor.

Introduction

Interferon alpha (IFNα) is a critical cytokine that creates an antiviral state in surrounding cells as well as inhibits viral replication within infected cells. IFNα has also been identified as a potential neurotoxin in diseases such as HIV-associated neurocognitive disorders (HAND) (Rho, Wesselingh et al. 1995), Aicardi-Goutieres syndrome (Goutieres, Aicardi et al. 1998), systemic lupus erythematosus (Shiozawa, Kuroki et al. 1992) and multiple sclerosis (Traugott and Lebon 1988, Fritz-French and Tyor 2012, Tzartos, Khan et al. 2012). Additionally, neuronal toxicity has been implicated in hepatitis and cancer patients being treated with high-dose IFNα therapy where patients have shown neuropsychiatric side effects, such as subcortical dementia, that dissipate after terminating treatment (Valentine, Meyers et al. 1998, Kirkwood, Bender et al. 2002, Schaefer, Engelbrecht et al. 2002). Identifying pathways involved in IFNα neurotoxicity could lead to potential therapies for CNS inflammatory diseases as well as treatments directed to the side effects of IFNα therapy.

Perhaps the most compelling evidence of IFNα induced neurotoxicity has been in animal (Sas, Bimonte-Nelson et al. 2009) and clinical studies of HAND (Rho, Wesselingh et al. 1995). Importantly, IFNα levels in the cerebrospinal fluid of HIV-infected patients are significantly higher in patients with dementia compared to patients without dementia (Rho, Wesselingh et al. 1995). Studies using a SCID mouse model of HAND indicate that increased IFNα levels in the brains of HAND mice correlate with cognitive and neuronal dysfunction (Sas, Bimonte-Nelson et al. 2007) and that treatment with neutralizing antibodies to IFNα significantly decreases neuropathology (Sas, Bimonte-Nelson et al. 2009).

This substantial, corroborative data points to IFNα most likely playing a role early on in HAND when neuronal death is not prominent and toxicity is still reversible. Despite the advent of combination antiretroviral therapy (cART) the prevalence of HAND continues to increase in HIV-infected patients, affirming the need for more specific treatments targeted to HAND. Therefore, we have focused on IFNα as a potential target for treatment in HAND.

The mechanism of IFNα induced neurotoxicity has yet to be established. Here we begin to investigate the cell signaling pathways involved using a cortical neuron culture model (Kaech and Banker 2006). We examine the roles of cytoskeletal proteins, type 1 interferon receptor engagement (IFNAR), and the *N*-methyl-D-asparate receptor (NMDAR), which has been previously implicated in IFNα induced neurotoxicity and in HAND pathogenesis (Sas, Bimonte-Nelson et al. 2009). IFNα initiates an antiviral response by binding to the IFNAR, and therefore we expected that IFNAR binding would be required for IFNα induced neurotoxicity. However, many other potential neurotoxins are produced after IFNα exposure and it is also possible that IFNα directly or indirectly employs receptors other than the IFNAR. In the present study we show that blocking the IFNAR only partially protects neurons from dendritic damage caused by IFNα and that the NR2A subunit of the NMDAR also plays a significant role in toxicity IFNα. The NMDAR has been linked to IFNα toxicity in our previous studies as well as clinical studies where treating patients on IFNα therapy with mild NMDAR antagonists resulted in decreased side effects (Quarantini, Miranda-Scippa et al. 2006, Sas, Bimonte-Nelson et al. 2009). To further delineate the pathways involved in IFNα neurotoxicity, we tested the effects of IFNα on

neuronal cytoskeletal proteins, such as microtubule associated protein 2 (MAP2), as well as genes involved in synaptic plasticity. Further characterizing signaling molecules involved in IFNα induced neurotoxicity will help to identify potential therapeutic targets for inflammatory diseases of the CNS where IFNα is upregulated, such as HAND and the side effects of IFNα therapy.

Materials and Methods

Neuronal Cell Culture

Frontal, cortical neurons were dissected from E18 Sprague Dawley rat embryos (Charles River Laboratory). High-density cultures for protein and RNA extractions were prepared by plating neurons in poly-L-lysine (PLL) (Sigma) coated dishes at 5x10 cells/60mm dish and incubated at 37°C with 5% CO_2 in plating medium (MEM supplemented with 10% FBS, glucose 0.6% wt/vol, HEPES). After 2hrs, plating medium in the high-density cultures was exchanged for neurobasal medium (neurobasal medium supplemented with B27 and GlutaMax) (Invitrogen). Low-density neuronal cultures prepared for co-culturing with glia were plated on PLL coated glass coverslips at $5x10^5$ cells/10cm dish as described in Kasech and Banker (2006). Two-hours after plating, the low-density neuronal cultures on the glass slides were flipped over onto a dish containing a glial monolayer grown in neurobasal medium. Cytosine-D-arabinoside (Ara-C) (1 μM) (Fluka) was added to both high- and low-density cultures at day 3 after plating to prevent glial cell proliferation. Media was exchanged every 7 days.

Treatment and Reagents

Rat IFNα (PBL Interferon) was diluted in 0.9% saline at a dose range of 300-900 IU/μl and a single dose was administered to cultures on day 14. Control groups were given equal volumes of saline. For high-density cultures, treatment was left on cultures for different time points varying from 20mins to 48hrs prior to RNA or protein extractions. Low-density neurons co-cultured with glia on

coverslips were removed from the glia and treated with IFNα or saline for 2 hours. The neurons were then returned to the glia for long-term culturing. Type I interferon receptor (IFNAR) antibody (eBioscience) or IgG1 K isotype control (eBioscience) or saline was administered at 1 to 50 μg/μl 20 minutes prior to IFNα treatment. NMDAR antagonists TCN 201 (Tocris) and ifenprodil (Tocris) was administered at 0.1 to 10 μM 20 minutes prior to IFNα treatment.

Protein Extractions and Western Blot

Protein was harvested from the high-density neuronal cultures using RIPA buffer with 10 μL/ml protease inhibitors (Thermo Scientific), incubated on ice for 10 min, and spun down at 14000 rpm for 10 min. The lysate was removed and total protein concentration was determined by a Pierce® BCA Protein Assay Kit. The lysate was aliquoted and stored at -80°C. Five micrograms of each sample was then loaded on a 7.5% polyacrylamide gel. Protein was then transferred to PVDF membrane run at 120V for 40mins. The anti-MAP2, anti-Phospho-MAP2 (Thr1620/1623), anti-Phospho-MAP2 (Ser136), anti-Phospho-STAT1, anti STAT1 (Cell Signaling), anti-ERK (Cell Signaling), anti-Phospho-ERK (Cell Signaling), anti-Phospho-CREB (Cell Signaling) and GAPDH (Millipore) antibodies were used as primary antibodies at 1:1000 each. Western Blots were probed according to the manufacturer's protocol. The membrane was then washed and incubated with either goat anti-rabbit IgG HRP-linked secondary antibody at 1:1000 (Cell Signaling) or goat anti-mouse IgG HRP-labeled secondary antibody at 1:1000 (Perkin Elmer). The membrane was then exposed

to X-ray film using Supersignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

Real-time PCR

High-density neuronal cultures were used to extract RNA according to the RNeasy Mini Kit (Qiagen) protocol. cDNA synthesis was performed using iScript cDNA Synthesis Kit (Biorad). Levels of rat interferon-stimulated gene 15 (ISG15) were analyzed using real-time PCR with Biorad C1000 Thermocycler and GAPDH was used as our house keeping gene and an endogenous control for experiments. ISG15 and GAPDH primers were designed using Assays-by-Design (Applied Biosystems). A rat synaptic plasticity PCR array (Qiagen) was also used and manufacturer's protocol followed. Levels of the target gene were normalized against the endogenous gene to assess relative quantification. The relative changes in gene expression were analyzed using $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen (Livak and Schmittgen 2001).

Image acquisition and processing

The low-density neuronal cultures plated on the glass coverslips were removed from the glial co-culture and washed in PBS before fixing cells with cold methanol for 20 minutes at -20˚C. Coverslips were rinsed in PBS and TBS_{50} before permeabilizing the cells in 0.3% Triton X-100/TBS_{50} for 5 minutes. Cells were then rinsed in 0.1% Triton X-100/TBS_{50} before blocking in blocking buffer (2% BSA, 2% FBS (Sigma), 0.1% Triton X-100, in TBS_{50}) for 1-2 hrs. Coverslips were then rinsed in buffer (2% BSA, 0.1% Triton X-100 in TBS_{50}) and then incubated in primary anti-MAP2 antibody (Millipore) at 1:300 at room

temperature for 1 hr. Secondary fluorescein goat anti-rabbit IgG (Vector) was then added at 1:500 in the dark after rinsing for 30 min. Coverslips were rinsed and placed on slides with Vectashield fluorescence protection (Vector). Slides were viewed using an Olympus IX71 and photographs were taken using ImagePro Express 6.0. ImagePro was also used to measure dendritic length and branching.

Electrophysiology on dissociated rat cortical neurons

After 16- 21 days in vitro, neurons were transferred by glass coverslip (12 mm round) onto the perfusion chamber of a patch-clamp electrophysiology apparatus. Artificial cerebrospinal fluid (in mM: 127 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.4 NaH_2PO_4, 25 glucose, bubbled with 95% O_2, 5% CO_2, pH 7.4, osmolarity 310-320 mOsm) was perfused over the monolayer of cells at a rate of 0.75 – 1.5 mL/min. IFNα was superfused via bath application. Neurons were allowed to equilibrate for at least 2 minutes between solution changes. Healthy neurons with smooth borders were visually identified under the 40X water immersion IR/DIC objective (Olympus BX51WI) and a patch-clamp microelectrode (resistance 3 – 8 MΩ) filled with intracellular solution (in mM: 145 potassium gluconate, 3 KCl, 2 $MgCl_2$, 0.2 EGTA, 10 HEPES, NaATP, pH 7.25, osmolarity 280-290 mOsm) was used to form a gigaohm seal in the conventional way. Whole-cell configuration was established to gain electrical access to the inside of the cell. Signals were amplified with a MultiClamp 700B and recorded with pClamp11 (Axon Instruments, Foster City, CA). Action potentials (APs) were recorded in current clamp configuration and post-synaptic currents were recorded in voltage clamp configuration (holding potential: -70 mV). Height of

APs, firing frequency, and post-synaptic current frequency were determined using custom analysis scripts written in MATLAB (MathWorks, Natick, MA). Biophysical indicators of neuronal health (series resistance, whole-cell capacitance, and resting membrane potential) were periodically monitored throughout data gathering to insure stationarity.

Statistics

Analysis of real-time PCR results and dendritic length and branching data was completed using two-way ANOVA. Analysis between treatment groups was done with *post hoc* Tukey tests using SPSS Statistics 19 software. Significance was set at $p\text{-value}<0.05$ for all analyses.

Results

IFNα is toxic to neurons co-cultured with glia

Previously, we showed that IFNα administration had a dose-dependent effect on neuronal dendritic morphology in rat dissociated neurons from the frontal cortex (Sas, Bimonte-Nelson et al. 2009). Here we used a new culture system involving rat primary neurons co-cultured with a monolayer of glia that results in neurons with robust dendritic morphology that could be cultured for extended time points. To study the effect of IFNα on primary rat neurons co-cultured with glia, neurons were treated with various doses of IFNα and dendritic morphology was measured to assess total length of dendrites per neuron and number of dendritic branches. Neuronal dendritic length and branching was significantly reduced after treatment with 300 IU of IFNα at 48 hours after treatment, confirming previous results (Figure 4.1A). For the present study, a more comprehensive time course study was done to assess when dendritic morphology damage begins, where previously only one time point (48 hours) was observed (Sas, Bimonte-Nelson et al. 2009). A significant decrease in dendritic length and branching occurs at 24 hours and continues for 72 hours, but no effect is seen at 12 hours after IFNα exposure (Figure 4.1B).

IFNα does not have a direct effect on MAP2 expression

Previous data in the lab (Sas, Bimonte-Nelson et al. 2009) and other studies (Masliah, Roberts et al. 2004) suggested that IFNα may have an effect on neuronal cytoskeletal proteins like MAP2. To begin to determine the effect of IFNα on cytoskeletal proteins, cortical neurons were treated with 300 IU of IFNα

and protein extracted from high density cultures at time points between 20 minutes and 48 hours. Protein extracts were analyzed by immunoblotting with MAP2 antibodies. Surprisingly, Western blot analysis revealed that IFNα has no effect on total MAP2 protein (Figure 4.2A). These results were also confirmed with a MAP2 specific ELISA at all time points (Figure 4.2E). The activation of MAP2 by phosphorylation is critical for neuronal integrity and we wanted to test whether the phosphorylation state of MAP2 was affected by IFNα treatment. Immunoblotting was utilized with MAP2 antibodies that recognize residues Ser136 and Thr1620/1623. Phosphorylation at these sites modulate association of MAP2 with microtubule and therefore affect stability and development the cytoskeleton in neurons. The phosphorylation state of MAP2 was not affected at either residue based on western blot analysis (Figure 4.2B). Finally, MAP2 mRNA levels were examined with real-time PCR. MAP2 mRNA levels were found to be unaffected by IFNα treatment at all time points (Figure 4.2C). MAP1a, another cytoskeletal protein implicated in HAND, was examined and mRNA levels were also found to be unchanged in IFNα treated cultures (Figure 4.2D).

Additionally, a gene array designed to look at genes associated with synaptic plasticity involving long term potentiation, extracellular matrix processing, neuronal receptors, and postsynaptic density was utilized. Preliminary data at 20 minutes after IFNα exposure revealed no change in gene expression between treated and untreated cultures.

JAK/STAT pathway activated in neurons treated with IFNα

Activation of the JAK/STAT pathway after IFNα treatment has not previously been investigated in primary cortical neurons. For protein and RNA analysis, high density neuronal cultures were utilized. Neurons were treated with 300 IU of IFNα and protein extracted at 20 minutes, 2, 12, 24, and 48 hours. Neurons showed a peak of phosphorylated STAT1 at 20 minutes after a single IFNα treatment that persisted for 48 hours based on western blot analysis (Figure 4.3A). Interferon-stimulated gene 15 (ISG15) is upregulated after IFNAR activation and used as a marker in this system to assess downstream JAK/STAT pathway activation. ISG15 mRNA was elevated in cultures treated with IFNα 20 minutes after treatment (Figure 4.3B). ISG15 levels remained elevated in neuronal cultures treated with IFNα for at least 48 hours.

Blocking IFNAR blocks the JAK/STAT pathway

Although IFNα is likely to be exerting some neurotoxic effects through its receptor, previous data and data from current studies indicate receptor associations that are complex (Sas, Bimonte-Nelson et al. 2009). In addition, IFNα stimulates a number of substances that could be neurotoxic, therefore, many or all IFNα effects could be indirect and independent of the IFNAR. In order to determine the requirement of the IFNAR for IFNα induced neurotoxicity, the effectiveness of an IFNAR-blocking antibody was examined in culture. IFNAR engagement induces the JAK/STAT pathway after IFNα binds the receptor. Based on immunocytochemistry the IFNAR is ubiquitous throughout neurons (Figure 4.4B). Neurons pretreated with IFNAR-blocking antibody prior to IFNα treatment showed a decrease in phosphorylated STAT1

after 20 minutes compared to neurons treated with an isotype matched control to the IFNAR antibody or IFNα only treated cultures (Figure 4.4C). To ensure that the pathway was efficiently blocked, ISG15 gene expression was examined. ISG15 expression in IFNAR antibody pretreated cultures was equivalent to saline treated cultures indicating that the JAK/STAT pathway was effectively blocked by the IFNAR antibody (Figure 4.4A).

Blocking IFNAR is partially protective against IFNα induced neurotoxicity

After determining that the IFNAR antibody is efficient in blocking the JAK/STAT pathway in neuronal cultures, we assessed the ability of the IFNAR antibody to block IFNα induced neurotoxicity. Neuronal cultures were pretreated with either IFNAR antibody, an isotype control, or saline. After 20 minutes, neurons were exposed to IFNα or saline and cultured for 48 hours. Dendritic measurements and analysis of IFNAR antibody treated cultures showed partial protection against IFNα induced neurotoxicity based on total dendritic length and branching (Figure 4.5). Cultures treated with varying doses of IFNAR antibody prior to IFNα exposure were significantly different than cultures treated with IFNα alone. Additionally, IFNAR antibody treated cultures were significantly different from untreated cultures, suggesting that other pathways, aside from the IFNAR related pathway, are involved in mediating neuronal toxicity after IFNα exposure.

Blocking NR1/NR2A-containing NMDAR more protective than blocking NR1/NR2B

Previous studies found that blocking the NMDAR with MK801 partially protected neurons from IFNα induced neurotoxicity (Sas, Bimonte-Nelson et al. 2009). MK801 is a non-competitive NMDAR antagonist. In our current studies, blocking the IFNAR only partially protected neurons from IFNα toxicity. Considering these factors, we then investigated the role of the NMDAR in IFNα neurotoxicity in more detail. Here, we examined the effects of neurons pretreated with TCN201 (NR1/NR2A antagonist) (Edman, McKay et al. 2012)and ifenprodil (NR1/NR2B antagonist) (Reynolds and Miller 1989) prior to IFNα exposure (Figure 4.6). Neuronal cultures were treated with doses ranging from 0.1 to 10 uM of either TCN201 or ifenprodil 20 minutes before adding IFNα. Cultures were analyzed 48 hours later for dendritic length and branching. Neuronal cultures pretreated with 10 uM of TCN201 were found to be significantly different than cultures treated with IFNα alone indicating a partial protective effect. Ifenprodil was found to have no protective effects against IFNα.

IFNα does not affect CREB/ERK protein levels or phosphorylation

Neuronal cultures treated with IFNα showed no effect on total ERK protein levels based on immunoblotting (Figure 4.7). Additionally, phosphorylation of ERK was not affected by IFNα treatment in neurons. Additionally, phosphorylation of CREB or total CREB was not found to be altered (Figure 4.7).

IFNα interferes with neuronal communication

Exposure of dissociated neurons in low density culture to IFNα 1000 U / mL resulted in slowing of spiking activity and a decrease in synaptic transmission without significant changes in biophysical parameters (membrane resistance, membrane time constant, action potential height, threshold to firing) suggesting that IFNα may interfere with neuronal communication (Figure 4.8 and Table 4.1). The decreased synaptic transmission and spiking activity was found to be reversible based on data showing spiking levels returning to normal after IFNα was washed off (Figure 4.8D).

IFNβ has no toxic effect on neurons

We investigated the effect of IFNβ on neurons co-cultured with glia. Treating neurons with 100 IU, 300IU, or 900 IU of IFNβ had no effect on the neurons up to 72 hours. However, if neurons were treated with IFNβ and IFNα at the same time, neurons were protected from IFNα induced neurotoxicity, suggesting competitive binding of the IFNAR (Figure 4.9).

Discussion

Previous studies showed that neuronal cultures treated with neutralizing antibodies to IFNα were completely protected against IFNα neurotoxicity (Sas, Bimonte-Nelson et al. 2009). However, due to the complex findings in these studies and multiple actions of IFNα, it was not firmly established whether IFNα was directly acting through its receptor to adversely affect neurons or whether the effects were largely or completely indirect. In this study, we blocked the IFNAR and the subsequent JAK/STAT signaling pathway. We found that pretreating neurons with an IFNAR blocker was less protective against IFNα induced neurotoxicity than previous studies using IFNα neutralizing antibodies, based on only 65% retention of dendritic length in the current study compared to 97% retention as seen previously. Similarly, IFNAR blocker pretreated neurons showed more loss in dendritic branching compared to neurons treated with neutralizing antibodies after IFNα exposure. The results reported in the present study indicate that IFNα is inducing neurotoxicity through direct engagement of its receptor, but that there are additional, important mechanisms involved in IFNα induced neurotoxicity.

Also, we found that despite IFNβ engaging the same receptor as IFNα, treating neurons with IFNβ showed no signs of toxicity based on dendritic arborization assessments and, notably, IFNβ was protective against IFNα induced neurotoxicity. This data is consistent with numerous studies that have found differential signaling pathways between IFNα and IFNβ after IFNAR binding (MacDonald, Kuhl et al. 1990, Platanias, Uddin et al. 1996, Marijanovic, Ragimbeau et al. 2007). It is also consistent with the clinical observation that

treatment of patients with IFNβ does not result in cognitive impairment. Finally, the fact that IFNβ protects from IFNα *in vitro* neurotoxicity may have important treatment implications.

The NMDAR has been implicated in IFNα neurotoxicity (Katafuchi, Take et al. 1995). Sas et. al. previously showed that neurons treated with AP5 or MK801 (NMDAR antagonists) during IFNα exposure were partially protected from toxicity based on dendritic length and branching measurements (Sas, Bimonte-Nelson et al. 2009). The NMDAR is a glutamate-gated cation channel composed of NR1 and NR2/3 subunits that plays a vital role in synaptic plasticity and cognitive function. The NR2 subunit is expressed as four specific isoforms and this subunit controls the electrophysiological properties of the NMDAR. Additionally, there is a NR2B to NR2A switch that occurs in the NMDAR as the neuron develops and matures (Wang, Bosy et al. 1995). Our results found that neurons pretreated with a novel NR1/NR2A specific antagonist, TCN 201, (Edman, McKay et al. 2012) were partially protected against IFNα induced neurotoxicity. Neurons pretreated with a NR1/NR2B antagonist, ifenprodil, (Reynolds and Miller 1989)were not as protected against IFNα exposure. These results suggest that our neuronal cultures represent a more mature phenotype, perhaps more consistent with adult brain neurons. The data also confirm and extend previous observations by Sas et. al. that the NMDAR is involved in IFNα neurotoxicity. Further and importantly, the data indicates the NR2A subunit of the NMDAR is specifically involved and is a possible therapeutic target for IFNα mediated neurotoxicity. Stimulation of the NMDAR is known to activate the CREB/ERK pathways. However, based on data shown, the CREB/ERK pathway

does not appear to be affected. Excessive activation of the NMDAR has been most notably implicated in HAND/HIVE, but has additionally played a role in other neurological diseases including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and Huntington's disease (Kaul, Garden et al. 2001, Kalia, Kalia et al. 2008). Therefore, our new data extends previous findings and emphasizes the potential that finding treatments for NMDAR mediated toxicity could benefit multiple neurological diseases.

Data shown here corroborates the IFNAR receptor blocking data indicating that neuronal damage caused by the interaction of IFNα with its receptor is not restricted to NMDAR pathways since TCN 201 only partially restored the neuron's baseline health in the presence of IFNα. It is possible that the cellular cascade initiated by IFNAR activation causes neuronal damage via other pathways (e.g., disrupting the cell's metabolism). It is also possible that IFNα itself has neurotoxic actions independent of its receptor mediated effects. We further demonstrate the complexity of IFNα neurotoxicity because the electrophysiological recordings reveal a general slowing of synaptic activity in cultured neurons acutely exposed to IFNα (see Figure 8).

Previous human neuropathological (Masliah, Roberts et al. 2004) and animal data (Sas, Bimonte-Nelson et al. 2009) strongly suggested that MAP2 is decreased in the CNS during HAND. MAP2 is a cytoskeletal phosphoprotein required for neuronal differentiation and stabilization and a critical component of dendrites (Sanchez, Diaz-Nido et al. 2000). Activation of the NMDAR is also known to play a role in MAP2 degradation (Buddle, Eberhardt et al. 2003). We hypothesized that elevated CNS levels of IFNα are directly affecting MAP2

protein levels resulting in decreased dendritic arborization. Surprisingly, we found that total MAP2 protein and RNA levels do not appear to be affected by IFNα. MAP2 phosphorylation is also known to control binding of microtubules to the cytoskeleton and dendritic stabilization, however, we found that MAP2 activation was also not affected by IFNα treatment. Our data implies that IFNα alone does not directly affect MAP2 levels. In HAND there are many other postulated neurotoxins that could be responsible for decreases in MAP2 that have been reported (Kaul, Garden et al. 2001). In addition, IFNα might have other effects on structural proteins as suggested in the Masliah gene array study on HAND patients (Masliah, Roberts et al. 2004). Future studies will continue to look at possible direct effects of IFNα on signaling pathways and proteins associated with neuronal dendritic integrity.

Our studies demonstrate that IFNα induced neurotoxicity is multifaceted. IFNα is engaging its specific receptor in addition to affecting surrounding neuronal receptors like the NMDAR. Data indicates that IFNα is having both direct and indirect effects on neurons in the CNS resulting in decreased dendritic arborization. Multiple neurotoxins are stimulated by IFNα including additional cytokines and reactive oxygen species which also have adverse effects on neurons (Fritz-French and Tyor 2012). Further characterization of the mechanisms of IFNα neurotoxicity is critical for unraveling the complexity of its inflammatory effects in the CNS. Identifying potential targets for IFNα neurotoxicity could lead to specific treatments for patients suffering from CNS inflammatory diseases, especially HAND. Additionally, IFNα remains the backbone of hepatitis treatment and complications resulting directly from IFNα treatment involving

cognitive dysfunction continue to occur (Okanoue, Sakamoto et al. 1996, Lieb, Engelbrecht et al. 2006). Blocking IFNα could prevent the downstream cascades that result in damage to neurons and eventually cognitive dysfunction that is seen both in CNS inflammatory diseases and in patients on IFNα therapy.

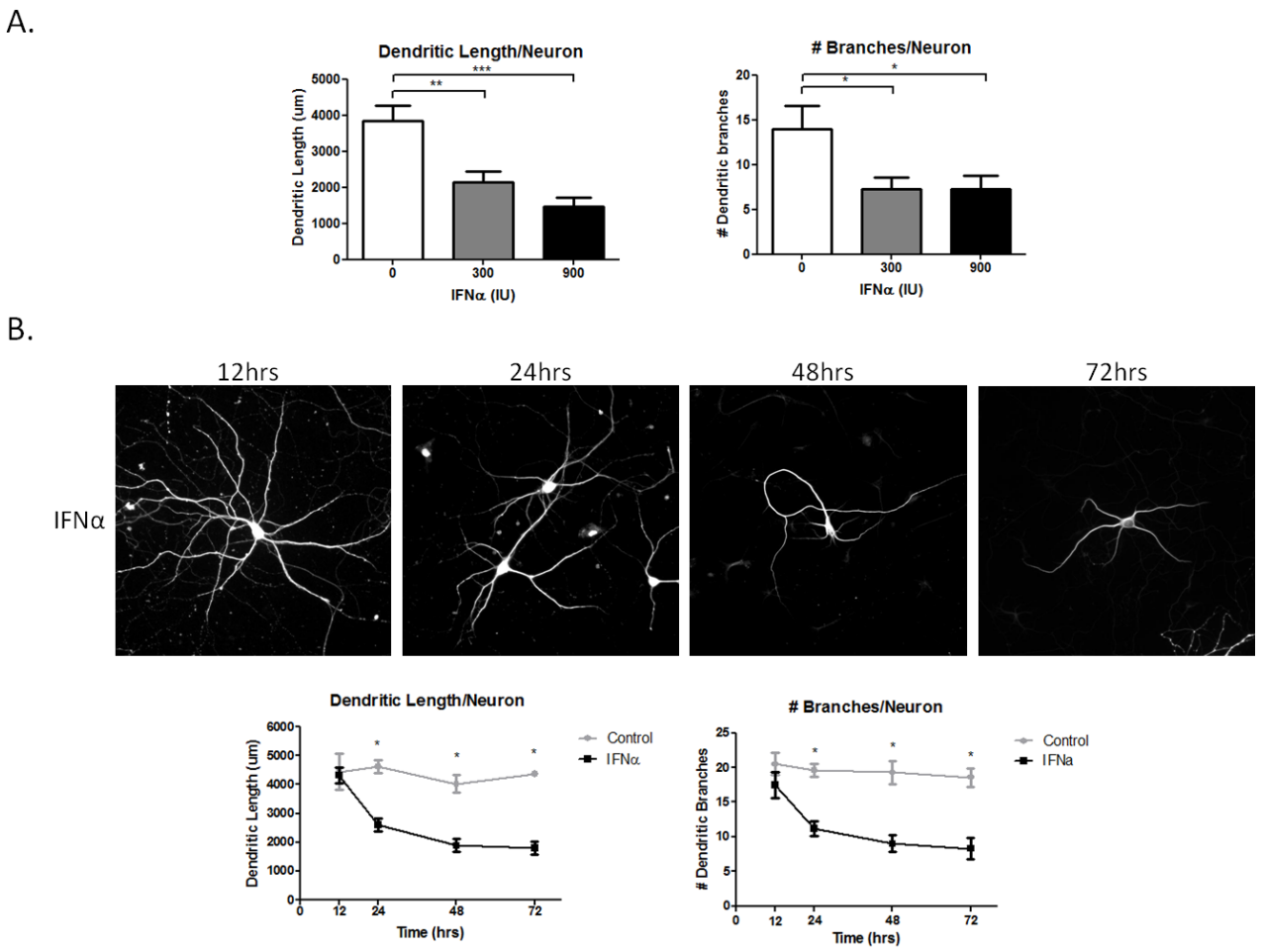
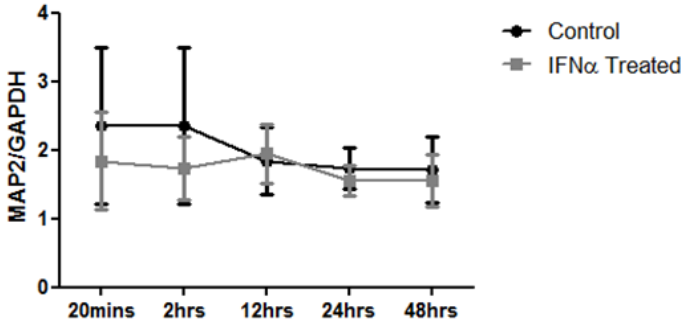
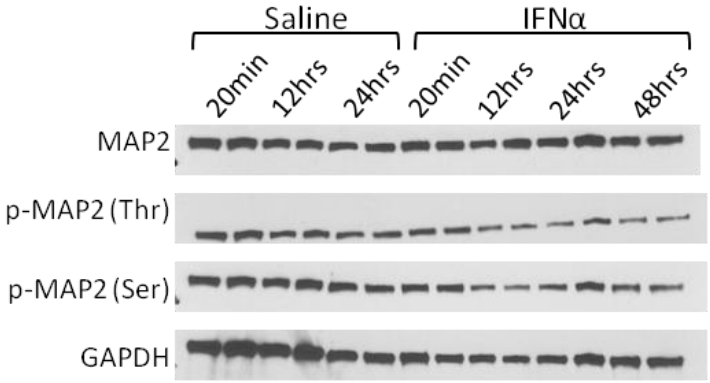


Figure 4.1. Neurotoxicity occurs between 12-24 hours after exposure to IFNα. Neurons were treated with increasing doses of IFNα and neuronal integrity was assessed by measuring dendritic length and branching for 48 hours (*p<0.05, **p<0.001, ***p<0.0001)(A). IFNα toxicity was seen at doses as low as 300 IU. Additionally, a time course study was done to establish when toxicity begins (B). IFNα induced toxicity is observed at 24 hours after a single dose of IFNα and persists for 72 hours. No toxicity is seen at 12 hours post treatment. Graphs include data from three separate experiments.

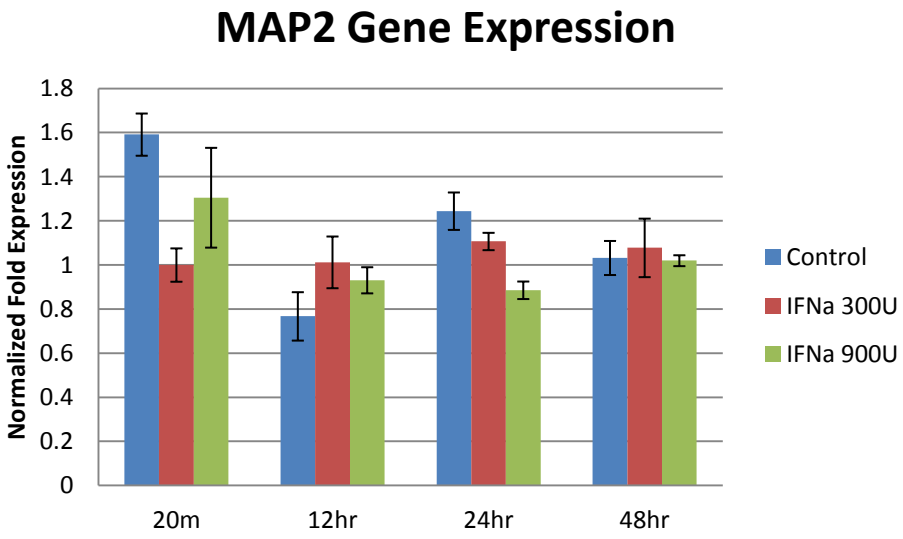
A.


MAP2/GAPDH
4
3
2
1
0
20mins
2hrs
12hrs
24hrs
48hrs
Control
IFNα Treated

B.


Saline
IFNα
20min
12hrs
24hrs
20min
12hrs
24hrs
48hrs
MAP2
p-MAP2 (Thr)
p-MAP2 (Ser)
GAPDH

C.


MAP2 Gene Expression
Normalized Fold Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20m
12hr
24hr
48hr
Control
IFNa 300U
IFNa 900U

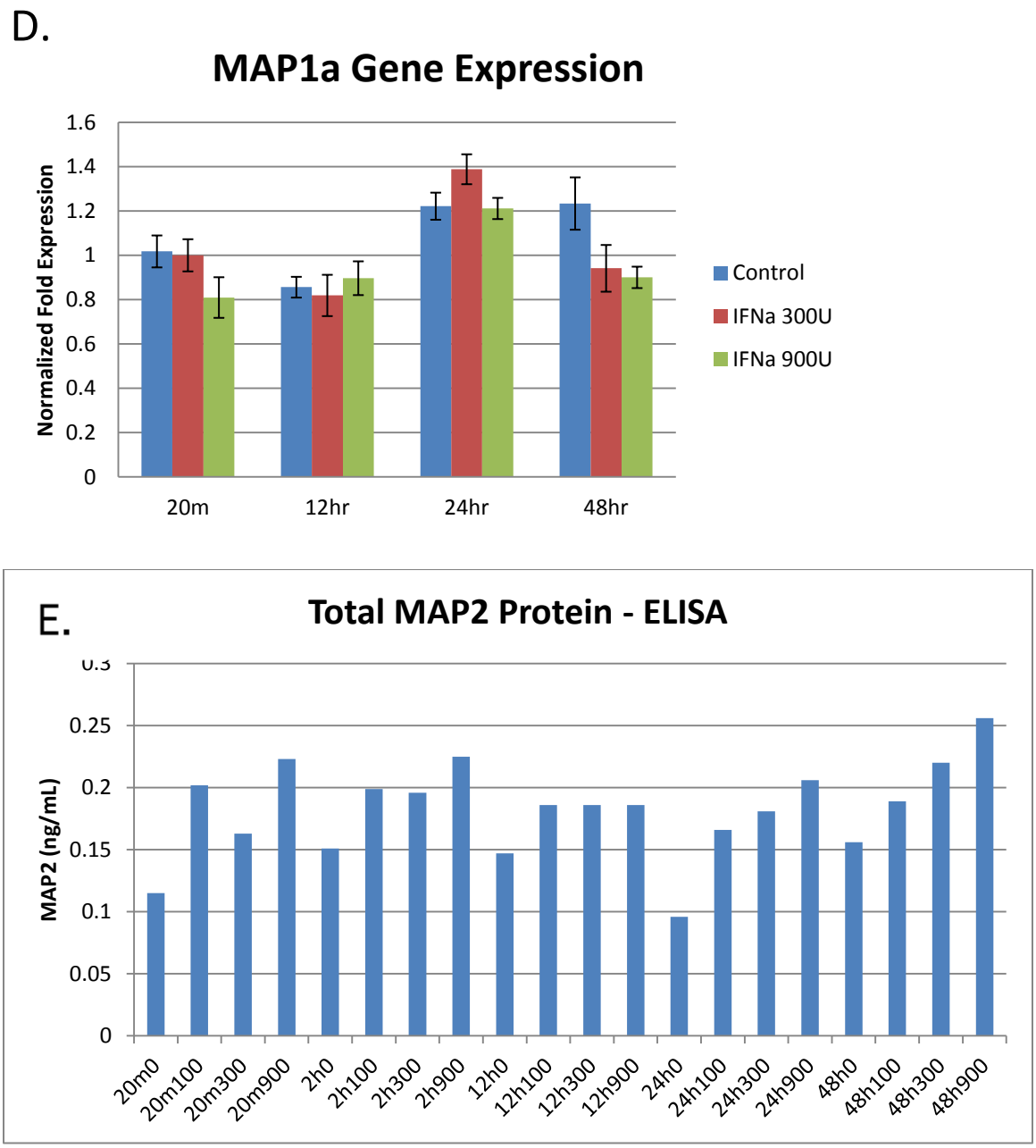


Figure 4.2. MAP2 protein and gene expression levels were not decreased in cultures treated with IFNα. IFNα has no direct effect on total MAP2 protein levels or MAP2 phosphorylation. Neurons were incubated with either saline or 300 IU of IFNα for 20 mins - 48 hours and protein extracts were analyzed for expression of MAP2, phospho-MAP2-Thr1620/1623 (p-MAP2 (Thr)), phospho-

MAP2-Ser136 (p-MAP2(Ser)), and GAPDH (A). Samples were run in duplicate on the western blot and densitometric ratios are the mean of three experiments (B). Neuronal cultures treated with varying doses of IFNα at various time points were assessed for MAP2 (C) and MAP1a (D) gene expression with real-time PCR. There was not a significant difference in gene expression for any dose or time point. MAP2 ELISA results showed no significant changes in MAP2 after IFNα treatment (E).

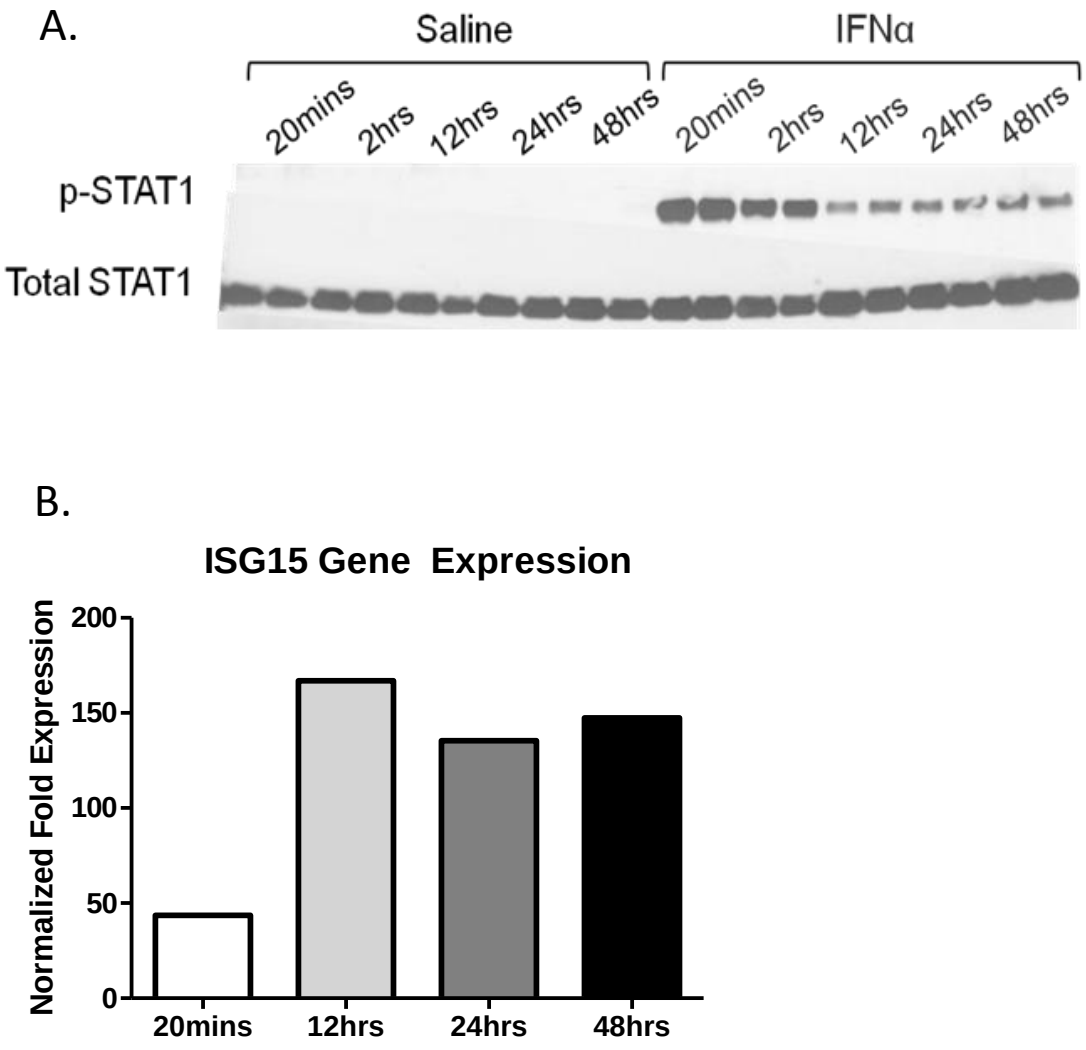


Figure 4.3. STAT1 is phosphorylated in neurons treated with IFNα (A). Protein extracts from neurons treated with IFNα at various time points were analyzed by immunoblotting for STAT1 and phospho-STAT1 (p-STAT1) expression. p-STAT1 peaked at 20 mins post-treatments and signal persisted for 48 hours indicating IFNAR activation. Samples were run in duplicate lanes on western blot. ISG15 gene expression was found to be upregulated after 20 minutes of a single treatment of IFNα (B). Gene expression remained elevated for up to 48hrs.

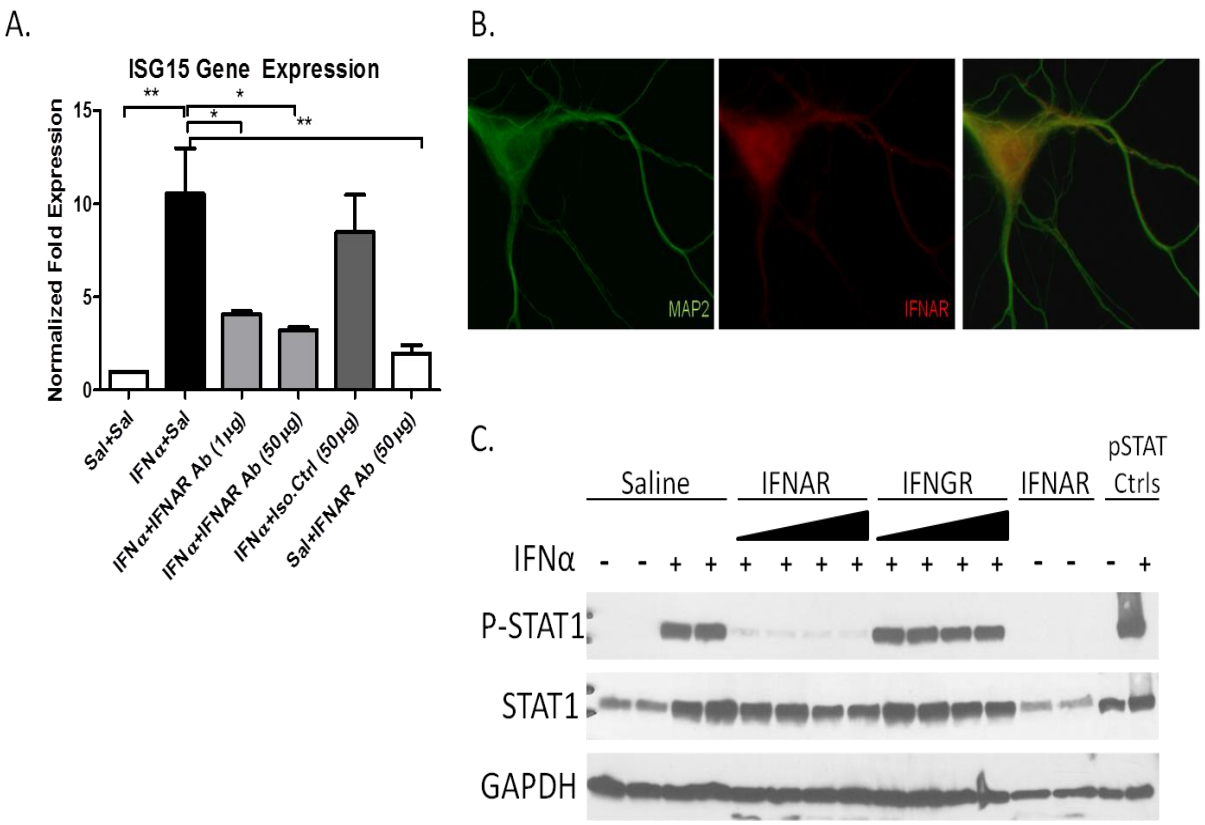
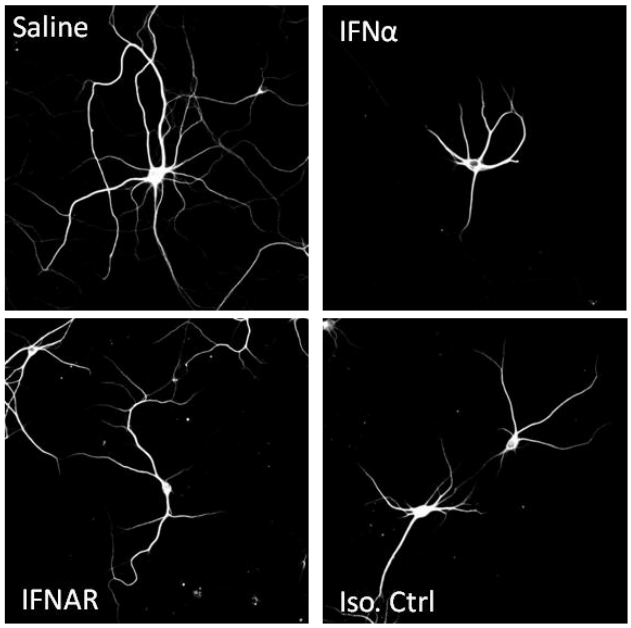


Figure 4.4. Neurons were pretreated with increasing doses of saline, an IFNAR blocker, or an isotype control (IFNGR). Cultures were then exposed to either saline or IFNα for 20 minutes. ISG15 expression was found to be decreased in cultures pretreated with IFNAR blocker compared to IFNα alone treated cultures (*$p<0.05$, **$p<0.001$) (A). Immunocytochemistry was utilized to show ubiquitous expression of IFNAR in neurons (B). JAK/STAT signaling is inhibited in cultures treated with IFNAR blocker. p-STAT1 expression was decreased in cultures pretreated with the IFNAR blocker prior to IFNα treatment compared to cultures treated with IFNα alone STAT1 and p-STAT1 controls were run with the protein extracts as positive controls (C). Experiments were conducted in triplicates.



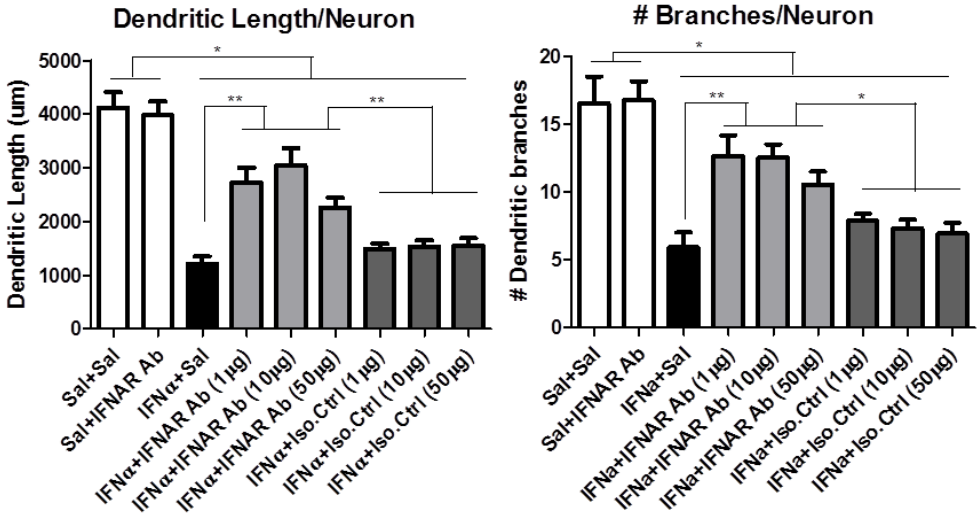


Figure 4.5. Blocking the IFNAR partially protected the neurons from IFNα toxicity. Neurons were pretreated with saline, an IFNAR blocker (1ug, 10ug, or 50ug), or an isotype control (IFNGR) (1ug, 10ug, or 50ug) and then exposed to IFNα for 48 hours. Dendritic length and number of branches was significantly

improved in cultures pretreated with IFNAR Ab at all doses prior to IFNα exposure compared to cultures treated with IFNα alone or an isotype control. However, cultures pretreated with IFNAR Ab were not completely protected and were significantly different than saline treated cultures (*$p<0.05$, **$p<0.001$).

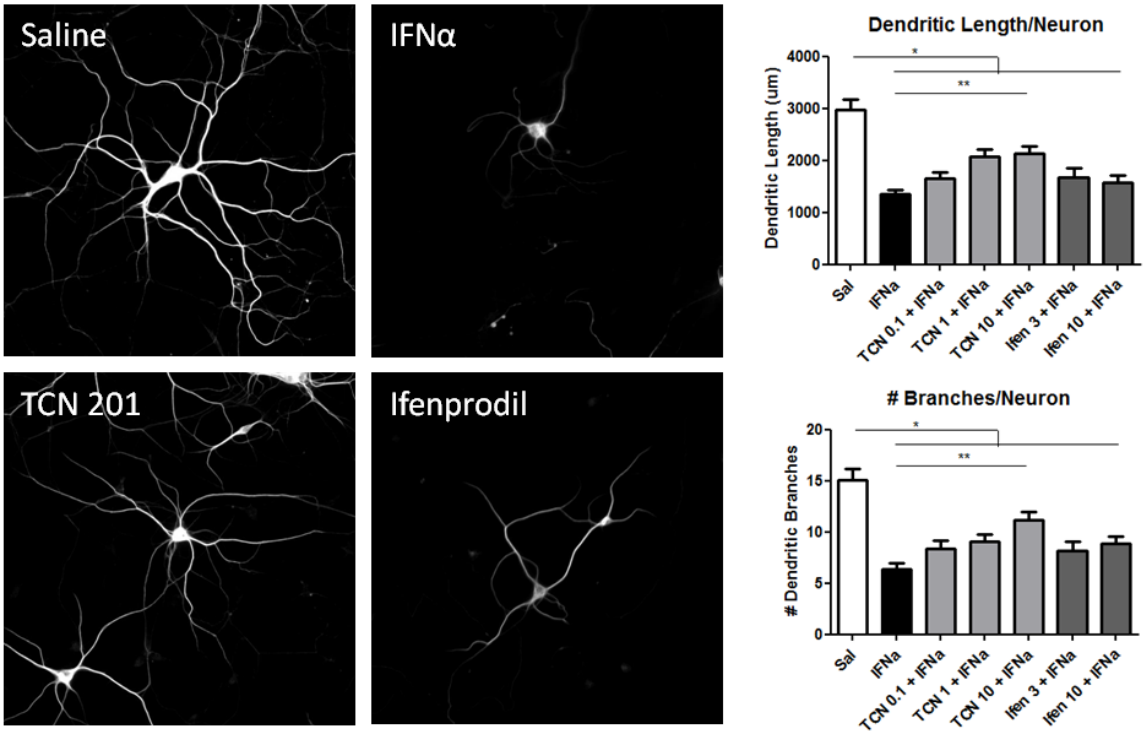


Figure 4.6. Blocking the NR2A specific subunit of NMDAR was partially protective against IFNα toxicity. Cultures were pretreated with either TCN 201 (0.1uM, 1uM, or 10uM) or ifenprodil (3uM or 10uM) and then exposed to IFNα for 48 hours. TCN 201 at 10uM had a protective effect against IFNα that was not seen in lower doses or with ifenprodil. However, TCN201 was not completely protected as dendritic length and branching was still significantly different than saline treated cultures (*$p<0.05$, **$p<0.001$), suggesting partial involvement of the NR2A subunit of NMDAR in IFNα induced neurotoxicity. Images are representative of three separate experiments that were combined in the graphs.

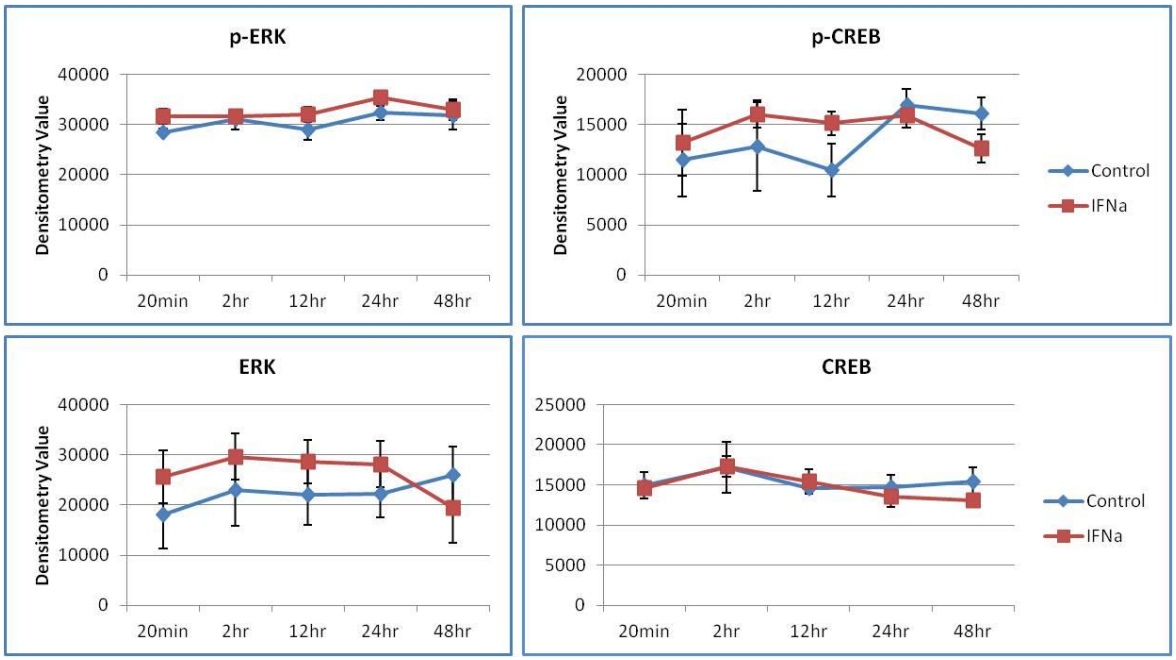


Figure 4.7. Graphs illustrate densitometry results from immunoblots representing p-ERK or ERK and p-CREB and CREB protein expression from neurons exposed to IFNα. Total ERK protein levels and ERK phosphorylation was not significantly affected by IFNα treatment. Total CREB protein levels and CREB phosphorylation was not significantly affected by IFNα treatment.

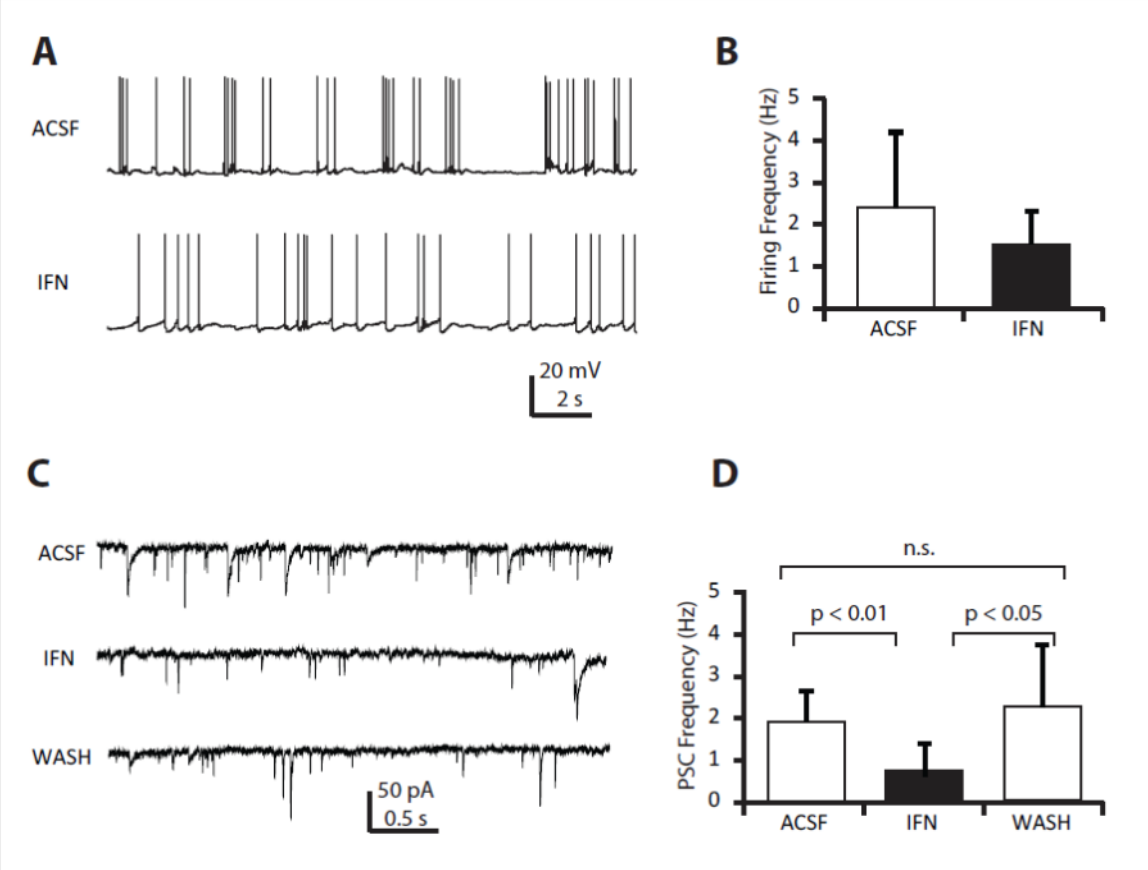


Figure 4.8. Exposure of dissociated neurons in low density culture to 1000 U / mL resulted in slowing of spiking activity and a decrease in synaptic transmission. A. Bursts and doublets of action potentials (APs) are common in the control condition but transition to single AP spikes with application of IFNα. B. Bar graph showing the average firing frequency for 4 neurons exposed to both interferon and artificial cerebrospinal fluid (Mean ± SD: ACSF 2.78 ± 1.88 Hz, IFN 1.63 ± 0.86 Hz; p = 0.17). C. Voltage clamp records of a neuron exposed to IFNα reveal a decrease in synaptic input to the cell that is recovered upon washout. D. Bar graphs depicting the decrease in frequency of post-synaptic currents in control, IFNα application, and washout conditions (Mean ± SD: ACSF

1.98 ± 0.50 Hz, IFN 0.73 ± 0.69 Hz, washout 2.24 ± 1.54; n = 6, p-values as designated on graph, n.s. = not significant). (Data provided by Jonathan Fidler and Dr. Paul Garcia.)

Parameter	ACSF	Interferon-α	n
R_m (MΩ)	175.2 ± 67.9	183.6 ± 71.5	6
Tau_m (ms)	12.5 ± 6.6	12.8 ± 6.2	5
AP_{height} (mV)	26.6 ± 9.8	26.7 ± 10.1	5
V_{thresh} (mV)	-43.5 ± 4.4	-41.8 ± 2.8	6

Table 4.1. No significant changes in biophysical parameters noted due to interferon exposure suggesting that interferon may interfere with neuronal communication but not be directly neurotoxic to neuronal membranes. Abbreviations: R_m (MΩ), membrane resistance; Tau_m (ms), membrane time constant; AP_{height} (mV), action potential height; V_{thresh} (mV), voltage threshold (to firing). (Data provided by Jonathan Fidler and Dr. Paul Garcia.)

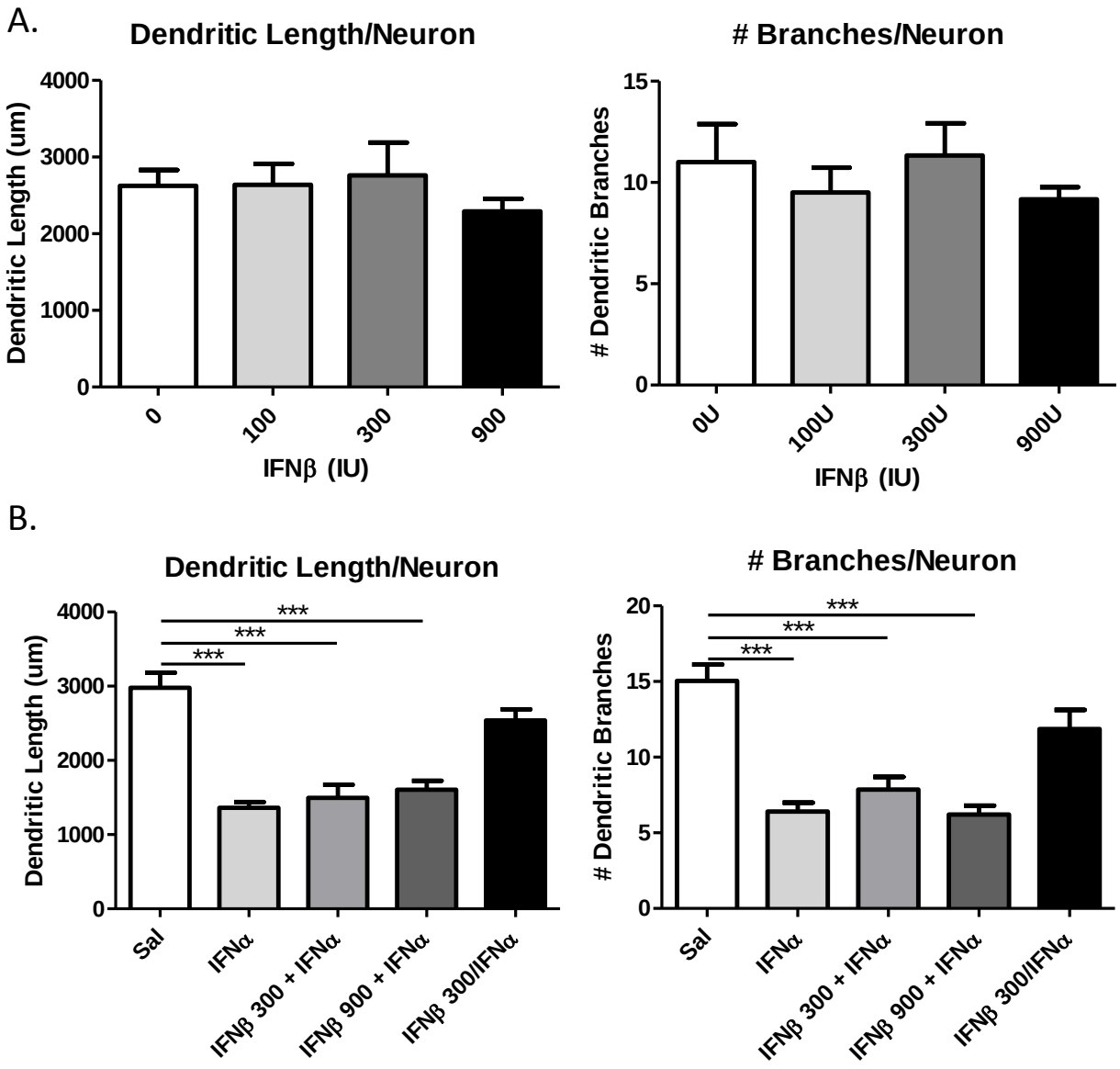


Figure 4.9. Neuronal cultures displayed no significant decrease in dendritic length or branching after exposure to increasing doses of IFNβ (A). Neurons pretreated with IFNβ 20 minutes prior to IFNα exposure showed decreased dendritic length and branching (IFNβ 300 + IFNα and IFNβ 900 + IFNα) (B). However, when cultures were treated with both IFNβ 300 IU and IFNα 300 IU

at the same time, neurons displayed no significant decrease in dendritic length or branching, indicating a possible protective effect of IFNβ against IFNα induced neurotoxicity.

Chapter 5:

Discussion and Implications of IFNα Neurotoxicity

Summary

Interferons were discovered in the 1950's when they were first identified as viral inhibitors. During an acute viral infection, interferons are immediately upregulated and secreted by all nuclear cell types in order to initiate a sufficient immune response to the infection. In most acute infections, the interferons, mostly type I interferons, are efficient in suppressing and helping to clear the virus within days. With the danger signals gone, upregulation of interferons is significantly reduced. However, during chronic infections, such as HIV, IFNα levels are continuously upregulated for extended periods of time and can become toxic. Specifically, when levels of IFNα are upregulated in the central nervous system to high enough amounts for an extended time period, cognitive dysfunction is evident.

As described in Chapter 1, HIV enters the CNS early in infection, infecting residential CNS cells like microglia, macrophages, and astrocytes. These cells then secrete various inflammatory cytokines, chemokines, and viral proteins. Because HIV does not typically infect neurons, the persistent inflammatory environment is believed to be the main contributing cause of decreased dendritic arborization on neurons that results in HIV- associated neurocognitive disorders. As mentioned previously in the Rho study (Rho, Wesselingh et al. 1995), severity of cognitive dysfunction was directly correlated to IFNα levels in the CNS. Despite the advent of antiretroviral therapies, the prevalence of HAND continues to increase due to the aging population of people infected with HIV (Alfahad and Nath 2013). Therefore, developing more effective and specific therapeutics to

HAND is necessary. Substantial clinical, *in vivo*, and *in vitro* data clearly shows that IFNα plays a critical role in damaging neurons during inflammatory diseases of the CNS, including HIV and others. Therefore, investigating different therapies targeted to IFNα and determining the mechanisms of IFNα neurotoxicity, in order to identify other potential therapeutic targets, is needed.

Sas et. al. previously demonstrated that not only were IFNα levels upregulated in an HIVE/SCID mouse model, but that treating HIVE/SCID mice with neutralizing antibodies to IFNα prevented HIVE associated pathology and behavioral abnormalities (Sas, Bimonte-Nelson et al. 2009). However, using neutralizing polyclonal antibodies in humans would not be an ideal treatment because of the potential for serum sickness. and monoclonal antibodies to IFNα that could be used would necessarily only recognize a limited number of IFNα subtypes. B18R is a recombinantly produced vaccinia virus gene product that mimics the type 1 interferon receptor (IFNAR). B18R binds all 14 IFNα subtypes with a higher affinity than the IFNAR itself. Unlike neutralizing antibodies, an immune response is less likely due to very little homology shared between B18R and IFNAR. The safety of inhibiting IFNα has also been demonstrated in humans who produce inhibiting antibodies either naturally or as a consequence of IFNα therapy (Gringeri, Musicco et al. 1999). Inhibiting IFNα during a viral infection may seem counter intuitive, however, both the Sas et. al. studies and the B18R study demonstrated in Chapter 2, that HIV viral loads were not affected when IFNα was inhibited. We do not expect decreasing levels of IFNα would prevent patients from mounting an appropriate immune response to a viral infection

because of type II and III interferons, but the possibility remains as B18R binds both type I interferons. Only clinical trials in humans with HIV will determine whether B18R is safe.

HIVE/SCID mice treated with B18R showed decreased IFNα signaling in the brain, based on downregulation of interferon stimulated genes (ISGs). Immunohistochemistry staining shows the presence of B18R in the brains of treated mice demonstrating that B18R crosses the blood brain barrier, binds IFNα, and prevents downstream signaling of IFNAR engagement. Most importantly, HIVE/SCID mice showed decreased activation of mononuclear phagocytes, including macrophages and microglia. B18R treatment also prevented decreased neuronal degradation in HIVE/SCID mice. Together, this data demonstrates that the decreased pathology associated with HIVE is a result of decreased IFNα levels in HIVE/SCID mice. Despite the positive results in treating mice with B18R and the likelihood of little to no side effects expected in human patients, we began to determine the mechanism of neuronal toxicity associated with IFNα in order to identify other potential therapeutic targets.

IFNα neurotoxicity is multifaceted as described in the *in vitro* experiments in Chapter 3. We know from previous studies (Sas, Bimonte-Nelson et al. 2009) and recent studies that IFNα has a dose dependent toxic effect on dendritic arborization in neurons. We also know that the JAK/STAT pathway is activated as expected and when this pathway is blocked using an IFNAR inhibitor, neurons are partially protected. Pretreating neurons with a NMDAR subunit specific inhibitor, TCN201, partially protected the cells from IFNα induced neurotoxicity.

Concepts

Our hypothesis of how IFNα causes neurotoxicity based on the data we have collected thus far shows a specific time course of events (immediate, intermediate, and late effects) that leads to toxicity in neurons and eventually cell death (Figure 5.1). When a neuron is first exposed to IFNα, there is an *immediate effect* seen. We know this because when IFNα is added to neurons during patch-clamp experiments, decreased firing is seen within seconds. At this time the exact mechanism(s) leading to this finding are unclear. For example it is unclear if IFNα is mediating this affect through direct binding to its own receptor (IFNAR) or through binding to a receptor that is yet to be identified. At any rate this immediate electrophysiological occurrence is followed by an *intermediate* event that is seen within minutes. Within 20 minutes, we show that the JAK/STAT pathway is activated based on phosphorylation of STAT1 and upregulation of ISG15 expression. Data presented in Chapter 4 indicate that this intermediate affect is due to IFNAR engagement by IFNα. After 24 hours, there is a *late effect* where a visible decrease in dendritic length and branching is evident *in vitro*. The events that culminate in this finding of dendritic simplification, which certainly must form the foundation of behavioral abnormalities in mice and cognitive dysfunction in humans, are complex. Glutamate levels are likely elevated causing increased NMDAR activation. This is evidenced by the resulting partial neuronal protection seen when the NMDAR is inhibited. There are likely increased calcium levels, increased production of reactive oxygen species (ROS) and an increase in firing and excitotoxicity. It is likely that the long-term presence of inflammatory substances such as these on neurons results eventual apoptosis and neuronal cell

death. This results in more severe cognitive dysfunction (ie, dementia), which is irreversible. In addition, HIV-infected astrocytes likely become sick and can no longer regulate glutamate levels, therefore contributing to excess glutamate and excitotoxicity in neurons.

Future Directions

Future studies should include more extensive electrophysiological studies that will include multiple time points to tease out what happens during acute and chronic exposures to IFNα. Additional experiments will assess glutamate, calcium and ROS levels after IFNα exposure to neurons. Currently we are using gene arrays to determine if signaling pathways, in addition to the IFNAR pathway, are affected by IFNα in neurons. These pathways include synaptic plasticity, spine formation, and neuronal ion channels. These studies would provide direction for determining the mechanism of how IFNα signaling causes decreased dendritic arborization. Time lapse experiments with confocal microscopy are planned to determine the visual course of decreasing dendritic length. Visualizing how dendritic length and branching are decreasing (beading, active retraction, or other mechanisms) would inform about subcellular localization of events and possibly cell signaling pathways that might be involved.

We also plan to conduct extended studies designed to show reversible effects. Based on clinical studies, side effects associated with cognitive dysfunction in patients on IFNα therapy diminished when therapy was terminated. Therefore, in both our *in vitro* and *in vivo* studies, we plan to

demonstrate IFNα induced neurotoxicity, and then provide an IFNAR, IFNα or NMDAR inhibitor treatment and determine if neurotoxicity is reversible. Based on clinical studies, we predict IFNα will be reversible. Additional *in vivo* studies include conducting behavior studies, as previously done in our lab, using B18R in HIVE/SCID mice. Utilizing the object recognition test (ORT), we can conduct a study where we initially observe cognitive impairments in our HIVE/SCID mice, administer B18R, then subject the mice to the ORT again. We predict improvements in behavior compared to initial testing.

There are other directions in which the IFNα research could lead and a few have been highlighted above. For example, confocal imaging could allow us to determine the proximity of NMDA receptors to IFNARs. It is possible that receptor proximity influences the outcomes we have observed. Determining the mechanism of IFNα induced neurotoxicity is complex and we have only begun to assess its complexity.

Clinical implications

Treatments in HIV infected patients involving blocking NMDAR, ROS, TNFα, and other inflammatory cytokines have been found to be insufficient in reversing or preventing HIV-associated neurocognitive disorders. These treatments were typically tried in patients with HAD, the most severe form of HIV-associated neurocognitive disorders, which is likely a more irreversible condition than milder forms of HIV-associated neurocognitive disorders such as Mild Neurocognitive Disorder. IFNα is known to upregulate inflammatory

cytokines, ROSs, chemokines, and quinolinic acid early on in HIV infection. Blocking IFNα early in infection might prevent and/or decrease upregulation of inflammatory and potential toxic substances, therefore preventing the cumulative toxic environment that eventually leads to neuronal cell death. IFNα is most likely expressed early in HAND patients. Incorporating an IFNα inhibitor, such as B18R, into antiretroviral treatments in HIV patients may improve cognitive problems in HAND patients and ultimately prevent neuronal destruction. Additionally, blocking IFNα in other inflammatory diseases of the CNS may be found to be beneficial in improving cognitive function and preventing neuronal damage.

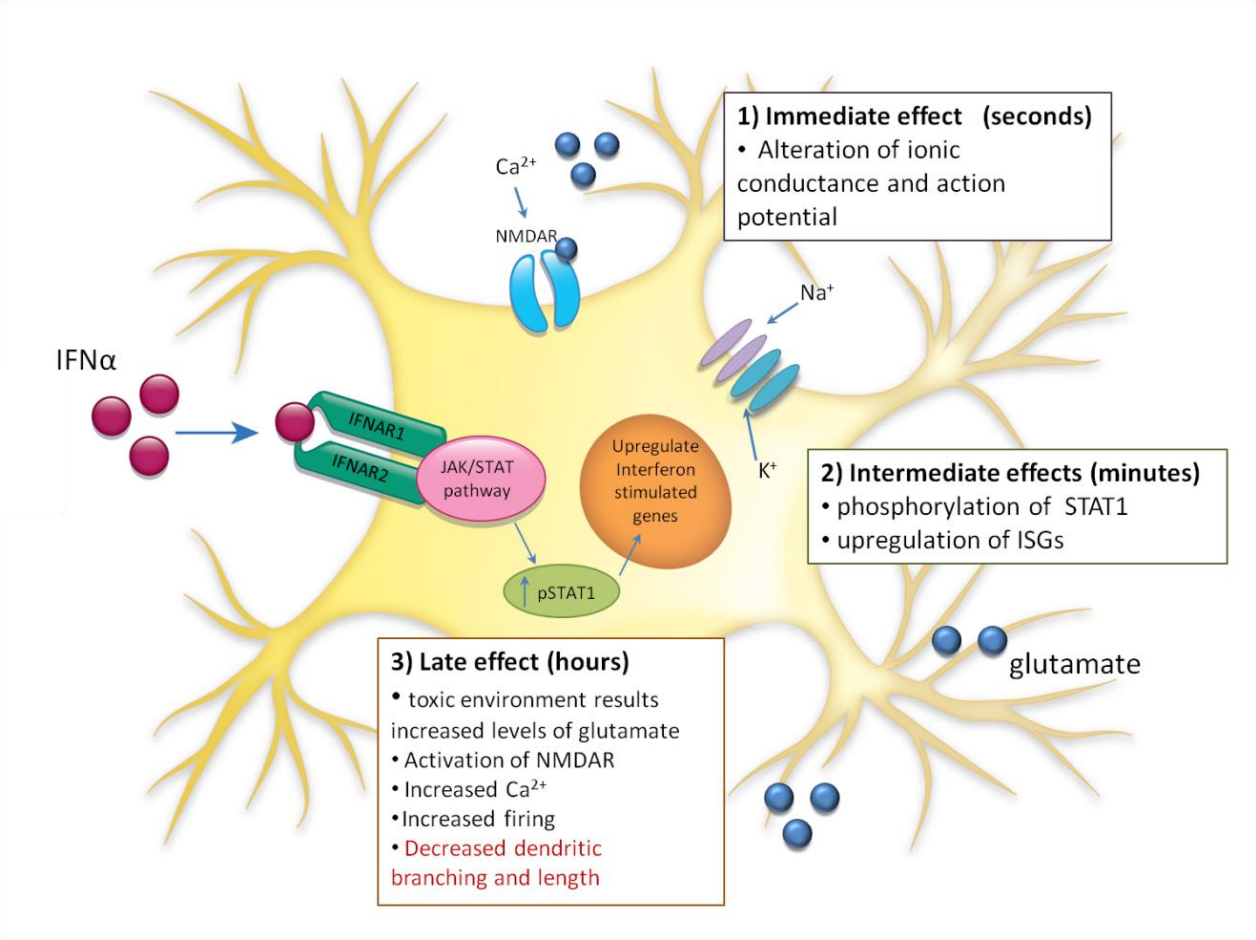


Figure 5.1. Concept diagram demonstrating the predicted time course of events of IFNα induced neurotoxicity, including immediate, intermediate, and late effects. 1) IFNα has immediate effects on neurons causing an alteration of ionic conductance and action potential evident through reduced firing. 2) Within minutes of IFNα exposure, the type I interferon receptor signaling cascade is activated including phosphorylation of STAT1 and upregulation of interferon stimulated genes. 3) Eventually, a chronic inflammation most likely results in decreased uptake of glutamate by glia and increased activation of NMDAR,

increased calcium intake, and increased firing. The prolonged exposure of IFNα eventually results in decreased dendritic branching and length on neurons.

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