INFECTION OF NEURAL STEM CELLS WITH MURINE LEUKEMIA VIRUSES
INHIBITS OLIGODENDROGLIAL DIFFERENTIATION:
IMPLICATIONS FOR SPONGIFORM NEURODEGENERATION

A thesis submitted
To Kent State University
In cooperation with
Northeast Ohio Medical University
In partial fulfillment of the requirements for the
Degree of Master of Sciences

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May, 2012
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LIST OF ABBREVIATIONS

AIDS: Acquired immune deficiency syndrome
ALS: Amyotrophic lateral sclerosis
BSA: Bovine serum albumin
CAII: Carbonic anhydrase II
CasBrE: Wild Mouse Virus, MLV
CCD: Charge-coupled device
CEC: Cytoplasmically effaced cells
CNPase: 2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS: Central nervous system
dpi: Days post-inoculation
dpt: Days post-transplantation
DMEM: Dulbecco’s modified Eagle medium
DNA: Deoxyribonucleic acid
dsDNA: double-stranded DNA
EGF: Epidermal growth factor
EGFP: Enhanced green fluorescent protein
env: Envelope, retroviral gene
Env: Envelope protein
ffu: Focus-forming units
Fr57E: NN ecotropic MLV
FrCasE: NV ecotropic MLV

gag: Group antigen, retroviral gene
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescent protein
gp70: Surface-expressed subunit of the Env glycoprotein
GRP: Glia-restricted precursor cell
H&E: Hematoxylin and eosin
HAART: Highly active anti-retroviral treatment
HAM/TSP: HTLV-1 associated myelopathy/tropical spastic paraparesis
HIV: Human immunodeficiency virus
HTLV-1: Human T-cell leukemia virus I
IF: Immunofluorescence
ip: Intraperitoneal
IRW: Inbreeding Rocky Mountain Laboratories white mice
mAb: Monoclonal antibody
MBP: Myelin basic protein
mCAT-1: Murine cationic amino acid transporter
MLV: Murine leukemia virus
mTmG: Membrane-tomato (RFP), membrane-GFP transgenic mouse
NG2+: Chondroitin-sulfate proteoglycan NG2; nerve/glial antigen-2
NN: Non-neurovirulent murine leukemia virus
NPH: Neurosphere

NSC: Neural stem cell

NV: Neurovirulent murine leukemia virus

OL: Oligodendrocyte

OPC: Oligodendrocyte progenitor cell

P0: Postnatal day 0, day of birth

PBS: Phosphate-buffered saline

PDGFRA: Platelet-derived growth factor receptor alpha

PFA: Paraformaldehyde

PiT-2: Phosphate transporter-2

PLP: Proteolipid protein

pol: Polymerase, retroviral gene

RBD: Receptor binding domain of the envelope protein

RFP: Red fluorescent protein

RNA: Ribonucleic acid

RT: Room temperature

SU: Surface-expressed subunit of the envelope protein, gp70

TM: Transmembrane subunit of the envelope protein

Tx-100: Triton-X 100 detergent
ACKNOWLEDGEMENTS

I would like to thank my advisor, Bill Lynch, for encouraging me to pursue my interests in higher education. I would not be where I am today without his support and his willingness to take chances. He has challenged me to reach my fullest potential, and I am very grateful. I would also like to thank the other members of my committee, Shobhana Sivaramakrishnan for her scientific insight and advice all throughout the last few years and Mark Simmons for his willingness to provide feedback on the thesis and experiments I have completed.

To Ying Li, I am grateful for her meticulous attention to detail, and for completing the preliminary experiments of this project. I would like to thank Sandra Cardona for her guidance and patience. She has been a mentor and friend throughout this experience, and I have learned a great deal from her. I would like to thank other members of the laboratory who have contributed to the work presented here, especially Alvin Das, Krystal Renszel and Thomas Mbimba.

To my colleagues and friends: Will Hamlet and Ryan Longenecker, thanks for challenging and engaging me. After talking to each one of them, I have come away with a greater understanding of science, and ever more curiosity.

I would also like to mention all the people here at NEOMED and Kent State University who have gone out of their way to help me and to brighten my day, especially Karen Greene and Carolyn Miller, Judy Wearden, Erin Bailey, Denise McBurney, Sharon
Usip, Sarah Stone, Laura Link, and everyone at the CMU and Physical Plant. I have been fortunate enough to interact with these outstanding people on a daily basis for the last three years.

I want to thank my parents for investing themselves in my education throughout my entire life. They were my first teachers, and helped me fall in love with learning. And finally, I need to thank my husband Bill. When I hear him describe my research to other people, it makes me very proud.

Jaclyn Dunphy

March 27, 2012

Kent, Ohio
INTRODUCTION

Human retroviruses such as the human immunodeficiency virus (HIV) and human T-cell leukemia virus-I (HTLV-1) are capable of inducing progressive neurodegenerative diseases with significant co-morbidities, in addition to causing acquired immune deficiency syndrome (AIDS) and cancer (Barmak et al., 2003; Wiley et al., 1991). Of particular note is that with the advent and broad application of highly active anti-retroviral therapy (HAART) for AIDS treatment, the incidence of neuroAIDS has increased to almost 50% of the HIV-infected population (Power et al., 2009). Despite the enormity of this disease problem, the molecular and cellular basis for the neurodegenerative changes that account for the clinical manifestations in neuroAIDS and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) remain largely unresolved. One reason for the delayed progress on these problems is that the viral replication of these human viruses is restricted at multiple levels in easily manipulated, tractable animal model systems such as mice. As a result, investigators have resorted to using animal model systems employing mouse retroviruses with varying degrees of similarity to the human retroviruses, with the expectation that similar or parallel mechanisms are likely to be operating, and that analysis of these simplified models will provide instruction for more focused testing in expensive and experimentally difficult primate models. One such murine model, outlined below, is the focus of this thesis, where I attempt to distinguish between direct versus indirect effects of neuropathogenic
murine leukemia viruses (MLVs) on a subset of glial cells within the brain. The parallels with the human viruses will again be broached in my discussion of the results.

In the 1970s, Murray Gardner and his colleagues performed a study of wild mice trapped at several sites in southern California. Mice were housed in the laboratory over the course of 35 months, and it was discovered that mice from the Lake Casitas region spontaneously developed lymphomas (cancer) and/or a progressive paralytic disease resembling amyotrophic lateral sclerosis (Andrews and Gardner, 1974; Gardner et al., 1976a; Gardner et al., 1973; Gardner et al., 1976b). A surprising 10.5% of mice from this region exhibited lower limb paralysis, whereas no mice from other trapping sites became paralyzed (Gardner et al., 1976a; Gardner et al., 1976b). A type-C RNA tumor virus was isolated from these animals, and found to be indigenous to the wild mouse population. The virus, named Wild Mouse Virus or CasBrE, causes tremors in the hindlimbs that progress to the trunk and head, and eventually results in paralysis. Within two months of clinical onset of symptoms including weight loss, dehydration and incontinence, the animals die (Gardner et al., 1976a). They succumb to the disease when the neurons innervating the diaphragm are affected, or their paralysis makes it impossible to forage for food.

In the wild, the virus can be transmitted from mother to offspring in the milk. Among adults, the virus can be spread during sexual intercourse or fighting (Gardner et al., 1979). Infection of susceptible mouse strains with CasBrE virus results in slow, progressive spongiform neurodegeneration with an onset of approximately 180 days (reviewed in Portis and Lynch, 1998). Vacuolar pathology is observed in both the
white and grey matter of the anterior horn of the lower spinal cord, the dentate gyrus of the cerebellum, and other nuclei of the brainstem. Regions of pathology exhibit intense gliosis (Gardner et al., 1976a; Oldstone et al., 1977; Portis and Lynch, 1998). Using electron microscopy, type-C viral particles were observed in the extracellular spaces and budding from endothelial cells, neurons and oligodendrocytes of paralyzed animals (Oldstone et al., 1977). Some non-neuronal cells showed a general lack of cytoplasmic structure (Andrews and Gardner, 1974). At the cellular level, post-synaptic processes of neurons are the first structures to become vacuolated, followed by cellular effacement of glial cell bodies.

Murine leukemia viruses (oncornaviruses) are simple retroviruses whose genome consists of two single strands of positive-sense RNA that must be reverse transcribed into DNA and integrated into the host chromatin to complete the virus life cycle. The MLV genome consists of three genes: \textit{gag}, \textit{pol} and \textit{env}. The \textit{gag} gene encodes the structural capsid and matrix proteins, the \textit{pol} gene encodes protease, reverse transcriptase and integrase enzymes, and the \textit{env} gene encodes the envelope protein, a transmembrane protein which confers binding, fusion and entry of the viral particle into a host cell (Coffin et al., 1997).

The retroviral life cycle begins with a virion binding to a receptor on the surface of a cell. The binding of the envelope protein to the receptor initiates fusion of the viral membrane to the plasma membrane of the cell. The capsid of the virus containing viral proteins and the genome enter the cytoplasm, where the RNA is reverse-transcribed into DNA by the viral enzyme reverse transcriptase. The dsDNA of the virus then enters the
nucleus as the nuclear membrane breaks down during cell division. The viral genome is randomly and permanently integrated into the host cell’s genome. The viral genes are transcribed and translated using host cell machinery and the proteins are either made in the endoplasmic reticulum (envelope protein) or the cytoplasm (capsid, matrix, integrase, protease, reverse transcriptase). The proteins assemble at the plasma membrane where the envelope proteins are already embedded. Once the viral RNA, structural proteins and enzymes are encapsidated, the virus buds off from the membrane (Coffin et al., 1997).

The CasBrE virus was first cloned and sequenced by Jolicoeur and colleagues in 1983 (Jolicoeur et al., 1983) making it possible to create chimeric viruses between neurovirulent and non-neurovirulent viruses to identify which viral genes harbored the neurovirulence determinants. Initial neurovirulence mapping by this group suggested that the neuropathogenic sequences of the virus reside within the $env$ gene (DesGroseillers et al., 1984). However, more recent studies have suggested that these original studies actually mapped CNS tropism determinants rather than neurovirulence per se (Li et al., 2011). Nonetheless, later studies by Portis et al showed that neurovirulence did indeed map to the CasBrE $env$ gene (Portis et al., 1990), and that other sequences that influence viral replication and neuroinvasiveness could be mapped to the 5' untranslated region of the viral genome. Using this knowledge it was demonstrated that a combination of these distinct viral characteristics could dramatically increase the speed and penetrance of disease. For example, the CasBrE envelope is highly neurotoxic, but the virus itself is weakly neuroinvasive, and thus disease occurs only after a long incubation time with low incidence (Portis et al., 1990). In contrast, a strain of the Friend virus, FB29, is weakly
neuropathogenic, but highly neuroinvasive. Introduction of the \textit{env} sequence from CasBrE into the Friend virus (FB29 strain) genome resulted in the chimeric virus FrCasE which is capable of inducing fatal spongiform pathology in 100\% of susceptible animals in less than three weeks (Portis et al., 1990). Another Friend virus (clone 57) whose \textit{env} gene was completely non-neuropathogenic was used to generate isogenic virus Fr57E that serves as a non-neurovirulent MLV (NN) control for studies with the highly neurovirulent MLV (NV) FrCasE since both are highly neuroinvasive and differ only in their \textit{env} sequences (Askovic et al., 2000; Dimcheff et al., 2006). Subsequent studies by Li \textit{et al}, have demonstrated that the \textit{env} gene alone is sufficient for inducing neurodegeneration (Li \textit{et al.}, 2011), although how Env protein is neurotoxic remains unresolved.

MLV Env protein is a trimer of heterodimers, where each dimer is composed of two proteins, the surface-expressed protein (SU) which contains the receptor binding domain (RBD) (Heard and Danos, 1991; Kabat, 1989) and the transmembrane protein (TM), which mediates membrane fusion (Rein et al., 1994). The crystal structure of the receptor binding domain (RBD) of the Env protein from the Friend virus has been determined (Fass et al., 1997), and based on that structure, the RBD of the FrCasE Env protein has been modeled, revealing only subtle structural differences (R. Davey, personal communication). Once the SU engages the receptor the TM facilitates viral membrane fusion through conformational changes in the trimer that expose the fusion peptides (FP) to the host cell membrane. The tropism of the MLV is determined by SU, whose receptor binding function determines the tissue specificity and host range of the
virus (Coffin et al., 1997). Ecotropic MLVs are able to enter mouse cells by binding to
the mCAT-1 receptor, a murine cationic amino acid transporter (Albritton et al., 1989;

Several different CNS cell types have been demonstrated to be targets of
ectropic MLVs in vivo, and include postnatally dividing neurons, endothelia, microglia,
OLs, OPCs and pericytes. Astrocyte infection has been reported to be virtually absent or
rare from FrCasE (Czub et al., 1994; Lynch et al., 1991) and CasBrE (Gravel et al., 1993)
infected animals, respectively, despite the fact that these cells are readily infected in vitro
(Lynch et al., 1994). Importantly, the infected cell types are known to proliferate in
neonates, while the motor neurons that appear to be the major cell type undergoing
vacuolar neurodegeneration are post-mitotic by this developmental stage and are not
productively infected. These findings suggest that spongiosis results indirectly from
infection of the glia, although formal proof for this hypothesis has been difficult to
obtain. In the rapid FrCasE model, where pathogenesis arises quickly and in stereotypic
fashion, it has been reported that microglial infection most closely correlated with
spongiosis. However, infection of microglia by the neurovirulent amphotropic virus is
not associated with spongiosis, unless the amphotropic virus is pseudotyped by an
ectropic Env protein that allows expanded viral tropism. Essentially identical results
were observed for OLs infected by amphotropic virus, suggesting that some other cell
type is critical for pathogenesis. In contrast, it has been suggested by Clase et al that
infection of OLs by FrCasE or Mo-MLVts1 {a mutant NV that originated from Moloney
MLV; (McCarter et al., 1977)} results in directly toxic effects on these cells, reflected by
the appearance of CECs (Clase et al., 2006). In this thesis I have attempted to resolve this issue by examining the relationship of OPC infection \textit{in vitro} and \textit{in vivo} to the \textit{in vivo} cellular pathology and the differentiation state of these cells.

As cells in the brain differentiate they generally become more restricted in their ability to proliferate. Neural stem cells (NSCs) give rise to macroglial cells and neurons in the developing brain, which includes astrocytes, OLs, NG2+ cells and neurons. Throughout the differentiation process, cells express different cell lineage markers. While the exact mechanism of neural cell differentiation is still controversial, and differs between various brain structures, certain cell types can be characterized by specific protein expression. For example, many astrocytes express intermediate filament protein GFAP (glial fibrillary acidic protein) (Bignami et al., 1972). The expression of this protein is not absolutely specific to astrocytes, but in combination with cell morphology, this marker can be used to reliably identify astrocytes. Conversely, cells differentiating along the OPC pathway, such as NG2+ cells and non-myelinating oligodendrocytes, express the Olig2 transcription factor (Nishiyama et al., 2009). Kang \textit{et al} reported that NG2+ cells are not only a precursor cell, but are also abundant in the grey and white matter of the adult CNS, where they remain mitotically active and retain the ability to differentiate into oligodendrocytes (Kang et al., 2010). Nonetheless, as cells differentiate their protein expression profile changes. It has been reported that as OPCs differentiate, they down-regulate the expression of glutamate receptors (De Biase et al., 2010). Mature OLs, the CNS cell type that is responsible for myelinating neuronal axons express various
markers including PLP (proteolipid protein), MBP (myelin basic protein), CAII (carbonic anhydrase II), and CNPase (2’, 3’-cyclic nucleotide 3’-phosphodiesterase).

As NSCs differentiate, they become restricted from developing into neurons, and at this stage are known as GRP (glia-restricted progenitors). These cells express the NG2 proteoglycan on the cell surface and can differentiate into astrocytes or oligodendrocytes 
\textit{in vitro}, depending on cell culture conditions (Stallcup and Beasley, 1987). The next step in the pathway toward oligodendrocytes is the OPC, which has several names including synantocyte, polydendrocyte, or NG2+ cell (Butt et al., 2005; Butt et al., 2002; Nishiyama et al., 2009). It has recently been shown that more than 90% of NG2+ cells express the Olig2 transcription factor, and that Olig2 activity is required for oligodendrocyte lineage specification (Ligon et al., 2007). NG2+ cells are cycling cells which act as precursors to oligodendrocytes, but are also present in the adult brain (Nishiyama et al., 2009).

The purpose of this work is to determine what effect MLV infection has on OL lineage cells that undergo degenerative changes. It remains controversial whether the infection of OPCs is directly neurotoxic, or whether these cells are damaged as a consequence of changes in other cells that initiate a cascade of events facilitating neuronal excitotoxicity. In the exploration of this question, we have generated data indicating that MLV infection of OPCs is not neurotoxic, but rather interferes with the cellular differentiation of these cells, maintaining them as excitable glia and therefore highly vulnerable to the ongoing excitotoxicity responsible for neuronal degeneration.
MATERIALS AND METHODS

Animals. Mice were bred and housed in the Comparative Medicine Unit at NEOMED, with all procedures employed being approved by the NEOMED Institutional Animal Care and Use Committee. Wild-type IRW (inbred Rocky Mountain Laboratories white) mice are highly susceptible to neuropathogenesis associated with neurovirulent MLV infection. Rosa26-tdTOMATO-EGFP mice (herein referred to as mTmG) were obtained from Jackson Laboratories (Strain No. 007676) and bred to heterozygosity with wild-type IRW mice. mTmG mice ubiquitously express a chicken β-actin promoter-driven red fluorescent protein (RFP) in all tissues, including the brain (Muzumdar et al., 2007). These mice exist in the 129 mouse strain which is fully susceptible to MLV infection and neurodegeneration (Lynch, unpublished). Another strain expressed enhanced green fluorescent protein (EGFP) driven by the proteolipid protein (PLP) promoter, which resulted in fluorescent detection of both non-myelinating and myelinating oligodendrocytes, in addition to a subset of NG2+ glia (Fuss et al., 2000; Mallon et al., 2002; Miller et al., 2009). PLP-EGFP mice were a generous gift from W. Macklin. These mice were obtained on a CBA/B6 background and were backcrossed for a minimum of nine generations onto the IRW background to assure full susceptibility of the mice to MLV infection.
Cells and Viruses. NIH 3T3 cells were cultured in DM10N medium {Hi-glucose (4500mg/L) DMEM, HyClone; 10% Newborn Calf Serum, HyClone; 1% penicillin/amphotericin/ streptomycin, Mediatech} on 10 cm Nunc tissue culture plates. All cells were passaged with trypsin approximately three times per week. FrCasE (Portis et al., 1990) and Fr57E (Dimcheff et al., 2006) viruses were grown in NIH 3T3 cells as outlined previously and resulted in virus stocks with titers of 5-20 x 10^5 focus forming units (ffu)/mL of medium (Dimcheff et al., 2006) for use in subsequent studies. NIH 3T3 cells that were producing viral supernatants to be used to infect neurospheres (NPHs) were grown in serum-free NPH medium, described below.

Analysis of brain sections from intraperitoneally infected mice

A. Virus Inoculation. Neonatal mice were intraperitoneally (ip) infected with replication competent ecotropic viruses, either Fr57E for FrCasE. A dose of 50 µl of virus (2.5-10 x 10^4 ffu) was injected into the peritoneal cavity of each mouse pup using a 25 ga syringe. The virus replicates in the periphery and is able to enter the brain through the bloodstream until P6 when the development of the blood-brain barrier no longer allows the passage of virus (Czub et al., 1991).

B. Tissue Processing for Paraffin Embedding. Infected and uninfected IRW and PLP-EGFP mice were anesthetized using isoflurane and decapitated. The brains were harvested and immersion fixed in 10% buffered formalin overnight. Whole formalin-fixed mouse brains were divided into five sections using a brain matrix and allowed to fix for an additional 2 hrs in 10% buffered formalin. Tissues were dehydrated
in graded alcohols and xylene for 18 hrs, then embedded in paraffin (Paraplast/Paraplast Plus, Fisher Scientific) and sliced at 5 µm on a Leica microtome and mounted onto charged glass slides (Fisher Superfrost Plus Slides).

C. Histopathological Analysis of CECs. To examine tissues for histopathological changes, paraffin-embedded sections were stained using hematoxylin and eosin as outlined previously (Czub et al., 1991). Briefly, sections were deparaffinized using SafeClear II (Fisher) and xylene, and then rehydrated using graded alcohols. Samples were then rinsed with deionized water briefly and immersed in Harris’ modified hematoxylin solution for 2 min. The slides were rinsed with flowing tap water for 5 min and stained with eosin for 30 s. The tissue was dehydrated with graded alcohols and xylene, and then mounted with Permount (Fisher) and a No. 1.5 coverslip. Results were visualized and documented using an Olympus BX40 microscope equipped with a digital ColorCube 12 CCD color camera. Pathology incidence was determined and all mice were assigned a mean pathology score on a scale of 0 to 4.

D. Examination of CECs using Cell Lineage Marker Immunostaining: Thick Sections. Infected PLP-EGFP mice were anesthetized using isoflurane and decapitated. The brains were immediately harvested and immersion fixed in 10% buffered formalin overnight, followed by storage in PBS (phosphate-buffered saline) at 4° until sectioning. Brains were cut into 50 µm thick sections on a vibratome, permeabilized with 0.1% Tx-100 (Triton X-100) in PBS for 10 minutes, washed, and then incubated with 3% BSA (bovine serum albumin) in PBS. All antibodies used for immunostaining were diluted in Mac’s buffer. Rabbit-anti Olig2 (Chemicon, 1:500) was incubated with the slices at
room temperature (RT) for 1 hour. After washing with PBS, the slices were incubated for 1 hour at RT with fluorescent secondary antibody, Donkey anti-Rabbit AlexaFluor 594 (Molecular Probes, 1:1000). Slices were mounted on glass slides with glycerol:PBS, then coverslipped and imaged using a confocal microscope (Olympus Fluoview 500).

E. Double Immunolabeling for Env Protein and Cell Lineage Markers: Paraffin-Embedded Sections. All double-labeling on paraffin sections was performed as previously described (Li et al., 2011). Briefly, 5 μm thick paraffin-embedded sections that had been mounted on Superfrost Plus slides were deparaffinized in xylene and rehydrated in graded alcohol followed by equilibration in water. Antigen retrieval was performed in Sodium Citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 10 min in an autoclave. The slides were cooled to RT and incubated in 3% BSA for 20 min.

To visualize the Fr57E Env protein, Goat-anti Friend Env (1:10,000; gift from Roland Friedrich) was incubated on the slides for 1 hr at RT. One hour incubation with Donkey anti-goat Biotin (Jackson ImmunoResearch Laboratories, 1:500) was followed by Streptavidin AlexaFluor 594 (Molecular Probes, 1:1000) for 20 min. To visualize the FrCasE Env protein, primary mAb (monoclonal antibody) 697 (McAtee and Portis, 1985) was diluted 1:5 and incubated with the slides for 1 hr, followed by secondary antibody Donkey anti-mouse AlexaFluor 594 (Molecular Probes, 1:1000) for 1 hr. To detect PLP-EGFP expression, primary antibody Rabbit anti-GFP was incubated for 1 hr (abcam, 1:2000). Secondary antibody anti-Rabbit AlexaFluor 488 (Molecular Probes, 1:2000) was applied for 1 hr.
To perform cell lineage marker immunostainings, Rabbit anti-Olig2 (Chemicon, 1:500), Rabbit anti-NG2 (Chemicon, 1:200) or Rabbit anti-CAII (Chemicon, 1:2000) primary antibodies were incubated on the slides overnight at 4°C. For Olig2 and NG2 lineage markers, the same secondary antibody, Donkey anti-rabbit biotinylated antibody (Jackson ImmunoResearch Laboratories, 1:500) was incubated for 1 hr at RT. Streptavidin AlexaFluor 594 tertiary antibody (Molecular Probes, 1:1000) was incubated for 20 min at RT, and the slides were washed with PBS before the Env staining. Fluorescent secondary antibody Donkey anti-rabbit AlexaFluor 594 (Molecular Probes, 1:1000) was used with the CAII staining. For identification of Fr57E Env, Goat anti-Friend Env gp70 primary antibody (1:10,000) was used with a Donkey anti-goat IgG AlexaFluor 488 fluorescent secondary antibody (Molecular Probes, 1:1000). For identification of FrCasE infected cells, undiluted mAb 697 was used to detect Env protein, and labeled with Goat anti-mouse IgG 488 (Molecular Probes, 1:1000). All fluorescent images were captured on an Olympus Fluoview 500 confocal microscope equipped with Argon 488 nm, He-Ne 543nm, and He-Ne 633nm lasers.

**Generation of infected neurospheres from neonatal mice**

**A. Neurosphere (NPH) Cultures.** Primary neural cells were isolated from neonatal (P0-P1) mouse cortices, and enzymatically dissociated with papain using the Neural Tissue Dissociation Kit (Miltenyi). Primary cells were infected with virus on the day of isolation via spinoculation, wherein viral supernatants from NIH 3T3 cells grown in NPH medium were centrifuged at 2000 rpm at 4°C for 2 hrs prior to the addition of 1.0
x 10^6 primary neural cells per well in Nunc 6-well multi-dishes. The cells were maintained as NPH in culture with serum-free DMEM/F-12 medium (Invitrogen) containing B-27 supplement (Gibco), 10 ng/mL EGF (epidermal growth factor, Chemicon), and penicillin/streptomycin (Gibco). Infected cells were grown in vitro for one to three weeks before transplantation and were passaged using the Neural Tissue Dissociation Kit (Miltenyi) approximately every five days (Singec et al., 2006). Either mTmG or PLP-EGFP transgenic mouse strains were used to generate primary NSC cultures.

B. Assessment of Virus Infection of Cultured Neurospheres. Uninfected and infected spheres were seeded onto poly-D-lysine coated multi-dishes, and incubated for 2 hrs, then fixed with 4% PFA for 20 min. The spheres were permeabilized with 0.1% Tx-100 for 20 min, then stained with Mouse anti-Nestin (Chemicon, 1:500), anti-FrCasE Env mAb 697 (undiluted) or Goat anti-Friend Env (1:5000) and Rabbit anti-NG2 (Chemicon, 1:100), Rabbit anti-Olig2 (Chemicon, 1:500), or Rabbit anti-PDGFRα (SantaCruz Biotechnologies, 1:300) for 1 hr. Secondary antibodies for FrCasE Env or Nestin were Donkey anti-mouse AlexaFluor 555 (Molecular Probes, 1:1000) or for Fr57E Env were Donkey anti-goat AlexaFluor 555 (Molecular Probes, 1:1000). Secondary antibodies for cell lineage markers were Donkey anti-rabbit AlexaFluor 647 (Molecular Probes, 1:1000), which were incubated together for 1 hr at RT.

C. Transplantation of Infected NSCs into Neonatal Mice. NSCs were transplanted essentially the same as described previously by our laboratory for C17.2 NSCs (Lynch et al., 1996). Briefly, on the day of birth, IRW animals were subjected to
cryoanesthesia and the brain was visualized by transillumination of the head in order to provide landmarks associated with the vasculature to orient injections into the brainstem and lateral cerebral ventricles. Infected and uninfected NPH cells were dissociated and resuspended in PBS at $5 \times 10^4$ cell/$\mu$L to which 0.1% trypan blue had been added to assess viability as well as to localize the inoculum after injection. Six injections of 100 nL each were made bilaterally (3 per side) corresponding to just rostral to the cerebellum, the inferior colliculus and the superior colliculus. In addition, 200 nL injections were made into each lateral ventricle, resulting in a total of 1 $\mu$L of cell suspension inoculated into each brain. Pups were returned to maternal care until being sacrificed at 7 and 14 days post-transplantation (dpt), at which time the mice were perfused transcardially with freshly prepared 4% paraformaldehyde (PFA) in PBS. The brains were harvested and post-fixed in 4% PFA overnight, then stored in PBS until they were cut to a thickness of 50 $\mu$m on a vibratome.

D. Circulating-virus Titration Assay. At the time of sacrifice, blood samples were collected from animals transplanted neonatally with Fr57E or FrCasE infected NSCs. The blood was spun in a microcentrifuge at 13,000 rpm for 5 min, and then the serum was removed and stored at -80°. NIH 3T3 cells were seeded into 6-well multi-dishes at a density of $1 \times 10^5$ cells per well with 8 $\mu$g/mL polybrene in 2 mL of DM10N medium. Serum was added to each well in 10-fold serial dilutions. Plates were incubated for 24 hrs, and then the medium was replaced. The cells were confluent by day 3, and then fixed with 3.7% formaldehyde for 5 min. They were incubated in Mac’s buffer for 20 min, then immunostained with antibodies that recognize Env proteins (Ab
48 for Fr57E and mAb 697 for FrCasE) for 1hr at RT. After the cells were washed with PBS, secondary antibody Goat anti-mouse Alkaline Phosphatase (Promega, 1:5000) was incubated for 1hr. Western Blue Substrate was applied to the cells for 20 min, and foci were counted in each well to determine the titer.
RESULTS

Oligodendrocyte progenitor cells (OPCs) but not mature oligodendrocytes (OLs) undergo cytoplasmic effacement

Neurovirulent MLV (NV) infection of the CNS has been reported to induce two types of histopathological changes. The first type is neuropil vacuolation, which has been attributed to the swelling of or loss of structure in post-synaptic processes (Lynch et al., 1991; Nagra et al., 1993a). In the most rapid disease models, this type of spongiosis arises beginning 10 days after neonatal infection (Portis et al., 1990). The second NV-induced histopathological change is the appearance of cells showing a loss of cytoplasmic structure but with intact nuclei. These cytoplasmically effaced cells (CECs) have been ultrastructurally characterized as degenerating glia (Andrews and Gardner, 1974). More recent studies have suggested that the CECs were oligodendrocytes because they express the Olig2 transcription factor (Clase et al., 2006).

To define the nature of the cells undergoing cytoplasmic degeneration, we first compared when these cells became apparent in the brainstems of animals infected in the rapid FrCasE model of virus-induced spongiform neurodegeneration. As shown in Figure 1, CECs were frequently observed in animals at 14 dpi, a time that is just prior to the onset of clinical symptoms in these mice. These pathological changes in cellular morphology were observed in all areas of the brainstem, spinal cord and motor cortex.
Comparison with age matched controls and animals infected with the isogenic NN Fr57E showed no CECs regardless of the CNS area surveyed.
Figure 1. Histological Examination of MLV Infected Animals 14 Days Post-inoculation. Animals were intraperitoneally (ip) inoculated on the day of birth (P0) with no virus, NN Fr57E, or NV FrCasE. Mice were sacrificed 14 days post-inoculation (dpi) and brain tissue was paraffin-embedded and stained with hematoxylin and eosin. Images were collected from the pontine reticular nucleus, a region within the brainstem that is susceptible to FrCasE-induced spongiosis. Fr57E virus results in only mild spongiform pathology while FrCasE causes cytoplasmic effacement (arrows) of cells identified as degenerating glia in addition to severe neuropil vacuolation. Bar = 50 μm.
Having established that CECs are abundant by 14 dpi, our next experiments sought to re-address the oligodendroglial identity and developmental state of the cells that lose cytoplasmic structure. We chose to employ two experimental methods to identify cell types. The first involved the use of a transgenic mouse model that expresses a fluorescent marker, EGFP (enhanced green fluorescent protein), driven by the PLP (proteolipid protein) promoter, which is active in oligodendrocytes. PLP is the most abundant protein present in myelin, and indicative of a mature oligodendrocyte capable of myelinating neuronal axons (Mallon et al., 2002; Trapp et al., 1997). EGFP+ cells in the CNS of PLP-EGFP mice can be segregated into either EGFP\textsuperscript{high} or EGFP\textsuperscript{low} populations. The EGFP\textsuperscript{high} cells have been characterized as OLs, with both myelinating and non-myelinating phenotypes (Fuss et al., 2000). EGFP\textsuperscript{low} cells have been reported to include certain neuronal populations, but this assessment as neurons also relies on cell morphology details to draw this conclusion. Without this consideration, cells transitioning from OPCs to mature OLs that are EGFP\textsuperscript{low} could be incorrectly categorized as neurons (Miller et al., 2009). The cells that do not express any EGFP include NSCs, astrocytes, endothelial cells, microglia, and cells in the OL lineage that precede the oligodendrocyte stage. The second approach involved using immunofluorescence (IF) for common OL lineage markers. Since the CECs were suggested to be OLs, we immunostained for the Olig2 protein. Olig2 is a basic helix-loop-helix transcription factor that is required for OL specification and thus is an early/intermediate cell lineage marker for OPCs that is detectable throughout several stages of oligodendrocyte
differentiation and by developing motor neurons (Ligon et al., 2007; Zhou and Anderson, 2002).

These methods were applied to FrCasE infected mice at 15 dpi, a time coincident with the very onset of clinical symptoms. Thick sections (50 µm) from formalin-fixed PLP-EGFP mouse brains were immunostained for Olig2 that was visualized by incubation with AlexaFluor 594-coupled anti-rabbit IgG secondary antibody. Images captured by confocal microscopy were examined with and without adjusting the background image intensity in order to reveal the detailed cellular morphology of the CECs relative to Olig2 immunostaining and EGFP expression. Three populations of OPC/OL cells were identified that included Olig2+/EGFPneg, Olig2+/EGFP+ and Olig2neg/EGFP+ (Figure 2 panels A & B). CECs were only observed within the Olig2+/EGFPneg cell populations (panel A) and represented approximately 50% of the CECs identified. This finding suggested that the CECs were OPCs rather than OLs. Alternative explanations for this observation include that EGFP could be down-regulated by MLV infection, or infection could induce ectopic Olig2 expression. Examination of EGFP+ cells in FrCasE mice showed the occasional appearance of cytoplasmic vacuoles within the EGFP+ population (Figure 2, panels C-F), suggesting a mild or alternate pathology in cells that were either transitioning to mature OL lineage cells or were neuronal since these cells expressed intermediate levels of EGFP and showed various cellular morphologies. This finding was in general consistent with the idea that immature cells were more susceptible to degeneration.
Figure 2. OPCs Undergo Cytoplasmic Effacement as a Result of NV Infection of the CNS. Transgenic mice expressing EGFP driven by the PLP (proteolipid protein) promoter were infected neonatally with NV FrCasE virus and analyzed 15 days later for Olig2 (red) and PLP-EGFP (green) coexpression in the brainstem. Thick sections (50 μm) were immunostained for the transcription factor Olig2 (A). Fifty percent of cells exhibiting cytoplasmic effacement expressed Olig2 (arrows), but do not colocalize with PLP-EGFP. Olig2 staining colocalizes with PLP-EGFP in some cells (arrowheads), but these cells do not appear to be undergoing cytoplasmic effacement. Also, some PLP-EGFP cells appeared to have some small intracellular vacuoles located at the cell soma (C, D, E, F).
Evidence for ecotropic MLV infection of OPCs but not OLs in the brainstem

We next sought to understand the relationship between virus infection and the OL lineage of the CECs. To accomplish this we examined control and MLV infected brains for expression of Env protein along with early, intermediate, and late oligodendroglial markers by double immunostaining. The NG2 antigen was used to identify early OPCs as NSCs express this marker once they become committed to polydendrocyte or OL lineages (Li et al., 2011; Nishiyama et al., 2009). The Olig2 antigen was used to define OPCs, as outlined above. As these cells undergo terminal differentiation into mature oligodendrocytes they lose NG2 and Olig2 markers and begin to express mature oligodendrocyte antigens such as CAII (carbonic anhydrase II) (Ghandour et al., 1980a; Ghandour et al., 1980b).

Examination of brains infected with either FrCasE or Fr57E at 15 dpi showed that NG2+ and Olig2+ cells expressed Env proteins from their respective viruses (Figure 3). Note that the staining for NG2 and Env showed only limited overlap within the positive cells suggesting that the antigens localize to different cellular compartments (panels A & D). This is clearly the situation for Env and Olig2 in the confocal images as shown in panels B & E, which are found in the secretory pathway and the plasma membrane, and the nucleus, respectively. In these experiments (at least 3 animals per group) 33.2% of FrCasE (n = 916) and 37.8% of Fr57E Env-expressing cells (n = 498) also expressed NG2+ protein. Similarly, Olig2, a later developmental marker, was expressed by 31.2% of FrCasE Env+ cells (n = 409) and 32.8% of Fr57E Env+ cells (n = 697). In contrast, Env expression was rarely observed in CAII+ cells (panels C & F), with frequencies of
0.9% for FrCasE Env + cells (n = 377) and 2.9% for Fr57E Env+ cells (n = 1417).

These quantitative results are summarized in Table 1.
Figure 3. OPCs but not Mature CAII-expressing OLs Show Infection by Ecotropic MLVs in the CNS after Intraperitoneal Inoculation. Neonatal IRW mice were ip inoculated with either FrCasE (A, B, C) or Fr57E virus (D, E, F). After 15 days, the mice were sacrificed, and the brains were paraffin embedded and assessed by immunostaining for the cell lineage markers NG2, Olig2, or CAII (green) and Env protein (red). Viral envelope protein can be detected in one-third of NG2+ cells and Olig2+ oligodendrocyte lineage cells (arrows, A, B, D, E) regardless of the neurovirulence of the MLV. Colocalization of mature oligodendrocyte marker (carbonic anhydrase II) or CNPase (data not shown) and retroviral Env protein occurs rarely (C, F).
Table 1. Glial Expression of FrCasE or Fr57E Env in the Brainstem of Intraperitoneally Inoculated Mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>FrCasE Env (mAb 697)</th>
<th>Fr57E (Gt anti-Friend Env sera)</th>
</tr>
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<tbody>
<tr>
<td>NG2</td>
<td>33.2 ± 2.3 (n = 916)</td>
<td>37.8 ± 7.3 (n = 498)</td>
</tr>
<tr>
<td>Olig2</td>
<td>31.2 ± 1.7 (n = 409)</td>
<td>32.8 ± 6.6 (n = 697)</td>
</tr>
<tr>
<td>CAII</td>
<td>0.9 ± 0.7 (n = 377)</td>
<td>2.9 ± 1.4 (n = 1417)</td>
</tr>
</tbody>
</table>

*Values expressed were the percentage of Env-positive cells positive for the glial cell marker observed in the brainstem sections surveyed, ± the SD of the mean, followed by the total number of Env-positive cells counted in parentheses. Analysis was performed on images collected from a confocal microscope. mAb 697 was used to detect FrCasE Env protein.*
Since the NG2 and Olig2 markers are not mutually exclusive, but rather, can be expressed simultaneously as cells make the transitional change into more mature phenotypes, it was possible that some portion of these cell populations represented overlap. However, because it was not clear that this distinction would provide any meaningful new information we did not undertake this secondary analysis. These results indicate that both NV and NN infected OPCs, but in neither case did we see evidence that these progenitor cell populations transitioned efficiently to mature OLs. Whether this represents an effect of MLVs on OPC differentiation, cell viability or simply an inability to detect Env in OLs remain open questions. While we did not systematically evaluate the infection of CECs, we did not observe Env expression in CECs by increasing the gain on images captured to evaluate Env/OPC marker colocalization. Nonetheless, the fact that the OPCs represent a population of cells prone to cytoplasmic effacement, we cannot formally rule out the possibility that MLV infection leads to the loss of cytoplasmic structure. Why the phenotype is the same for NN and NVs represents an additional conundrum.

As another means to determine the developmental status of the cells that express Env protein we examined PLP-EGFP animals infected with MLVs, and immunostained the tissues for Env protein. Through this alternative approach to cell lineage marker immunostaining, we found very few FrCasE Env+ (0.34%; n=866) and Fr57E Env+ cells (1.05%; n=762) that were EGFP+ (Figure 4), consistent with the previous results using CAII as the marker for OL maturation. The main difference between the FrCasE and Fr57E infected brains at this time point was the presence of severe vacuolar degeneration
in the FrCasE infected brain. Whether the ongoing neurodegeneration could account for
the small differences noted is not clear since Env-infected cells expressing mature OL
markers were so rare. Instead, it is likely that the MLV Env protein does not directly
induce glial degeneration, but rather prevents progenitor cells from differentiating into
mature OLs.
Figure 4. MLV Env Protein is Rarely Expressed in PLP-EGFP Oligodendrocytes. Neonatal PLP-EGFP mice were ip inoculated with ecotropic viruses (Fr57E or FrCasE) and examined for Env protein (red) 18 (Fr57E) or 15 (FrCasE) days later. Colocalization of Env and PLP-EGFP was rarely observed, but an example for each an Fr57E infected animal (Top panel, arrows), and a FrCasE infected animal (Bottom panel, arrows) are shown. 1.05% and 0.34% of PLP-EGFP cells were Env positive after Fr57E (n= 762) and FrCasE (n=866) inoculation, respectively.
**MLV infection of neural progenitor cells *in vitro* is not toxic**

Unlike HIV, the MLVs do not typically induce direct cytotoxicity of infected cells except for two variants of Friend virus that cause hemolytic anemia (Sitbon et al., 1986) and brain hemorrhages (Park et al., 1993). Previous results from our laboratory have demonstrated that a variety of MLVs can infect the NSC cell line C17.2 without cytotoxicity of these cells either *in vitro* or *in vivo* (Lynch et al., 1996). However, because these cells have been conditionally immortalized with the *v-myc* gene (Ryder et al., 1990), the effects of the MLVs may not be expressed or apoptotic pathways may be averted {cf., (Ceccatelli et al., 2004)}. Therefore, primary NSC cultures were generated from susceptible IRW-strain mice that were either wild-type or expressed the PLP-EGFP transgene. The NSCs were exposed to FrCasE or Fr57E and cultured without serum yet supplemented with growth factors (such as epidermal growth factor, EGF) and other components to maintain the NSCs in a proliferative and undifferentiated state. These cultures are known as neurospheres (NPH) because the cells do not attach to their growth substrate, and when they are grown at a low density, each sphere is a clonal population of dividing stem cells.

MLV infected and uninfected NPHs were visually examined daily for expansion of sphere size and number over a period of greater than 3 weeks in culture. In more than 30 separate NPH culture experiments, no clear differences indicative of MLV-induced cell death or growth arrest were observed. To assess whether cells in the NPHs were indeed being infected by the MLVs, and to characterize the NPHs for expression of NSC and OPC markers, dissociated NPH cells were grown briefly in serum-containing
medium to induce cellular attachment, allowing for easy immunostaining for Env and cellular antigens. As shown in Table 2, analysis of PLP-EGFP NPHs, showed that close to 90% of the cells were Env+ for both FrCasE and Fr57E, and that greater than 95% expressed the NSC marker Nestin, an intermediate filament protein not found in mature cells. These cells also expressed at very high levels (>90%) markers for OPCs including NG2, Olig2, and PDGFRα. In postnatally dividing cells in vivo, the PDGFRα (platelet-derived growth factor receptor alpha) protein is expressed by cells in the developmental state immediately preceding oligodendrocytes (Mallon et al., 2002). These findings suggest that infection of NPH in culture with NN and NV does not affect cell growth, viability, or NSC/OPC marker expression.
Table 2. Characterization of Cell Lineage Marker Expression by PLP-EGFP Neurospheres Infected Ex Vivo with MLV

<table>
<thead>
<tr>
<th></th>
<th>Uninfected PLP-NPH</th>
<th>Fr57E-PLP-NPH</th>
<th>FrCasE-PLP-NPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Env</td>
<td>–</td>
<td>91.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nestin</td>
<td>87</td>
<td>95.2</td>
<td>97.5</td>
</tr>
<tr>
<td>NG2</td>
<td>97.3</td>
<td>95.3</td>
<td>96.5</td>
</tr>
<tr>
<td>Olig2</td>
<td>95.9</td>
<td>96</td>
<td>95.8</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>95</td>
<td>91.1</td>
<td>100</td>
</tr>
<tr>
<td>EGFP</td>
<td>15</td>
<td>13.2</td>
<td>13.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values shown were the percentage of cells expressing the cell lineage marker/total number of dissociated NPH cells counted.

<sup>b</sup> Goat-anti Friend serum was used to detect Fr57E Env expression.

<sup>c</sup> mAb 697 was used to detect FrCasE Env expression.
The findings on attached cells were supported by parallel immunostaining experiments on intact NPHs where multiple cell markers were localized simultaneously (Figure 5). However, because of poor antibody penetration into the interior of the spheres, quantitative analysis of antigen colocalization was not undertaken. Nonetheless, it was clear that both FrCasE and Fr57E MLV Envs were colocalized with NSC and OPC markers in the intact NPHs. Interestingly, approximately 15% of the cells in these cultures expressed detectable levels of EGFP regardless of virus infection, although the levels of expression were visually lower with MLV infection. Whether this represented promoter leakiness, or in vitro cellular differentiation is not known, but since the cells did not have morphologies characteristic of mature oligodendrocytes, EGFP expression was likely an artifact of growth in culture. Nonetheless, these in vitro results suggest that MLV infection was not directly cytotoxic to NPHs despite the fact that these cells share antigenic features with those undergoing neuropathogenic changes within the CNS. Moreover, the frequency of Nestin, NG2, Olig2 and PGDFRα-expressing cells was similar among the treatments, suggesting that MLV infection did not drastically alter the stem-like properties of NPHs. Whether these cells may have to transition to a later stage of development for the expression of MLV cytotoxicity remains a possibility, however, attempts to develop either oligosphere or OL cultures in our laboratory were not successful regardless of the infection status of the initiating NPH cultures.
Figure 5. Primary Neural Stem Cells From PLP-EGFP Mice Infected *In Vitro* with Ecotropic MLVs Express Various Cell Lineage Markers Including Nestin, NG2, Olig2 and PDGFRα When Cultured as Neurospheres. Primary neural cultures were induced to grow as spheres in serum-free, B27-supplemented media containing EGF. The cells were infected with virus on the day of isolation, and grown in culture for several days. Neurosphere cultures were fixed and stained with Nestin, NG2, Olig2, and PDGFRα coupled to fluorescent antibodies. Images were collected on a confocal microscope. Scale bar = 50 μm.
MLV infection of NSCs *ex vivo* is productive, but does not impair their engraftment and survival within the developing CNS

Because Clase *et al* suggested that infected OLs degenerate and die as a direct result of their infection in the CNS, we re-examined this hypothesis *in vivo* using the NSC-based brain chimera strategy pioneered in our laboratory (Lynch *et al.*, 1996). Specifically, we assessed whether MLV infection reduced NPH viability after transplantation into developing brains by using NPHs generated from mTmG RFP+ reporter mice. Examination of IRW mice 7 and 14 days post-transplantation (dpt) with dissociated Fr57E, FrCasE or uninfected NPHs showed readily observable RFP+ cells at both time points (*Figure 6*) irrespective of the MLV infection of the NPHs. As early as 7 dpt, RFP positive cells possessed ramified morphologies indicative of cellular differentiation, with no obvious differences in cell morphologies between groups. Analysis at 14 dpt showed transplanted cells that had taken on more mature cellular morphologies. Most notable was the presence of RFP+ processes consistent with myelinating cells present in the brains receiving uninfected NPHs (*Figure 6, panel D*). RFP+ cells showing myelinating morphologies were only occasionally seen in animals receiving FrCasE- and Fr57E-infected NSCs (cf **panel F**). Instead these mice showed cells with morphologies consistent with astrocytes (*Figure 6, brackets*), that were seen with all NPH groups. These cells showed the development of endfeet on blood vessels, ensheathment of neuronal cell bodies and synapses, and a restriction of cellular processes to reflect their discreet tiling domains.
Figure 6. Transplantation of *Ex Vivo* Infected mTmG Neurospheres into Naïve Neonatal Mice Results in Engraftment of mTmG-expressing Cells After 7 or 14 Days. In the brains of mice receiving uninfected NPH it is possible to locate cells with long fibrous processes characteristic of oligodendrocytes (arrowheads, D & F). Regardless of treatment with virus, neural stem cells transplanted into host brains can survive and engraft. The cells differentiate into morphologically distinct cell types, many resembling astrocytes (bracketed), due to the presence of endfeet aligned along blood vessels and fine processes.
To establish whether the MLV infection had an effect on the survival of the RFP+ NSCs, donor cell engraftment was quantitated using between 3 and 6 animals per experimental group. As shown in Figure 7, there was no statistical difference between MLV-infected and control groups. However, considerable variation of NSC engraftment efficiency was seen within each group (see Table 3). This result speaks in part, to the difficulty of effectively delivering cells within the brain. In this regard, 8 separate injections were made in each brain using an automated micro-syringe delivery device; however, the failure rate for delivery of the semi-viscous cell suspension could be greater than 50%. Therefore, the power to detect small changes in engraftment efficiency was not possible given the limitations of the current methodology. Nonetheless, we can conclude that regardless of virus exposure, RFP-expressing mTmG NSCs were capable of engrafting, surviving, and undergoing differentiation in host brains at 7 and 14 dpt. Using this transplant paradigm, it appeared that the MLV-infected NPH cells were biased away from differentiating towards cells with mature OL morphologies. While quantification of these differences was possible using this model, there were considerable limitations that made this difficult; for example there was extensive RFP+ cellular overlap and crowding at engraftment sites. Thus, we chose to address this question using a more refined approach where we would only detect maturing OLs, that is, we used the PLP-EGFP NPHs, characterized above.
Figure 7. Transplantation of MLV Infected mTmG Neural Stem Cells Results in the Engraftment of Donor Cells in a Host Brain. RFP+ cells were detected 7 dpt (A) and 14 dpt (B), regardless of the virus treatment. Data shown was the mean ± SD, where n = 3 to 6 animals per group. Kruskal-Wallis statistical tests were performed, A: P = 0.3802. B: P = 0.0125. Pairs of groups were directly compared using Dunn’s Multiple Comparison Test, and significant differences between the groups were noted by asterisks when P < 0.05.
Table 3. mTmG Neural Stem Cells Engrafted into Host Brains at 7 and 14 Days Post-transplantation

<table>
<thead>
<tr>
<th>Uninfected</th>
<th>Fr57E</th>
<th>FrCasE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7 dpt</td>
<td>14 dpt</td>
</tr>
<tr>
<td>145(^a)</td>
<td>1403</td>
<td>3090</td>
</tr>
<tr>
<td>11496</td>
<td>4638</td>
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<td>181</td>
<td>1934</td>
<td>1539</td>
</tr>
<tr>
<td></td>
<td>1123</td>
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4906 ± 4995\(^b\)
2567 ± 1645
2057 ± 964
1260 ± 669
7214 ± 3982
18970 ± 11795

The whole brain was cut into 50 μm slices, and each slice was sequentially screened for fluorescent cells on an Olympus BX51 microscope.

\(^a\) Values shown were the total number of mTmG RFP+ cells counted per brain at each time point.

\(^b\) Values shown were the mean number of cells per brain ± SD.
MLV infection of NSCs restricts their differentiation to OLs in the developing CNS

The next step was to determine if MLV-infected NPH cells had the same differentiation potential \textit{in vivo} as uninfected cells. Therefore, PLP-EGFP NPHs with and without Fr57E, or FrCasE were transplanted into P0 IRW mice and examined for GFP+ cells at 7 and 14 dpt. Animals transplanted with control uninfected PLP-EGFP NPHs showed the presence of GFP+ cells with both myelinating and non-myelinating morphologies at both time points (\textbf{Figures 8}). The percentage of GFP+ myelinating cells increased from 18% at 7 dpt to 48% at 14 dpt showing that the ex \textit{vivo} cultured cells were fully capable of becoming mature OLs. In comparison to controls, very few EGFP+ cells were observed in the brains of mice transplanted with NPHs that were infected with FrCasE or Fr57E (\textbf{Figure 9} \& \textbf{Table 4}). Because the frequency of GFP+ cells observed was so low in MLV-NPH transplants, some experiments were carried out with NPH cells that were labeled with the membrane permeant dye PKH-26 to assure that the transplanted cells persisted to the 7 and 14 day time points. No gross differences were observed in the PKH-26 cells persisting, between control and infected brains, so no specific quantification was carried out. This observation was consistent with the results generated upon transplantation of NPHs derived from mTmG brains and suggests that the ecotropic MLVs do not affect NSC viability, but do appear to interfere with the transition to a mature OL phenotype. Whether these cells remain in an undifferentiated, partially differentiated OPC state, or are shunted down a default pathway to become astrocytes or NG2+ glia remains to be investigated, but it appears that infected NPHs can take on mature astrocyte morphologies.
Figure 8. PLP-EGFP Neural Stem Cells Engraft in the Brains of Neonatal Animals, and Express EGFP When the PLP Promoter is Active. Uninfected PLP-NPH were transplanted into naïve animals, which were sacrificed after either 7 or 14 days. By 7 days post-transplantation (dpt), some uninfected PLP-EGFP cells were present throughout the brain. By 14 dpt, the oligodendrocytes had a mature phenotype, complete with long processes that produce myelin, and were capable of enwrapping several neuronal axons.
Figure 9. Transplantation of MLV Infected PLP-EGFP Neural Stem Cells Results in Fewer Cells Differentiating Into Oligodendrocytes than Uninfected Controls. PLP-EGFP cells were detected 7 dpt (A) and 14 dpt (B), but the number of cells that differentiate into OLs was significantly fewer when the PLP-NPH were exposed to MLVs *ex vivo*. Data shown was the mean ± SD, where n = 7 or 8 animals per group. Kruskal-Wallis statistical tests were performed, A: P = 0.0039; B: P = 0.0023. Pairs of groups were directly compared using Dunn’s Multiple Comparison Test, and significant differences between the groups were noted by asterisks when P < 0.05.
The whole brain was cut into 50 μm slices, and each slice was sequentially screened for fluorescent cells on an Olympus BX51 microscope.

Values shown were the total number of PLP-EGFP cells counted per brain at each time point.

Values were expressed as the mean number of cells per brain ± SD.

### Table 4. PLP-EGFP Neural Stem Cells Engrafted into Host Brains at 7 and 14 Days Post-transplantation

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Fr57E</th>
<th>FrCasE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 dpt 14 dpt</td>
<td>7 dpt 14 dpt</td>
<td>7 dpt 14 dpt</td>
</tr>
<tr>
<td>7 dpt</td>
<td>1049</td>
<td>698</td>
<td>0</td>
</tr>
<tr>
<td>14 dpt</td>
<td>779</td>
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<tr>
<td>7 dpt</td>
<td>135</td>
<td>320</td>
<td>1</td>
</tr>
<tr>
<td>14 dpt</td>
<td>73</td>
<td>968</td>
<td>208</td>
</tr>
<tr>
<td>7 dpt</td>
<td>175</td>
<td>885</td>
<td>2</td>
</tr>
<tr>
<td>14 dpt</td>
<td>44</td>
<td>617</td>
<td>2</td>
</tr>
<tr>
<td>7 dpt</td>
<td>262</td>
<td>159</td>
<td>0</td>
</tr>
<tr>
<td>14 dpt</td>
<td>360 ± 393</td>
<td>546 ± 331</td>
<td>31 ± 78</td>
</tr>
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</table>
The challenge with the current experimental methodology was that formal proof for the idea that viral protein expression was responsible for the differentiation block requires the demonstration that the transplanted NPHs expressed virus *in vivo*. However, it has recently been shown in our laboratory that many MLV-infected CNS cells of neuroectodermal origin express virtually undetectable levels of viral proteins (S. Cardona, unpublished). Thus, identifying the presence of virus in the engrafted NPH cells may not be possible. Therefore, novel methods need to be employed to complement the current approach to track the fate of the infected cells within the developing CNS.

A final critical aspect of these experiments that impacts the interpretation of these results was the fact that the MLV-infected NPHs themselves produced infectious virions. In this regard we observed a circulating virus titer in the 14 dpt mice transplanted with FrCasE-NPH and Fr57E-NPH that was $5.1 \pm 0.5$ and $7.5 \pm 0.1 \log_{10}$ ffu/mL, respectively (*Table 5*). Therefore, the transplanted cells released enough viruses into the host that they escaped into the periphery where they replicated. The expectation is that these cells would also result in extensive infection of the host CNS as has been previously demonstrated for engraftable C17.2 NSCs (Lynch et al., 1996). This infection might be expected to alter the niche for the transplanted NPH cells to interact. If this were the case one would expect to find a significantly lower level of engraftment and differentiation with FrCasE compared to Fr57E which does not induce histopathology (cf., *Figure 1*). Remarkably, we saw that FrCasE cells were better able to engraft at 14 dpt, and both MLVs prevented the appearance of mature GFP+ OLs, suggesting that MLV infection alters fate specification of OPCs.
Table 5. Circulating Virus Titer 14 dpt of Mice Transplanted with FrCasE-PLP-EGFP or Fr57E-PLP-EGFP Neural Stem Cells

<table>
<thead>
<tr>
<th>Titer (log\textsubscript{10} ffu/mL)</th>
<th>FrCasE-PLP-EGFP</th>
<th>Fr57E-PLP-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.61\textsuperscript{a}</td>
<td>7.60</td>
<td></td>
</tr>
<tr>
<td>5.08</td>
<td>7.53</td>
<td></td>
</tr>
<tr>
<td>5.76</td>
<td>7.40</td>
<td></td>
</tr>
<tr>
<td>5.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.13 ± 0.47\textsuperscript{b} 7.50 ± 0.1

Serum was collected 14 dpt from mice that had been transplanted neonatally with infected NPHs (FrCasE, n=4; Fr57E, n=3). NIH 3T3 cells were exposed to serial dilutions of the virus-containing serum, grown to confluence, then immunostained for Env protein.

\textsuperscript{a} Values shown were the virus titers from each individual mouse, log\textsubscript{10} ffu/mL.

\textsuperscript{b} Values were expressed as the mean titer (log\textsubscript{10} ffu/mL) ± SD.
DISCUSSION

MLVs are neurotropic retroviruses, capable of inducing fatal neurodegeneration primarily affecting motor system neurons. In addition to neuropil vacuolation, there is a population of cells that lose cytoplasmic structure as a result of CNS infection by NV. We found that the cells that undergo cytoplasmic effacement are OPCs but not mature OLs. We also found that OPCs are capable of expressing both NN and NV Env protein, but mature OLs are not. This raised the possibility that MLVs are cytotoxic to cells, but when we addressed this directly, we found no evidence that MLVs die as a direct result of infection either \textit{in vitro} or \textit{in vivo}. Rather we found that MLV infection alters the differentiation fate of progenitor cells, preventing them from maturing into OLs. The molecular mechanism of MLV-induced alterations in differentiation remains to be explored.

These present findings contradict the prior conclusions of Clase and colleagues indicating that NVs Moloney ts-1 and FrCasE kill OLs as a direct result of infection (Clase et al., 2006). In our results we show that both NV FrCasE and the NN Fr57E abundantly infected OPCs but only rarely infected OLs consistent with the idea of MLV infection induced OPC/OL cytotoxicity. However, neither glia nor neurons degenerate upon CNS infection by Fr57E, so one would have to postulate a different means of cell death associated with Fr57E infection of OPCs that was histopathologically invisible and clinically insignificant. We think this is unlikely. The findings for Fr57E were consistent
with prior data from our laboratory showing that infection of OPCs and mature OLs occurs with the neurovirulent but tropically restricted 4070A amphotropic virus under conditions where the virus does not result in neuropathogenesis (neither glial nor neuronal) (Li et al., 2011). Were MLVs directly oligotoxic, 4070A should have induced CECs through infection of NG2, Olig2 and CAII expressing glia. Instead, this virus needs to be targeted to other CNS cell types to express its neuropathogenic potential. Because 4070A was reported to be readily found in mature OLs, our results suggest that ecotropic, but not amphotropic MLVs may affect OPC differentiation.

An alternative explanation for the lack of infected mature OLs that were observed (e.g., CAII+ or PLP-EGFP+) could be MLV-induced down-regulation of OL markers. However, such a change in OPC/OL marker expression would ostensibly constitute the functional equivalence of altered OL differentiation. It was also possible that mature OLs suppressed Env expression making it difficult for us to detect these cells using sensitive immunologic techniques. Importantly, we did not see a difference in the numbers of engrafted NSCs cells with and without MLV-infection, under conditions where their identification and persistence was independent of MLV expression, supporting the idea that NPH cells were not directly killed by MLVs. Again, these experiments were done under conditions with and without ongoing neurodegeneration. What was affected by MLV infection of NSCs was their transition from OPCs to myelinating and non-myelinating OLs within 2 weeks of transplantation, regardless of viral neurovirulence. Moreover, our ex vivo analysis of NSC viability failed to reveal any overt virus-induced toxicity, despite the fact that in culture the infected NPH cells expressed multiple OPC
markers. Thus, ecotropic MLV interference with OPC differentiation seems a more likely explanation than cytotoxicity for the absence of infected OLs.

A number of studies have demonstrated that HIV-1 and HTLV-1 are capable of infecting the neural stem/progenitor cell pool in the CNS of humans. These viruses induce either cell death, maintenance as quiescent undifferentiated neural progenitors, or impairs neuronal or glial differentiation; all of which result in impaired neurogenesis, and a decreased ability for the brain to repair itself after injury or throughout the aging process {reviewed in (Das and Basu, 2011)}. Similarly, murine cytomegalovirus infection of NPHs leads to astrocyte differentiation and limited migration upon transplantation into CNS ventricles. The experiments undertaken herein, however, are the first to demonstrate that retrovirus infection of NSCs prevents their maturation into oligodendrocytes. Based on our observation that ecotropic MLV infected cells differentiated into phenotypically mature astrocytes, the infected NSCs may follow an astrocyte specification pathway. Alternatively, they may remain arrested as progenitor cells due to blockage of ERK1-mediated mitogenic signal-producing transduction pathway as reported for HIV-1 infected NSCs (Krathwohl and Kaiser, 2004). Distinguishing between these two possibilities may be critical for understanding the details of the neuropathogenic process.

In considering how differentiation arrest might influence neuropathogenesis, it is important to consider that as cells differentiate, they transiently express many different cell markers. In this study we have used several of them for defining the cell lineage and the stage of differentiation. In this regard, work from the Bergles laboratory has
concentrated on characterizing the expression of glutamate receptors, glutamate transporters and voltage-gated sodium channels in NG2+ cells as they differentiate into oligodendrocytes (De Biase et al., 2010).

The NG2+ cell has recently become known as the fourth glial element (reviewed in Nishiyama et al., 2009), and there is still controversy surrounding the developmental fate and role of these cells in the brain. NG2+ cells compose approximately 5% of the cells in the adult CNS, apparently acting as stand-by progenitor cells that can replace oligodendrocytes when necessary (Dawson et al., 2003; Pringle et al., 1992). NG2+ cells have received a lot of attention recently because they were found to be excitable, capable of firing action potentials; a phenomenon previously thought only to occur in neurons (Karadottir et al., 2008). The purpose of the action potential is undetermined, but it has been suggested that it may induce the NG2+ cells to differentiate into oligodendrocytes. Other researchers remain unconvinced that the NG2+ cells actually fire action potentials, despite the fact that they express voltage-gated sodium channels (De Biase et al., 2010). Another interesting characteristic of NG2+ cells is that they maintain synaptic contacts with neurons throughout cell division (Ge et al., 2009).

Previous work from our laboratory suggested that MLV-induced spongiosis reflects ongoing excitotoxicity based on the appearance of vacuolation associated with post-synaptic processes (Lynch et al., 1991; Nagra et al., 1993b). Excitotoxicity is associated with the presence of excess glutamate in synaptic regions for extended periods, which over-excites cells and leads to elevated intracellular Ca^{2+} levels that ultimately damages cells. Glutamate can bind to Ca^{2+}-permeable receptors on the
plasma membrane, which induces the influx of Ca$^{+2}$ into the cell, and can initiate signaling cascades which are known to activate Ca$^{+2}$-dependent proteases or to potentiate Ca$^{+2}$ release from intracellular stores. Interestingly, at the NG2+ cell stage, there are high levels of Ca$^{+2}$-permeable AMPA and NMDA receptors as well as voltage-gated sodium channels in these cells, which would increase their susceptibility to excitotoxic damage. These proteins are down-regulated as cells mature into oligodendrocytes, at which time they also lose synaptic connections with neurons (De Biasi et al., 2010). Therefore, as OPCs develop along the OL lineage, they become less susceptible to infection and less likely to undergo cytoplasmic effacement. Since MLV infection arrests cells as OPCs, there will be an overabundance of cells prone to excitotoxic damage. Thus while MLVs do not appear to induce cell death or lysis in infected cells, they may act to increase the pool of glial targets that suffer bystander effects when they are located near other infected cells.

It is also possible that MLVs alter differentiation by biasing infected progenitor cells toward the astrocyte lineage. Whether there is an excess of these cells or if infected cells have altered functional properties could influence OPC viability. Astrocytes are known to release the gliotransmitters glutamate, D-serine and ATP, all of which can open Ca$^{+2}$ channels on neurons and glia. Astrocytes perform a number of additional essential functions within the brain, such as providing a scaffold during development, regulation of synaptogenesis, uptake of glutamate from the synapse, synthesis of glutamine and GABA from glutamate, formation and regulation of the blood-brain barrier, chemical signaling through gap junctions, and regulation of the cellular microenvironment by maintaining
pH homeostasis and buffering potassium (Zhang and Barres, 2010). A mechanism involving excitotoxicity has been implicated in many disease models, but remains only one of many possibilities. Thus, if the MLV directs infected progenitors toward either NG2+ cells or astrocytes, it is easy to imagine how this could induce vacuolar pathology in the brain.

SUMMARY

Upon infection of the CNS with NV, the brain undergoes spongiform pathology, resulting in both neuronal and glial degeneration. In this thesis, I have explored the nature of the glial pathology in an effort to understand their contribution to neurodegenerative diseases. In this study I have identified the glial cells undergoing cytoplasmic effacement as OPCs. By assessing the cell types that express Env protein, the OPCs were identified as infected, but interestingly, mature OLs were not. This led us to investigate whether the MLVs induce direct oligotoxicity upon infection of OPCs or OLs, but we conclude that neither NV nor NN caused cell death of infected cells. Using a NSC based approach, we found that infected OPCs were not killed by MLVs, but rather, were prevented from maturation into OLs. The link between infection of OPCs and the occurrence of cytoplasmic effacement in these cells may be due to this altered differentiation or that infected OPCs cannot transition away from the excitable NG2+ glia that degenerate due to excitotoxic damage. Future studies could focus on the nature of the differentiation block, and whether it induces cells to remain in a quiescent progenitor state or to mature along cell-type specific pathways. The molecular mechanism for how
MLVs affect differentiation fate is not known, but could be critical for our understanding of spongiform neuropathogenesis.


