Neural stem cells as a delivery vector for chemokine expression in the central nervous system

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NEURAL STEM CELLS
AS A DELIVERY VECTOR
FOR CHEMOKINE EXPRESSION
IN THE CENTRAL NERVOUS SYSTEM

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
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in

Veterinary Medical Sciences
through the
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by
Mark Winston Stalder
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Table of Contents

Acknowledgments........................................................................................................ii

Abstract.............................................................................................................................v

Introduction..........................................................................................................................1

Review of Literature............................................................................................................3
  Innate Immune Response and Neurological Disease.......................................................3
  Fr98-Mediated Neurological Disease...............................................................................5
  CCL2/MCP-1.....................................................................................................................6
  CCL7/MCP-3.....................................................................................................................8
  CCL12/MCP-5...................................................................................................................10
  pSFF/C17.2 Murine Neural Stem Cell Chemokine Expression........................................11

Rationale and Significance...............................................................................................14

Materials and Methods....................................................................................................16
  CCL-pSFF Construct..........................................................................................................16
  Cell Culture......................................................................................................................16
    Cell Maintenance............................................................................................................16
    Transfection..................................................................................................................17
    Transduction................................................................................................................18
  Immunofluorescent Staining/Flow Cytometry................................................................18
  Western Blot Analysis.......................................................................................................20
  Mice.................................................................................................................................22
    C17.2 Inoculation............................................................................................................22
    Tissue Collection..........................................................................................................23
  Histology..........................................................................................................................23
    In Situ Hybridization......................................................................................................23
    CD3 Immunohistochemistry..........................................................................................26
    β-Galactosidase Immunohistochemistry........................................................................27
    Post-In Situ Immunohistochemistry................................................................................28
  RNA Preparation............................................................................................................29
    Isolation from Cultured Cells........................................................................................29
    Isolation from Primary Tissue........................................................................................29
    DNAse Treatment...........................................................................................................30
    Reverse Transcriptase PCR........................................................................................31
  Real-Time PCR Analysis................................................................................................32
    Primer Design...............................................................................................................32
    Plate Set-Up and PCR Reaction....................................................................................33
  Statistical Analysis.........................................................................................................34

Results..............................................................................................................................35
  C17.2 In Vitro Chemokine Expression.............................................................................35
Abstract

Increased expression of cytokines and chemokines in the central nervous system (CNS) is closely associated with the development of retroviral-induced neurological diseases such as HIV-associated dementia, as well as other neuropathologies such as Alzheimer’s Disease and Multiple Sclerosis. The specific functions of many of these pro-inflammatory factors have yet to be elucidated in the disease process, and it is unclear whether the nature of their effects is protective, pathogenic, or both. Additionally, current models of chemokine function have inherent limitations, with direct injection resulting in a brief response that doesn’t accurately represent the effects of chronic production, and transgenic mice constitutively expressing chemokines by a large percentage of intrinsic brain cells from inception. This study takes the first step in developing a more representative in vivo system by which the direct of effects of the over-expression of individual CNS chemokines may be studied. C17.2 neural stem cells (NSC) were transduced with retroviral vectors containing the genes for CCL2, CCL7, CCL12, or the vector alone, and were inoculated into mice to generate a more accurate representation of the limited chemokine producing cell population that is seen with disease. The in vivo data generated herein suggests that this system is capable of consistently expressing comparable levels of these three chemokines, and characterizes the migration patterns of these cells into the major regions of the CNS. As no significant glial activation was seen concurrent with CCL over-expression, this study provides support for idea that the CCL chemokines may be involved in priming the CNS immune response, rather than its direct initiation. Additionally, ancillary histopathological data
suggests a possible role for CCL12 in the development of spongiform lesions in the presence of recombinant retrovirus infection.
Introduction

The up-regulation of central nervous system (CNS) cytokines and chemokines is closely associated with retrovirus-induced neurological diseases. For example, the development of HIV dementia, SIV encephalitis, and murine retrovirus-induced neurological disease correlate with increased expression of TNFα, CXCL10 (IP-10), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL2 (MCP-1), CCL7 (MCP-3), and CCL12 (MCP-5). However, the roles of these cytokines and chemokines in CNS disease have yet to be clearly elucidated. Studies involving the neurovirulent murine retrovirus Fr98 and chemokine receptor-deficient mice have suggested that CCL2, CCL7, and/or CCL12 may contribute to retroviral pathogenesis, but presently an in vivo system that accurately replicates the temporal, spatial, and quantitative expression of chemokines in the CNS during neurological disease does not exist. This project attempts to take the first step in developing a system useful for accurately studying the effects of a single over-expressed chemokine in the CNS, and to overcome the limitations of previous models that employed the use of transgenic mice and direct injection. Additionally, we attempt to characterize the effect of CCL2, CCL7, and CCL12 over-expression to determine the potential role of these genes in neuropathogenesis, and the activation of the immune response in the brain.

The actions of these chemokines in the CNS were investigated by over-expression of each gene of interest in the brain using C17.2 murine neural stem cells (NSCs) as delivery vectors. In short, these NSCs were transduced using a pSFF retroviral vector carrying the chemokine gene of interest, and then introduced into the CNS of neonatal IRW mice by way of intracranial inoculation via the lateral ventricles. The mice were
observed for any signs of clinical neurological disease until the brains were harvested at 7, 14, and 21 days post-inoculation. Histological analysis was performed on the tissues to assess NSC distribution, detection of inflammatory infiltration, activation of astrocytes and microglia, and the correlation of these pathologies with the localized expression of chemokines from the NSCs using in situ hybridization and immunohistochemical dual staining techniques. Molecular changes were quantified by real-time PCR mRNA analysis to confirm and characterize expression levels of CCL2, CCL7, and CCL12, and to check for up-regulation of astrocyte and microglia/macrophage activation markers.

As these chemokines have different effects on the peripheral immune response, we hypothesized that their individual over-expression in the CNS would have differing effects on the activation of microglia/macrophages and astrocytes, and the recruitment of inflammatory macrophages and T lymphocytes. The specific aims of this project are to characterize the use of retrovirally transduced C17.2 neural stem cells as an in vivo delivery vector for CNS chemokine study, and to examine the immunopathological response to over-expression of CCL2, CCL7, and CCL12.
Review of Literature

Innate Immune Response and Neurological Disease

Though the specific causes of numerous instances of neurological dysfunction have yet to be elucidated, extensive evidence has shown that clinical neurological disease is consistently correlated with an up-regulation of CNS pro-inflammatory cytokines and chemokines, as well as the activation of resident astrocytes and microglia/macrophages. These findings are evident in both viral and non-viral mediated pathologies such as Multiple Sclerosis (MS), Alzheimer’s Disease (AD), ischemia, Experimental Autoimmune Encephalitis (EAE), Simian Immunodeficiency Virus (SIV), MuLV-associated disease, and HIV-associated dementia (HAD) (Conant et al., 1998; Godiska et al., 1995; Karpus and Ransohoff, 1998; Luster, 1998; Orandle et al., 2002; Persidsky et al., 1999; Schmidtmayerova et al., 1996; Van, V et al., 1999). In AD, while inflammatory infiltration in the CNS has not been clearly demonstrated, activated astrocytes and microglia/macrophages are typically associated with plaques, as are increased levels of CCL2, CXCL10, and CCL4 (Akiyama et al., 2000; Ishizuka et al., 1997; Xia et al., 2000; Xia et al., 1998). These observations are also seen in MS, along with increased levels of CCL5 and CCL3 in chronic active lesions (Boven et al., 2000). The presence of CCL2 and CCL7 has also been described in acute and chronic active MS lesions, as well as in cerebral ischemia (Banisor et al., 2005; Chen et al., 2001; McManus et al., 1998; Simpson et al., 1998). In EAE, which is a model for MS, increased levels of CCL2, CXCL10, CCL5, and CCL7 have also been reported (Berman et al., 1996; Godiska et al., 1995). Studies with EAE have demonstrated that antibodies against CCL2
and CCL3 inhibit disease, which clearly indicates a role for cytokines and chemokines in neuropathogenesis (Karpus and Kennedy, 1997).

In instances of SIV, MuLV, and HIV infections of the CNS there tend to be conflicting reports as to the genesis of neurological dysfunction. While in some cases there is significant CNS inflammatory infiltration and neuronal apoptosis, these pathologies can not be consistently correlated to all incidences of neurological disease (Adamson et al., 1996; Glass et al., 1995; Kaul and Lipton, 1999; Marcondes et al., 2001; Persidsky et al., 1999; Portis et al., 1995). These viruses all infect similar cell types in the CNS, including microglia/macrophages, and are closely associated with astrocyte and microglia/macrophage activation (Adamson et al., 1996; Garden, 2002; Persidsky et al., 1999; Peterson et al., 2004; Portis et al., 1995). Likewise, in all cases, the host response to CNS infection includes significant cytokine and chemokine up-regulation, which would seem to indicate some involvement in the development of clinical disease. The observed increases in cytokine and chemokine levels are typically attributed to the activated astrocytes and microglia/macrophages (Garden, 2002; Orandle et al., 2002; Peterson et al., 2004). In SIV, encephalitic animals show elevated levels of TNFα, IL-1β, CXCL10, CCL2, CCL7 (Orandle et al., 2002; Sasseville et al., 1996; Sopper et al., 1996). In MuLV-infected mice there is significant up-regulation of TNFα, TNFβ, IL-1α, CCL2, CCL3, CCL4, CCL5, CCL7, CCL12, CXCL1, and CXCL10 (Peterson et al., 2001; Peterson et al., 2004). TNFα, TGFβ, IL-6, IL-1β, CXCL10, CCL2, CCL3, CCL4, and CCL5 are also expressed at high levels in the CNS of HAD patients (Cinque et al., 1998; Conant et al., 1998; Letendre et al., 1999; Perrella et al., 1992; Poluektova et al., 2001; Wahl et al., 1991; Wesselingh et al., 1993). These observations clearly suggest some
essential involvement of pro-inflammatory cytokines and chemokines in neurological disease, whether in a neuroprotective role, which has been suggested, or as mediators of pathogenesis.

**Fr98-Mediated Neurological Disease**

Neuropathogenesis induced by the murine retrovirus Fr98 correlates strongly with the increased production of pro-inflammatory cytokines and chemokines, providing a useful model to study the contribution of these immune factors to the disease process. In this model Fr98 infection of neonatal mice leads to severe clinical disease characterized by ataxia, seizures, and death, typically by days 14 to 16 post-infection, in nearly one hundred percent of cases. In contrast, CNS infection of a closely related murine retrovirus, Fr54, does not induce neurological disease (Peterson et al., 2001; Portis et al., 1995). The primary difference between Fr98 and Fr54 is in the envelope protein, indicating its important role in mediating pathogenesis. There are no differences, however, between the two viruses with regard to the types of cells infected, or the histopathology observed (Peterson et al., 2001). Both viruses primarily infect CNS microglia/macrophages and endothelial cells, and occasionally oligodendrocytes. The minimal pathology seen consists of astrogliosis and microgliosis, with some vacuolar and neuronal degeneration (Peterson et al., 2001; Portis et al., 1995; Robertson et al., 1997). Interestingly, similar levels of apoptosis gene expression are seen in both types of infection, and no inflammatory infiltrate is associated with either virus (Peterson et al., 2001). However, Fr98 infection differs from that of Fr54 with respect to increased viral load, and activation of astrocytes and microglia/macrophages. Fr98 infection also induces significant up-regulation of pro-inflammatory cytokines and chemokines in the
brain, including TNFα, TNFβ, IL-1α, CCL2, CCL3, CCL4, CCL5, CCL7, CCL12, MIP-2, and CXCL10, which is not observed in Fr54 infection (Peterson et al., 2001; Peterson et al., 2004). This demonstrates that the up-regulation is not due to generic host response to retroviral infection. It is important to note that the increased levels of cytokines and chemokines seen in Fr98 infection occur prior to the increases in viral load and onset of disease (Peterson et al., 2001). Additionally, decreased incidence of disease has been observed in CCL2 antibody blocking experiments using the Fr98 model (Peterson et al., 2004). In accordance with this observation, CCR2-/- mice infected with Fr98 have also shown a decreased incidence of disease, further suggesting the involvement of its ligands, CCL2, CCL7, and CCL12 in immune-mediated retroviral neuropathogenesis (Peterson et al., 2004).

**CCL2/MCP-1**

CCL2 is a member of the β-chemokine subfamily which is classified by four conserved cysteine residues, the first two of which are adjacent (Bacon et al., 2002; Leonard and Yoshimura, 1990; Yoshimura et al., 1989). By far the most well-characterized member of this subfamily, the actions of CCL2 are still not completely understood, as it seems to function in differing roles dependant upon the situation at hand. The main receptor for this protein is CCR2, but it is also known to bind CCR4, as well as the DARC and D6 chemokine receptors (Murphy, 2000; Peterson et al., 2004; Ransohoff and Tani, 1998). Upon stimulation by CCL2, CCR2 and CCR4, as is typical of G-protein-coupled receptors, cause a transient rise in cytosolic calcium levels (Bacon et al., 2002; Murphy, 2000; Ransohoff and Tani, 1998). CCL2 has been shown to stimulate and chemoattract monocytes, macrophages and microglia, as well as T cells,
mast cells, dendritic cells, basophils, and NK cells (Allavena et al., 1994; Carr et al., 1994; Eugenin and Berman, 2003; Fuentes et al., 1995; Gunn et al., 1997; Huang et al., 2002; Taub et al., 1995). It is also known to stimulate and direct the migration of astrocytes, and is a strong histamine-releasing factor for basophils (Andjelkovic et al., 2002; Bischoff et al., 1992). CCL2 can be expressed by fibroblasts and monocytes, and is induced in endothelial cells by IL-1β, IL-4, TNFα, and IFNγ (Rollins et al., 1990; Rollins and Pober, 1991). It is also produced by microglia and astrocytes, the main sources of CCL2 in the CNS, in response to stimulation by TNFα, IFNγ, IL-1β, TGFβ, CCL3, CCL4, and CCL5 (Hurwitz et al., 1995; Legoux et al., 1992; McManus et al., 2000; Peterson et al., 2004; Valente et al., 1998; Zhou et al., 1998). Concordantly, CCL2 also affects the production of other cytokines, enhancing the secretion of IL-4, IL-5, and IL-10, while antagonizing the secretion of IL-12, and TNFα (Chensue et al., 1996; Gu et al., 2000; Karpus et al., 1997; Matsukawa et al., 2000; Zisman et al., 1997).

Though its specific effects are not always obvious, the correlation between CCL2 and neurological disease has been well documented. It is a mediator of CNS inflammation in a number of conditions, including EAE, MS, Alzheimer’s, ischemia, and trauma (Berman et al., 1996; Che et al., 2001; Glabinski et al., 1996; Glabinski et al., 1995; Gourmala et al., 1997; Grzybicki et al., 1998; McManus et al., 1998; Sun et al., 2003; Van, V et al., 1999; Wang et al., 1995). However, transgenic mice with CNS-specific CCL2 expression showed little evidence of inflammation until stimulated with pertussis toxin, an observation that suggests that it may prime the CNS for infiltration, rather than directly recruiting inflammatory cells (Huang et al., 2002). With regard to virally induced CNS disease, HAD patients have demonstrated substantial increases in
CCL2 levels in brain tissue and cerebrospinal fluid, and polymorphism studies have indicated that a certain allelic variant may increase susceptibility to the disease (Conant et al., 1998; Gonzalez et al., 2002). However, while high CCL2 levels strongly correlate with HAD, it is not clear whether its effects are damaging or protective. HIV tat protein can induce the increased production of CCL2, and studies have shown that when added to astrocyte and neuronal cultures simultaneously with tat, CCL2 inhibited apoptosis. However, when added to the cultures after tat exposure it offered no protection (Conant et al., 1998; Eugenin et al., 2003; Gonzalez et al., 2002; Lim and Garzino-Demo, 2000).

Several knockout studies have shown that CCR2-/- mice have a reduced incidence of virally mediated clinical disease. This was observed with CNS infection of the Fr98 retrovirus, and also with mouse hepatitis virus (Chen et al., 2001; Peterson et al., 2004). Since CCR2 is the main receptor for CCL2 in the CNS, these results suggest a pathogenic role for this chemokine. Kinetic analysis of CCL2 expression during the course of Fr98 infection demonstrated an increase in production starting at day twelve post-infection, prior to the development of clinical signs, with continued expression persisting throughout the course of the disease (Peterson et al., 2004). Additionally, CCL2 antibodies administered to Fr98 infected mice were able to delay the onset of disease, demonstrating further its potential role in virally induced CNS dysfunction (Peterson et al., 2004).

**CCL7/MCP-3**

Originally identified from human osteosarcoma cells, the production of CCL7 in peripheral blood mononuclear cells (PBMC) has been shown to be induced by stimulation with IL-1β, IFNα, and IFNβ (Menten et al., 1999; Minty et al., 1993;
Opdenakker et al., 1993; Van et al., 1992). While its function in disease processes has not been well-defined, this member of the β-chemokine subfamily is an important mediator of inflammation (Schols et al., 1997). Numerous studies have shown that this protein is strongly chemotactic for monocytes and T lymphocytes, as well as eosinophils, basophils, NK cells, and dendritic cells (Allavena et al., 1994; Dahinden et al., 1994; Franci et al., 1995; Sozzani et al., 1994; Sozzani et al., 1995; Taub et al., 1995; Van et al., 1992). Stimulation of these cells by CCL7 results in a rapid and transient concentration-dependent increase in cytosolic free calcium, inhibition of adenylyl cyclase, as well as the release of histamine and leukotriene C4 (Dahinden et al., 1994; Franci et al., 1995). The effects of CCL7 on monocyte chemotaxis and basophil mediator release are comparable to CCL2, suggesting that the two chemokines utilize the same receptors and transduction pathways (Dahinden et al., 1994; Franci et al., 1995). Accordingly, this does appear to be the case, as CCL7 is now known to bind to CCR1, CCR2 (the main receptor for CCL2), and CCR3 (Bacon et al., 2002; Bajetto et al., 2002; Franci et al., 1995; Murphy, 2000).

With respect to disease, CCL7 has demonstrated anti-tumor properties, as well as anti-HIV activities against T-tropic viruses, most likely through competition with the virus for cellular co-receptor binding (Hu et al., 2002; Schols et al., 1997). In instances of neurological disease, its presence has been described in acute and chronic active MS lesions, as well as in cerebral ischemia and mouse hepatitis virus-mediated disease (Lane et al., 1998; McManus et al., 1998; Wang et al., 1999). Interestingly, there is also evidence that CCL7 production is up-regulated at time points prior to immune cell entry into inflammatory lesions in the CNS (Asensio and Campbell, 1997). This observation also holds true with respect to Fr98-induced disease, where the levels of CCL7
consistently rise several days before the appearance of clinical symptoms. However, these increased levels are maintained only for a few days, and levels return to normal around the time of the onset of disease (Peterson et al., 2004). While it is yet unclear which CNS cells are responsible for the up-regulation of CCL7 in the Fr98 model, or what its effect may be, the current data seems to suggest some role in the early phase of the innate immune response to the infection.

**CCL12/MCP-5**

Murine CCL12 is a member of the β-chemokine subfamily, and there is currently no known homologue in humans. Its roles in innate host defense and immune-mediated pathogenesis have not been well-characterized, but some general information has been elucidated. CCL12 is a product of activated macrophages and microglia, and its production can be induced by IFN\(\gamma\) and LPS exposure (Peterson et al., 2004; Sarafi et al., 1997). As with the other CCL chemokines, CCL12 is a mediator of inflammation. It is strongly chemotactic for monocytes, and has also been shown to stimulate eosinophil migration into the lungs (Gonzalo et al., 1998; Sarafi et al., 1997). It signals through CCR2, and produces a rapid and transient rise in cytosolic calcium in cells which express those receptors (Sarafi et al., 1997).

The only defined role for CCL12 in disease processes thus far is its involvement in recruiting the early mononuclear phase of the inflammatory response (Sarafi et al., 1997). This is evident in instances of inflammatory lesions in the CNS, where CCL12 is up-regulated at time points prior to leukocyte entry (McTigue et al., 1998). In a model of pulmonary nematode infection, CCL12 levels in the lungs were increased by day 7, peaked at day 10, and had returned to baseline by day 14. In this instance the point of
peak expression again preceded the peak recruitment of immune cells (Sarafi et al., 1997). In the case of Fr98-mediated CNS disease, activated microglia appear to be the likely source of the increased production of CCL12 (Peterson et al., 2004). Again, the kinetic observations remain consistent, with CCL12 levels peaking and returning to normal prior to the onset of disease. Interestingly, the increases in production becomes evident a day or so earlier than the increases seen with CCL2 or CCL7 (Peterson et al., 2004). This fact suggests the possibility of the involvement of CCL12 in the induction of the other two CCL chemokines, as well as a general role in the induction of immune-mediated neurological disease.

**pSFF/C17.2 Murine Neural Stem Cell Chemokine Expression**

The CNS chemokine over-expression in this project was accomplished using pSFF vector-transduced C17.2 murine neural stem cells. pSFF is a retroviral expression vector derived from the replication-defective spleen focus forming virus (SFFV). It has been shown to be capable of high-level expression in murine stem cell lines in vitro (Bestwick et al., 1988; Tumas et al., 1996). Following entry, the vector integrates stably into the host cell genome and is thereafter transferred to the progeny of the transduced cells (Tumas et al., 1996). In the construction of this vector the majority of the env gene has been removed, and the space created is used to insert the gene of interest (Figure 1A) (Tumas et al., 1996). To produce the retroviral particles carrying the gene of interest, vector DNA is introduced into a mixed culture of the retroviral packaging cell lines ψ2 and PA317, each of which expresses a different retroviral env gene (Figure 1B) (Bestwick et al., 1988). The ψ2 line releases ecotropic virions, while the PA317 line releases amphotropic virions (Mann et al., 1983; Miller and Buttimore, 1986). Each cell
Figure 1. pSFF vector (1A), and the pSFF/co-culture/C17.2 neural stem cell in vivo chemokine expression system (1B). The pSFF plasmid DNA containing the CCL insert is transfected into the $\psi^2$/PA317 retroviral packaging cell co-culture. The $\psi^2$ cells carry an ecotropic retroviral envelope gene, and are susceptible to amphotropic viruses. The PA317 cells carry an amphotropic retroviral envelope gene, and are susceptible to ecotropic viruses. The co-culture of the two packaging cell lines results in high titers of retroviral particles carrying the chemokine gene of interest. The co-culture supernatant is then collected and used to transduce the C17.2 neural stem cell line. Once positive gene expression is confirmed, the transduced C17.2 cells are inoculated into the CNS of neonatal mice via the lateral ventricles.
line is resistant to superinfection by the type of virus that it releases, but is susceptible to
infection by the virions produced by the other cell type (Bestwick et al., 1988). The
result of this co-culture mechanism is a “ping-pong” effect that produces rapid and
efficient amplification of replication-deficient pSFF retroviral virions capable of infecting
different host ranges. The high-titer supernatant containing the amplified vector can be
collected and stored for later use (Bestwick et al., 1988).

C17.2 murine neural stem cells (NSCs) are a well-characterized line of stable,
self-renewing cellular clones, originally derived from neonatal mouse cerebellum (Ryder
et al., 1990). These NSCs are immature, multipotent cells found in both developing and
adult nervous systems that are capable of differentiating into most neuronal and
neuroglial lineages, and have been successfully used for in vivo gene expression in a
number of investigations (Lynch et al., 1999; Snyder et al., 1992). In culture they are
easily expanded and genetically manipulated, and when infected with pSFF vectors,
readily acquire the gene of interest (Bestwick et al., 1988; Lynch et al., 1999). After
confirmation of proper gene expression, the NSCs can be introduced directly into the
CNS of neonatal mice. The C17.2 cells are migratory post-inoculation, so wide
dissemination of the gene product is achieved. Owing to the introduction of the lacZ
reporter gene in the C17.2 line, β-galactosidase is stably expressed and in vivo
localization can be conveniently confirmed through immunohistochemistry (Snyder et al.,
Rationale and Significance

The up-regulation of cytokines and chemokines in the CNS consistently correlates with the incidence of neurological disease, as does the activation of astrocytes and microglia. The chemokine CCL2 has been extensively studied in retrovirus-induced neurological disease including HIV-associated dementia, and while evidence strongly suggests it may have a role in mediating pathogenesis, that potential mechanism is not known. CCL7 and CCL12 up-regulation is also associated with neurological disease, but the functions of these chemokines have not been thoroughly examined, and it is not known what role, if any, they may play in retroviral pathogenesis. However, the kinetics of their expression in Fr98-induced disease suggests that they are in some way involved in the disease process.

Previous studies using transgenic mice to express CCL2 in the CNS do not accurately replicate chemokine expression in retrovirus-induced disease. In transgenic mice, large populations of cells constitutively over-express CCL2 from inception, which could result in tolerance and/or the development of compensatory pathways. In contrast, the proposed model presents a novel approach for investigating the questions at hand. With the use of C17.2 NSCs as an in vivo chemokine delivery system, more representative levels and spatial dissemination of CNS chemokine expression can be achieved. Thus, the major objectives of this project are to characterize the validity of the C17.2 NSC gene expression system as an accurate in vivo model for chemokine expression during neurological disease, and to determine whether or not increased levels of CCL2, CCL7, or CCL12 in the CNS are involved in the activation of astrocytes and microglia, or the general induction of neurological disease. Since CCL7 and CCL12
have not been studied extensively, this undertaking will help to further characterize the functions of these specific chemokines.
Materials and Methods

CCL-pSFF Construct

The CCL2, CCL7, and CCL12 genes were cloned and ligated into the pSFF retroviral plasmid by John Errett at Rocky Mountain Laboratories, NIAID, NIH. Briefly, RNA isolates from the CNS of Fr98-infected mice were collected upon onset of clinical disease. Samples were DNAse-treated and underwent reverse-transcriptase PCR to generate cDNA templates. CCL2, CCL7, and CCL12 cDNA was then amplified for cloning into the pSFF plasmid through PCR using the following primers:

CCL2-34f (with NheI site) TCATGCTAGCAGCACCAGCACCAGCCAAC
CCL2-539r (with ClaI site) TCACATCGATAGGCATCACAGTCCGAGTC
CCL7-21f (with NheI site) CAAGCTAGCTGAAGCCAGCTCTCTCACTC
CCL7-348r (with ClaI site) GCCATCGATGGTTTCTGTTCAGGCACATTTC
CCL12-1f (with NheI site) TCGTGCTAGCTTCGAAGTCTTTGACCTCAAC
CCL12-366r (with ClaI site) TCACATCGATAATATCACACTGCCCGTGG

Amplicons were purified in Qiagen columns, and then ligated into TOPO pcrII. The TOPO construct was then cut with EcoRI at either site of the insert and ligated into pSFF plasmid also cut with EcoRI. Proper directional insertion was verified by restriction digest and sequencing.

Cell Culture

Cell Maintenance. C17.2 murine neural stem cells were maintained in six well primaria culture plates (Falcon, non-pyrogenic, surface modified polystyrene) in growth media (DMEM high glucose with 1.5 g/L NaCO3, N2 Pyruvate, and 10% fetal bovine serum (Hyclone characterized FBS)). Cells were passed at a 1:10 ratio upon producing a
confluent monolayer on the bottom of the well. 1 ml of 0.25% trypsin was used to detach cells in each well, and the proper dilution was then added to 3 ml fresh growth media. The PA317 and ψ2 retroviral packaging cell lines were maintained and passed under identical conditions and procedures, with the exceptions that the growth media used for these cells was RPMI+NaHCO3 with 10% FBS, and the cells were grown in non-primaria six well plates (Greiner Bio-one, Cellstar, non-pyrogenic, TC-plate). All cell lines were grown in an incubator at 37º Celsius with 5% CO2.

**Transfection.** To produce the pSFF retroviral vectors containing the genes of interest, co-cultures of PA317 and ψ2 cell lines were first established by plating aliquots of 2.5 x 10^5 of each line to the same well. After 24 hours the cells were removed from incubation and transfected using Lipofectamine (Invitrogen) and the accompanying protocol. Briefly, plasmid DNA was diluted in serum-free cell culture media, as was Lipofectamine reagent. After a 5 minute incubation the diluted DNA and the diluted reagent were combined so that a DNA/Lipofectamine ratio of 1µg/3µl was achieved. After gently mixing, the solution was allowed to incubate for 20 minutes at room temperature (RT). Aliquots of the transfection mixture were then added to each of the wells containing the C17.2 cells with growth media. The plates were mixed by gentle rocking and then placed back in the incubator for a minimum of 24 hours before testing for transgene expression. To ensure successful transfection, the co-cultured cells were immunofluorescently stained using the protocol listed below, and examined under a fluorescent microscope for positive results. The antibody used was the monoclonal 34, which is an anti-retroviral gag antibody (Chesebro et al., 1981). Once positive results were obtained, the co-cultures were grown up in 175 cm² flasks (Greiner Bio-one,
Cellstar, non-pyrogenic, filter cap) with 35 ml of RPMI growth media in order to collect and store aliquots of the vectors. When cells were at roughly 75% confluency the growth media was removed and a fresh 10 ml supply was added, and the cells were allowed to grow for another 24 hrs. At that time the growth media was then collected and centrifuged for five minutes at 1500 RPM in order to remove any cellular debris. The supernatant was then divided into individual aliquots of 1 ml apiece and stored at -80º Celsius.

**Transduction.** C17.2 cells were transduced using the previously collected pSFF retroviral vectors virions containing the genes of interest (CCL2, CCL7, or CCL12), or with the empty vector itself as a control. First, 2.5 x 10^5 cells were plated out in multiple wells of a six well primaria plate. After 24 hours the cells were removed from the incubator and the growth media was removed. 1 ml of DEAE-dextran/DMEM growth media at a concentration of 8 µg/ml was added to each well of the plate, and allowed to incubate at 37º C for 45 minutes. The DEAE-dextran/DMEM growth media mixture was then removed, and an aliquot of pSFF vector virions was thawed, added to each well, and allowed to incubate at 37º C for 1.5 hrs. After that time fresh DMEM growth media was added, and the cells were maintained as previously described.

**Immunofluorescent Staining/Flow Cytometry**

For the purposes of flow cytometry all variations of the C17.2 cell line (including all transduced lines) were grown up in 175 cm² flasks (Greiner Bio-one, Cellstar, non-pyrogenic, filter cap) with 35 ml of DMEM growth media. Initially split at a 1:3 ratio, the cells were allowed to grow to confluency for 48 hrs before being harvested. To collect the cells, the growth media was removed and 2 ml of 0.25% trypsin was used to
wash the monolayer. 10 ml of 0.25% trypsin was then added to detach the cells into solution. The trypsin/cell mixture was then added to 15 ml of DMEM growth media in order to deactivate the trypsin. This mixture was then centrifuged for 5 minutes at 1500 RPM, and the supernatant discarded. The remaining pellet was dislodged, mixed, and centrifuged in 10 ml of 1X Phosphate-Buffered Saline (PBS) in order to wash away any remaining growth media. The supernatant was discarded, and the cells were brought up in 1 ml of 1X PBS and distributed equally into individual wells of a 96-well round bottom plate (Corning, costar, non-pyrogenic polystyrene) for staining. The plate was centrifuged as before to form a pellet of cells on the bottom of each well, and the PBS was discarded. Each well of cells was then brought up in 30 µl of FBS and allowed to incubate at room temperature (RT) for 10 minutes to establish protein/protein interactions and minimize cell loss during the staining process. After centrifugation and removal of the FBS, the cells were washed with 100 µl of 1X PBS, centrifuged as before, and allowed to incubate for 10 minutes at RT in 100 µl of 3.7% neutral buffered formalin for fixation. Each well was washed twice with 1X PBS as before, brought up in 0.1% saponin/1X PBS , and incubated for 20 minutes at RT in order to perforate the cell membrane. Following centrifugation and removal of the saponin, 50 µl aliquots of each primary antibody diluted in saponin (with the exception of the monoclonal antibodies which were diluted in culture media) were added to the appropriate wells and allowed to incubate for 1 hour at RT. The antibodies used included polyclonal rabbit anti-murine JE/CCL2 (ProSci, Inc.) at 0.67 µg/ml, polyclonal rabbit anti-mouse MCP-5/CCL12 (Leinco Technologies, Inc.) at 1 µg/ml, polyclonal goat anti-murine MCP-3/CCL7 (ProSci, Inc.) at 0.67 µg/ml, and the monoclonal mouse anti-FB29 gag antibody 34
After incubation, the wells were washed twice as before with 100 µl of saponin. 50 µl dilutions of the appropriate secondary antibodies were then added to each well and allowed to incubate at RT for 45 minutes. The secondary antibodies used include FITC conjugated goat anti-rabbit IgG (Biomeda) at a concentration of 10 µg/ml, FITC conjugated goat anti-mouse IgG (Zymed) at a concentration of 15 µg/ml, and FITC conjugated rabbit anti-goat IgG (Zymed) at a concentration of 15 µg/ml. After incubation with the secondary antibody, the cells were washed once as before with 100 µl of saponin, once with 1X PBS, and then were diluted in 400 µl of 1X PBS, transferred to 5 ml round-bottom tubes (Falcon, polystyrene, non-pyrogenic), covered with aluminum foil, and stored at 4º C until the time of use.

**Western Blot Analysis**

Western Blot protein analysis was performed on DMEM growth media supernatant from all of the C17.2 cell lines in order to confirm secretion of the chemokines. Cells were passed as usual at a 1:10 ratio into individual wells of a 6-well primaria culture plate containing 3 ml of DMEM growth media. After 48 hours the media supernatant was collected and stored at -20º C until time of use. After thawing samples, each was diluted 1:5 in 0.5% NP40/1X PBS. This mixture was then diluted 1:1 with Tricine Sample Buffer (Bio-Rad) containing β-mercaptoethanol as per product guidelines. Next, sample mixtures were vortexed and boiled for 5 minutes at 100º C in order to denature the native conformation of the proteins. 12.5 µl of each sample, as well as 10 µl of Polypeptide Standard (Bio-Rad), were then added to individual wells in a 26-well 16.5% Tris-Tricine pre-cast gel (Bio-Rad) immersed in 1X Tris/Tricine/SDS...
running buffer (Bio-Rad). The gel was run at 100 V for 100 minutes as per product guidelines. Following this step the gel was allowed to equilibrate for 15 minutes in chilled 1X Towbin buffer/20% methanol (transfer buffer). Fiber pads and filter paper were soaked in chilled transfer buffer in preparation for setting up the transfer cassette. A piece of immuno-blot PVDF membrane (Bio-Rad) was immersed in 100% methanol, then transfer buffer, and carefully placed over the gel in the cassette. After being placed on a stir plate and filled with chilled transfer buffer, an ice block was added to the transfer apparatus and the assembled cassette was properly inserted. The transfer was then run at 100 V for 60 minutes. The membrane was then removed and incubated on a rocker for 1 hour at RT in 5% milk/1X TBS 0.05% Tween in order to block the blot for protein/protein interactions. After blocking, the blot was placed in 20 ml of diluted primary antibody and allowed to incubate overnight at 4°C on a rocker. Primary antibodies were diluted in 5% milk/1X TBS 0.05% Tween and included polyclonal rabbit anti-mouse JE/CCL2 (Prosci, Inc.) at 0.2 µg/ml, polyclonal rabbit anti-mouse MCP-5 (Leinco Technologies, Inc.) at 0.2 µg/ml, and polyclonal goat-mouse MCP-3/CCL7 (Prosci, Inc.) at 0.2 µg/ml. Next, the blot was washed three times on a rocker in 50 ml of 1X TBS 0.05% Tween for 10 minutes at RT. The appropriate secondary antibody, having been diluted in 50 ml of 5% milk/1X TBS 0.05% Tween, was then added to the blot and allowed to incubate on a rocker for 1 hour at RT. Secondary antibodies used included Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG (Zymed) at a concentration of 0.03 µg/ml, and HRP-conjugated rabbit anti-goat IgG (Zymed) at a concentration of 0.03 µg/ml. Following secondary incubation, the blot was washed six times on a rocker in 50 ml of 1X TBS 0.05% Tween for 10 minutes at RT in order to
remove any excess antibody. To visualize the protein of interest, the blot was then incubated for five minutes at RT in 10 ml of a 1:1 mixture of Femto West SuperSignal Luminol Enhancer and Peroxidase buffer (Pierce). Excess detection buffer was allowed to drip off, and the blot was then wrapped in cellophane, using a roller to carefully remove all remaining enhancer/buffer. In a dark room the blot was placed in a cartridge with a sheet of film (CL-X Posure Film, Pierce) for 30-60 seconds. The film was then run through a processor and allowed to develop.

Mice

C17.2 Inoculation. The mouse strain used in this study was Inbred Rocky Mountain White (IRW). Within 48 hours after the birth of each litter the NSCs were harvested by the method hereafter described. Each of the different lines of C17.2 NSCs (untreated, empty pSFF-transduced, and CCL2, CCL7, or CCL12-transduced) were grown up in 175 cm² flasks (Greiner Bio-one, Cellstar, non-pyrogenic, filter cap) with 35 ml of DMEM growth media to approximately 95% confluency prior to inoculation. To collect the cells the growth media was removed and 2 ml of 0.25% trypsin was used to wash the monolayer. 10 ml of 0.25% trypsin was then added to detach the cells into solution. The trypsin/cell mixture was then added to 15 ml of DMEM growth media in order to deactivate the trypsin. This mixture was centrifuged for 5 minutes at 1500 RPM, and the supernatant then discarded. The remaining pellet was then dislodged, mixed, and centrifuged in 10 ml of 1X PBS in order to wash away any remaining growth media. The supernatant was again discarded, and the cells brought up in 1 ml of 1X PBS and placed on ice. In order to obtain the appropriate final concentration of 1.25 x 10^7 cells/ml the cells were counted using a hemocytometer, and diluted in 1X PBS. The final suspension
contained 0.001% Trypan Blue to assist in visualization during the inoculation. The resulting suspension was then kept on ice until inoculation, however for no more than 1 hour. After proper preparation of the NSCs, the neonates, too young for gaseous anesthetization, were placed in a deep hypothermic state in advance of inoculation. Using a microsyringe, a total of 8 µl (10^5 cells at the prepared concentration) was directly injected into the lateral cerebral ventricles (4µl in each side). The neonates were allowed to come back to temperature, and were placed back with the dam. For each experimental and control group there were 6 mice used per time-point.

**Tissue Collection.** Tissue samples were collected at 7, 14, and 21 days post-inoculation. At the time of collection, all animals were placed under deep anesthesia by way of IsoFlorane inhalation. Once anesthetized, the animals were exsanguinated through axial incision, and then terminated by cervical displacement in accordance with approved protocols. The entirety of the central nervous system tissue from the olfactory bulbs to the brain stem was then carefully removed and sliced into two sagittal halves. One half of the tissue was placed in 3.7% neutral buffered formalin for fixation and histological analysis, and the other half was flash frozen in liquid nitrogen and stored at -80º C for later molecular analysis.

**Histology**

**In Situ Hybridization.** All initial steps in this protocol were performed under RNAse-free conditions unless otherwise noted. Anti-sense DIG-labeled RNA probes were generated prior to this study. In brief, whole RNA was isolated from the CNS of Fr98-infected IRW mice. Following reverse-transcriptase PCR, the appropriate CCL sequences were amplified and ligated into a T7 plasmid. Using a DIG RNA labeling kit
the DNA plasmids were cut with the appropriate restriction enzyme to give a 5’ overhang. Restriction enzymes were then removed via phenol/chloroform extraction. The cleaned and cut plasmid DNA was then incubated for 2 hours at 37º C with T7 polymerase in order to generate the anti-sense probe. The remaining DNA was then removed by incubation with DNasel incubation for 15 minutes at 37º C, and the reaction stopped by the addition of 0.2M EDTA. The DIG-labeled probes were then purified using Qiagen RNA cleanup columns, eluted using RNase-free water, and stored at -20º C until further use. For the hybridization reaction, tissue samples were first baked overnight at 56º C to help fix the paraffin-embedded tissue sections to the slides. Next, the slides were placed in a glass Coplin jar and incubated overnight on a rocker in Xylenes to de-wax the sections. The tissue was then rehydrated through successive ethanol immersions (100%, 95%, and then 70%) for 5 minutes each on a rocker. The sections were then incubated for 20 minutes at RT in 4% paraformaldehyde to fix the tissue. After three rinses with 1X Tris-Buffered Saline (TBS) the slides were incubated in 0.2 M HCl for 10 minutes to denature tissue proteins, and then again rinsed three times with 1X TBS. In order to further denature the tissue proteins, the samples were incubated for 20 minutes at 37º C each in 100 µl of 10µg/ml Proteinase K (Invitrogen)/1X TBS/0.2 M CaCl2. After again washing with 1X TBS as before, the samples were then incubated in 1X TBS for 10 minutes at 4º C to deactivate any remaining Proteinase K activity. To prepare the tissue for immersion in organic solvent, the sections underwent dehydration through successive ethanol (70%, 95%, and 100%) on a rocker for 5 minutes each. After incubation on a rocker in chloroform for 20 minutes at RT, the samples were rehydrated in ethanol as before, then incubated in 2X SSC for 10 minutes. To prevent
any random nucleic acid interactions between the specific RNA DIG-labeled probe and the sample, each section was then incubated with 40 µl of Hybridization Buffer (2X SSC, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.02% SDS, 50% formamide), covered with a parafilm coverslip, and placed at 56º C for 1 hour in a humidity chamber containing 1X SSC/50% formamide. RNA probes were then diluted 1:100 from the stock solution into Hybridization Buffer. Excess buffer was drained from the slides and 20 µl of diluted probe was added to each tissue section. The slides were covered with a Hybridization coverslip (Grace Bio-Labs), placed on a heat block at 95º C to help remove any air bubbles, then incubated overnight at 56º C in the humidity chamber. The second day of the in situ protocol was performed under non-RNAse-free conditions. The slides were removed from the humidity chamber and placed in a plastic Coplin jar with 2X SSC for 20 minutes where the Hybridization coverslips were allowed to float off. In order to destroy any unbound and excess probe the slides were then incubated for 30 minutes at RT with 100 µl of RNAse A (Roche) at 20µg/ml in 0.5 M NaCl/0.1 M Tris pH 8.0 per section. Next the slides were rinsed with 2X SSC and then incubated twice for 30 minutes each in 50% formamide/1X SSC at 56º C. Following this, the slides were rinsed with 1X SSC, and then allowed to incubate for 15 minutes at RT in 1X SSC. To block non-specific interactions with the antibody the samples were incubated for 20 minutes at RT in 1X Blocking Buffer (diluted in 1X Maleic Acid solution, Roche Biochemicals) with a parafilm coverslip. To detect the DIG-labeled RNA probes the slides were incubated with 100 µl per section of an alkaline phosphatase anti-DIG antibody (Roche Biochemicals) diluted 1:500 in 1X Blocking Buffer for 1 hour at room temperature. After allowing the antibody to bind to the labeled-probe, the slides were rinsed once in
1X Blocking Buffer, and then three times in 1X TBS on a rocker for 15 minutes each. After allowing the slides to incubate in detection buffer (0.1 M Tris-HCl pH 8.2) for 10 minutes at RT they were allowed to develop in a filtered Fast Red (Roche Biochemicals) chromagen solution using a gasket coverslip for up to one week.

**CD3 Immunohistochemistry.** To detect the presence of CD3 positive lymphocytes in the samples that were collected, the tissues were processed, embedded in paraffin, cut and fixed to slides. In order to better adhere the sections to the slides they were all baked overnight at 56º C. To de-wax the slides they were incubated in Xylenes twice for 15 minutes each at RT. The sections were then rehydrated through successive ethanol incubations (100%, 95%, and 70% for 5 minutes each), then rinsed in 1X PBS. To unmask the antigen of interest for detection the slides were placed in a glass Coplin jar filled with Citrate Target Retrieval Solution (Dako), set in a bowl half-filled with water, covered with cellophane, heated in a microwave for 20 minutes at 50% power, and then again for 5 minutes at 50% power. After being allowed to cool for 20 minutes, the slides were placed in a plastic Coplin jar and incubated in 1% hydrogen peroxide/1X PBS for 20 minutes in order to deactivate any indigenous peroxidases that might cause background staining. In order to block any random protein/protein interactions involving the primary antibody the slides were then incubated in a humidity chamber with 175 µl of 1% normal goat serum/1X PBS with a parafilm coverslip at 37º C for 30 minutes. The polyclonal rabbit anti-human CD3 primary antibody (DakoCytomation) was diluted to a working concentration of 6 µg/ml in 1% normal goat serum/1X PBS. After draining the excess serum from the slides, 175 µl of the working antibody solution was added to each slide, covered with a parafilm coverslip, and then incubated in a humidity chamber for 1
hour at 37º C. Following incubation with the primary antibody the slides were washed twice with 1X PBS on a rocker at RT. After the HRP-conjugated goat anti-rabbit IgG (Zymed) secondary antibody was diluted to a working concentration of 5 µg/ml, 175 ul of the solution was added to each slide, which were then covered with a parafilm coverslip and incubated in a humidity chamber for 1 hour at 37º C. Slides were washed of excess antibody in 1X PBS as before. Metal-enhanced DAB (Pierce) was diluted 1:10 in stable peroxide substrate buffer (Pierce), vortexed, filtered through a 0.2 µM filter, and added to each slide, via a gasket coverslip, to allow any positively bound antigen/HRP-antibody to develop. Slides were then washed with ultra pure water, counterstained with hematoxylin, and mounted with glass coverslips using Aquamount (Lerner Laboratories).

**β-Galactosidase Immunohistochemistry.** For purposes of *in vivo* detection of pSFF C17.2 cells, immunohistochemistry was performed using an antibody specific for β-galactosidase, the lacZ reporter gene product. The protocol for detection of this antigen begins in an identical fashion to the CD3 protocol up through the ethanol incubations. At this point the slides were washed twice in 1X PBS/0.05% Tween for 5 minutes each. The slides were then placed in a plastic TissueTek container filled with 250 ml citrate retrieval buffer (4.5 ml of 0.1 M Citric Acid, 20.5 ml of 0.1 M NaCitrate dehydrate, 225 ml ultra pure water, brought to pH 6.0). The filled container holding the slides was then placed in a BioCare Decloaking chamber filled with 500 ml of ultra pure water and run for 20 minutes at a temperature setting of 120º C. After washing with 1X PBS-Tween twice for 10 minutes each on a rocker the slides were blocked in 175 µl of 3% normal goat serum/1X PBS-Tween with a parafilm coverslip in a humidity chamber for 30 minutes at RT. The rabbit anti-β-galactosidase primary antibody (Cappel) was diluted to
a working concentration of 30 µg/ml in 1% normal goat serum/1X PBS-Tween, added to each slide in a 175 µl volume, covered with a parafilm coverslip, and allowed to incubate for 30 minutes at RT in a humidity chamber. Slides were washed twice as before with 1X PBS-Tween and then incubated in 0.3% hydrogen peroxide/1X PBS-Tween to inactivate any indigenous peroxidases. The secondary antibody used was the same as for the CD3 protocol and was diluted to the same concentration (though in 1% normal goat serum/1X PBS-Tween) and was applied to each slide with a 175 µl volume, covered with a parafilm coverslip, and allowed to incubate for 30 minutes at RT in a humidity chamber. Following two washes in 1X PBS-Tween as before, the slides were allowed to develop in a 1:10 dilution of DAB/stable peroxide substrate buffer using gasket coverslips, washed with ultra pure water, counterstained with hematoxylin and mounted with a glass coverslip.

**Post-In Situ Immunohistochemistry.** In order to visualize the proximal effect of chemokine over-production in the CNS on glial activation, immunohistochemistry was performed on slides that had previously undergone the *in situ* protocol. The protocol for these samples was identical to that used for CD3 detection, with the exception that all the initial steps from Xylenes incubation through hydrogen peroxide incubation were skipped. The protocol began with the blocking step (again using 1% normal goat serum/1X PBS) and continued through development in DAB, counterstaining, and mounting. The primary antibodies used were rabbit anti-mouse Iba1 (Wako) at a concentration of 1 µg/ml for detection of microglia/macrophage activation and infiltration, and rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (DakoCytomation) at a concentration of 2.4 µg/ml for detection of astrocyte activation. The HRP-conjugated
goat anti-rabbit IgG secondary antibody was used for both of the aforementioned primaries at a concentration of 5 µg/ml.

**RNA Preparation**

**Isolation from Cultured Cells.** In order to establish the gene expression profiles of the C17.2 NSC lines, whole RNA samples were isolated from each cell line for use in real-time PCR. Cells were maintained as previously described in 6-well primaria culture plates with DMEM growth media. 48 hours after passing the cells at a 1:10 ratio two confluent wells of each line were collected by trypsinization, added to 5 ml of fresh growth media, and centrifuged at 1500 RPM for 5 minutes. The resulting pellets were then washed with 5 ml of 1X PBS and again centrifuged. Once the pellets were collected and washed, the RNA was then isolated using the materials and protocol provided with the Zymo Mini RNA Isolation II kit. First, the pelleted cells were homogenized by vortexing with 600 µl of ZR RNA Buffer. The resulting solution was transferred to a Zymo-Spin III Column which was placed in a 2 ml collection tube and then centrifuged at 14,000 RPM for 1 minute. This was followed by two successive washes and centrifugation with 350 µl of RNA Wash Buffer. The column was then transferred to a 1.5 ml microcentrifuge tube, 50 µl of RNAsae-free water was added, and the samples were again centrifuged as before to elute the RNA from the column. The samples were then placed at -80º C for storage until time of use.

**Isolation from Primary Tissue.** Whole RNA was isolated from the primary tissue extracted from the mice in order to establish *in vivo* gene expression profiles via real-time PCR analysis. For this process TRIzol Reagent (Invitrogen), and the accompanying protocol, was employed. The tissue was removed from the -80º C freezer and kept in a
bench-top cooler to prevent thawing until use. The first step was the homogenization of
the samples in 2 ml of TRIzol Reagent using a glass homogenizer. Each sample was then
separated into two tubes of 1 ml apiece to which 200 µl of chloroform was added and
shaken vigorously for 15 seconds. After allowing samples to incubate at RT for 3
minutes they were centrifuged at 10,000 RPM for 15 minutes at 4º C to achieve a phase
separation. The aqueous phase was then transferred to a new tube and 600 µl of
isopropanol was added to achieve precipitation of the RNA. Samples were allowed to
incubate at RT for at least 10 minutes, and then were centrifuged for 10 minutes at 10,000
RPM at 4º C to form a gel-like pellet of RNA. The supernatant was discarded, 1 ml of
70% ethanol was added, and the samples were vortexed in order to wash the pellet. After
centrifugation at 6000 RPM for 5 minutes at 4º C the ethanol supernatant was discarded,
and the pellet was allowed to dry for 10 minutes. To dissolve the RNA, 50 µl of RNAse-
free water was added to each sample, which were then placed on a heat block at 55º C for
10 minutes, and then vortexed. All samples were then stored at -80º C until time of use.

**DNAse Treatment.** In order to prepare the isolated RNA for real-time PCR, all cellular
and primary tissue isolates were treated with DNAse to remove any residual genomic
DNA. First, the samples were removed from the freezer and thawed. For each sample of
primary RNA 5 µl was placed in a 1.5 ml microcentrifuge tube, while 10 µl volumes
were used for the cellular samples. 10 µl of DNAse buffer (Ambion) was then added to
each sample, followed by 15 µl of DNAse I (Ambion). RNAse-free water was then
added to each sample to achieve a final volume of 100 µl. After gently flicking the tubes
to mix the solutions, the samples were allowed to incubate for 30 minutes at 37º C.
Following incubation, the samples were purified using the Zymo RNA Clean-Up Kit
materials and protocol. First, 400 µl of RNA-Binding Buffer was added to each sample and mixed well via pipette action. Each sample mixture was then transferred to a Zymo-Spin III Column, placed in a 2 ml collection tube, and centrifuged at 14,000 RPM for 1 minute. The waste in the collection tubes was discarded, the samples were washed twice with 350 µl of Wash Buffer, and each time centrifuged for 1 minute at 14,000 RPM. Columns containing the samples were then transferred to a fresh 1.5 ml microcentrifuge tube. 50 µl of RNAse-free water was added to each column and centrifuged at 14,000 RPM for 1 minute to elute the purified RNA. DNAse-treated RNA samples were placed at -80º C for storage until time of use.

**Reverse Transcriptase PCR.** Prior to use in real-time PCR analysis, DNAse-treated RNA samples were first subjected to reverse-transcriptase PCR in order to generate cDNA templates. Using the Bio-Rad iScript cDNA Synthesis kit a master mix was generated which contained for each sample 10 µl RNAse-free water, 4 µl 5X iScript Reaction Mix, and 1 µl iScript Reverse Transcriptase. 15 µl volumes of the master mix were then aliquotted into 48 wells of a 96-well plate (Fisher Scientific, DNAse, RNAse, and DNA-free)), to each of which was added 5 µl of each DNAse-treated RNA sample. The plate was sealed with a pierceable foil sealing strip, vortexed, and centrifuged at 1500 RPM for 5 minutes. The plate was then run on a thermal cycler (MJ Research PTC 200) under the following conditions: 25º C for 5 minutes, 42º C for 30 minutes, 85º C for 5 minutes, and then 20º C indefinitely (to maintain the integrity of the cDNA product). After the reaction was finished the plate was centrifuged as before, each sample was diluted in 80 µl of RNAse-free water in individual 0.5 ml tubes, vortexed, centrifuged, and then placed at -20º C for storage until time of use.
Real-Time PCR Analysis

Primer Design. mRNA reference sequences for each of the genes being examined were obtained at NCBI Gene. Primers were designed using the Primer3 website, and were blasted for specificity using the NCBI nucleotide blast non-redundant database. Each primer set was designed to be between 18 and 27 bases, with a G/C content between 20 and 80%, a Tm of 60º C, and with the desired amplicon being between 100 and 180 base pairs. The sequences of the primers used for each gene include:

- **Actb-29f**: CAGCTTCTTTGCAGCTCCTT
- **Actb-185r**: CACGATGGAGGGGAATACAG
- **AMuLVenv-f**: AGGCCTTAACCTCAACCAAT
- **AMuLVenv-r**: GGAAGTGGGCCGTACAGTTG
- **EnvMlvi4-1258f**: GCATGTTCATGATGTGAGG
- **EnvMlvi4-1442r**: CAAGAGTTGGGGTCAGAGG
- **F480.2-1321f**: ACAAGTGCTCTCCCTCGTGCT
- **F480.2-1430r**: AACATGGTCTTTCCACAGTC
- **FB29gag2-613f**: CCCGTGGCGGATTCTACT
- **FB29gag2-740r**: TCGGAGAAAGAGGGTTGTA
- **Gapdh2-152f**: AACGACCCCTTCATTGAC
- **Gapdh2-342r**: TCCACGACATACTCAGAC
- **Gfap-16f**: CGTTTCTCCTTGTCTGAATGAC
- **Gfap-112r**: TCGCCCGTGTCTCCTTGA
- **MCP1.2-178f**: CCCACTCACCTGCTGCTACT
- **MCP1.2-341r**: TCTGGACCCATTCCTTCTTG
Plate Set-Up and PCR Reaction. To make plate set-up more efficient, cDNA samples were first thawed, then dispensed 5 µl at a time into each well of 96-well plates (Fisher Scientific, DNAse, RNAse, DNA-free), sealed with optical plate-sealing tape (Bio-Rad), and stored at -20º C. At the time of use each plate was removed, thawed, centrifuged at 1500 RPM for 5 minutes, and then set in a 96-well plate bench-top cooling block while the reaction mix was prepared. The master mix used for these reactions was prepared in a UV sterilized PCR hood and contained the following reagent volumes per sample: 9 µl RNAse-free water, 17.5 µl 2X iTaq SYBR Green Supermix with Rox (Bio-Rad), 1.75 µl forward primer (at a 10µM concentration), and 1.75 µl reverse primer (at a 10µM concentration). The master mix was vortexed and added in volumes of 30 µl to each sample in the pre-prepared 96-well plate. After sealing the plate, it was vortexed and then centrifuged at 1500 RPM for 5 minutes. The reaction mixture was then transferred to a 384-well plate (Applied Biosystems) in triplicate, giving a final reaction volume of 10 µl per well. This plate was sealed with optical plate sealing tape (Applied Biosystems), centrifuged as before, placed in the ABI 7900, and was subjected to the following reaction conditions: 50º C for 2 minutes, 95º C for 3 minutes, then 40 cycles of 95º C for 15 seconds and 60º C for 1 minute, followed by a melting curve reaction of 95º C for 15 seconds, 60º C for 15 seconds, and then 95º C for 15 seconds.
Statistical Analysis

All statistical calculations were performed using Prism Graph Pad Software. 2-way ANOVA with Bonferroni post-test analyses were calculated with significance set at P<0.05.
Results

C17.2 In Vitro Chemokine Expression

C17.2 Transgenic mRNA Expression. To generate chemokine C17.2 NSCs expressing the proteins of interest, the cells were incubated in culture with pSFF vector virions containing genes for CCL2, CCL7, or CCL12, or the empty vector as a control. Positive transduction was confirmed by microscopic examination of cells fluorescently stained for CCL or retroviral gag protein expression. Following confirmation of transgene expression, six separate RNA isolates were collected for each of the C17.2 lines for analysis via quantitative real-time PCR. This technique allows for a quantitative comparison of cellular mRNA expression of the genes of interest relative to the production of the housekeeping gene β-actin. All values shown in Figure 2 have been calculated relative to the internal standard of β-actin mRNA expression within each cell line, thus compensating for any differences in the quantities of viable RNA collected in each isolate. Chemokine mRNA in each of the three CCL-producing lines was found to be expressed at levels beyond that of the β-actin housekeeping gene. More revealing, however, was the difference observed in CCL expression between each CCL-transduced line and the next highest value observed. The CCL2 C17.2 line expressed CCL2 mRNA at a relative level of 461-fold that of the next highest value observed, the CCL7 C17.2 line expressed CCL7 mRNA at a relative level of 1,112-fold that of the next highest value, and the CCL12 C17.2 line expressed CCL12 at a relative level of 60-fold the next highest value. Additionally, the CCL lines all expressed their respective chemokine mRNA at levels significantly higher than the other C17.2 lines in comparison. Interestingly, with regard to CCL2 and CCL7, all the lines expressed these genes at
Figure 2. Chemokine and retroviral gag mRNA expression in transduced C17.2 neural stem cell lines. Cells were harvested via trypsinization 48 hours after being plated. Total RNA was purified from the cells and analyzed for the indicated chemokine and retroviral mRNA by quantitative real-time PCR analysis. Variations within and between each sample are normalized through presentation relative to the housekeeping gene β-actin. Two-way ANOVA and Bonferroni post-test statistical analyses were performed for results of each of the three CCL genes. Where indicated (*) statistical significance was found with P<0.001 with respect to all other groups present in the comparison.

appreciably detectable levels, indicating that these genes may be innately expressed in the C17.2 line. With respect to CCL12 however, while both the CCL2 and CCL7 lines demonstrated detectable levels of expression, both the untreated C17.2 line and the pSFF line showed essentially no expression of this gene. The CCL2 and CCL7 lines also appear to be expressing CCL12 mRNA at a higher level than the basal levels of expression seen with the other two CCLs. It is possible that the presence of CCL2 and/or CCL7 in the cultures of those respective lines induced the production of CCL12 mRNA. As high levels of these chemokines were not present in the pSFF or untreated C17.2 cultures, this could explain the absence of CCL12 production.
As it is the only common transgene present between the transduced lines, levels of retroviral \textit{gag} mRNA expression were used as a molecular measure of relative levels of gene transfer between the individual lines. The observed levels of expression for this gene were similar between the CCL producing cell lines, and all the lines showed significantly higher expression as compared with the undetectable level observed in the untreated C17.2 cells. However, there was a disparity with regard to \textit{gag} mRNA levels detected in the empty pSFF vector control line, as the observed values were 9-fold lower, and significant, compared to the next highest level of expression recorded. At the time the initial experiment was conducted, this was the best rate of transduction we could achieve with the empty vector. As a preliminary study for the feasibility of this model, the low-level expressing pSFF-transduced cells were used for further experiments in this project. Since the initial experiment we were able to transduce C17.2 cells with the empty pSFF vector to produce a level of retroviral \textit{gag} production comparable to the other transduced lines.

\textbf{*C17.2 Recombinant Retrovirus Contamination.} During the course of this study it became apparent that at about three months post-inoculation, mice that had been inoculated with any one of the CCL2 C17.2, CCL7 C17.2, or CCL12 C17.2 lines were becoming sick and dying. At necropsy it was determined that their death was the result of multi-organ lymphosarcoma, a condition in mice that can be caused by recombinant retrovirus infection (Starkey et al., 1998). Upon this discovery, quantitative real-time PCR analysis was performed on RNA isolates from each of the C17.2 cell lines to test for the presence of either ecotropic or amphotropic retrovirus \textit{env} gene expression. Since the retroviral particles used to transduce the cell lines were designed to be replication-
Figure 3. Amphotropic and ecotropic retroviral env mRNA expression in C17.2 NSC lines. Cells were harvested, total RNA was isolated, and mRNA expression analyzed by quantitative real-time PCR as described in Figure 1. Variations within and between each sample were normalized through presentation relative to the housekeeping gene β-actin. Two-way ANOVA and Bonferroni post-test statistical analyses were performed for results of each of the retroviral env genes. Where indicated (*) statistical significance was found with P<0.001 and all samples being compared with the non-transduced C17.2 line.

deficient, in part due to the lack of an env gene, the presence in these cells of either of these retroviral gene products would indicate that recombination had taken place, and infectious, replicating virus was present. As Figure 3 indicates, every transduced C17.2 line used in this study was indeed contaminated with an infectious recombinant, and at significant levels compared to the untreated line, in which there was no evidence of any viral contamination. The pSFF C17.2 line contained evidence of both the ecotropic and amphotropic recombinants, as did the CCL12 C17.2 line at slightly higher levels. Both the CCL2 C17.2 and the CCL7 C17.2 lines were contaminated with only the ecotropic recombinant. As a result of these findings, it would be difficult to use retroviral gag
expression as an accurate point of comparison for gene transfer between the transduced lines since the infectious recombinant retroviruses would also induce \textit{gag} expression, thereby confounding the observed results.

\textbf{Flow Cytometry.} Immunofluorescent staining and flow cytometry analysis was employed to quantify the percentage of positively transduced cells within each transduced population. The anti-CCL antibodies utilized produced mixed and inconsistent results, and while each transduced cell line was clearly positive for the appropriate protein, percentages varied between the lines from 40-80\%, depending on when the flow run was conducted (data not shown). Earlier results were more consistent, and gave higher positive percentages for the CCL-producing cells, which would suggest the possibility of recombinant retroviral influence on the population as the infection proceeded with time.

Since a common antibody was needed to compare between the four transduced lines, the anti-retroviral \textit{gag} monoclonal antibody 34 was used to confirm positive transduction rates. The data obtained from this antibody indicated similar levels of transduction among the pSFF, CCL2, CCL7, and CCL12 C17.2 cell lines, with the percent positive cells ranging from 85-95\% (data not shown). However, the presence of the replicating, infectious recombinant retrovirus within the cultured cells (as shown in Figure 3) could confound the positive results seen for retroviral \textit{gag} expression, and may have influenced the overall make-up of the cultured population used in this analysis. Thus, the percent of cells positively transduced with the intended retroviral insert cannot be substantiated by the flow cytometry data obtained.
**Cellular Secretion of CCL Proteins.** As mRNA expression does not always correlate with protein production, we needed to demonstrate that the individual C17.2 lines were producing their respective CCLs, and confirm actual secretion of the chemokines. For this purpose, Western Blot analysis was performed on supernatant collected from the cultured C17.2 lines 48 hours after plating the cells. The same membrane blot was used for each of the three antibody incubations so that the results could be accurately compared. As shown in Figure 4, only culture supernatant from the appropriately transduced cell lines demonstrated the presence of the individual CCL of interest, and no errant or inherent CCL production was observed. All bands were observed at the expected molecular weight of ~9 kDa. The dual bands seen in the CCL2 blot were consistent with that protein’s reported tendency to persist in both the monomer and dimer forms (Zhang and Rollins, 1995). As such, the larger of the two bands is observed at approximately double the weight of the smaller monomer. A slight difference in intensity was observed between the protein levels of the monomer forms of the individual CCLs. This may be due to the necessary use of different secondary antibodies, the stripping process, or possibly the avidity or sensitivity of the different polyclonal antibodies. Additionally, the somewhat greater intensity of the CCL12 band may correspond to the slightly higher levels of mRNA expression observed in the quantitative real-time PCR results.

**In Vivo Chemokine Expression**

**Chemokine mRNA Expression in the CNS.** So that a proper comparison could be made between the experimental effects of the chemokine expressing C17.2 lines *in vivo*, and also to generally describe the *in vivo* characteristics of the system, the relative levels
Figure 4. Western Blot analysis of C17.2 neural stem cell culture supernatants. Growth media from each of the four transduced lines, and the untreated line, was collected for protein analysis following 48 hours of incubation. The samples were subjected to SDS-PAGE, blotted on a PVDF membrane, and incubated with polyclonal antibodies to each of CCL2 (A), CCL7 (B), and CCL12 (C) to confirm cellular secretion of the appropriate chemokine.

of transgenic chemokine expression in the CNS had to be established. For this purpose, CNS samples were collected at 7, 14, and 21 days following inoculation with the transduced cells. Brain tissue was divided into two sections at the mid-sagittal region, with half of each brain processed for RNA. The whole RNA isolated from these samples was then prepared for quantitative real-time PCR analysis as described in the methods section. CCL2 mRNA expression was significantly higher for the CCL2 C17.2-treated mice compared with the other groups for all time points tested, and increased with each interval possibly due to increasing migration of the cells into the brain tissue, or division of the inoculated cells (Figure 5A). CCL7 mRNA expression in the CCL C17.2-treated mice showed a similar pattern of increased chemokine expression with time (Figure 5B).
Figure 5. Chemokine mRNA expression in the central nervous system of neonatal Inbred Rocky Mountain White mice treated with one of four transduced C17.2 neural stem cell lines. All mice were intracranially inoculated through the lateral cerebral ventricles with 10^5 cells within 48 hours after birth. Total RNA was isolated from sagittal sections of brain samples collected at the indicated times post-inoculation. Relative mRNA expression for each of CCL2 (A), CCL7 (B), and CCL12 (C) was analyzed by quantitative real-time PCR. Variation within and between samples was normalized by presentation relative to the housekeeping gene β-actin. 5D represents relative CCL mRNA expression levels after adjusting for expression in the pSFF C17.2 treatment group. Two-way ANOVA and Bonferroni post-test statistical analyses were performed on all data shown, and where indicated (*) statistical significance was seen with P<0.05 with respect to all other experimental groups present in the analysis.

However, the CCL7 levels observed at the initial time point of 7 days p.i. showed little difference between the four groups. This suggests that CCL7-positive cells may migrate more slowly than the other cell types into the brain tissue. The CCL12 C17.2-treated mice also showed consistently higher CCL12 mRNA expression compared to the other groups (Figure 5C).
To compare relative increases in gene expression *in vivo*, the ratio of increased expression relative to the pSFF control group was calculated for each CCL. The adjusted comparison of CCL gene expression in the three CCL C17.2- treated groups across the observed time points also demonstrated that regardless of whatever effect the contaminating retroviruses may have had, the system reliably expressed the appropriate chemokine gene at comparable levels *in vivo* (Figure 5D). With the exception of CCL7 at 21 days p.i., the three chemokine genes were expressed at statistically similar levels at all time points. This observation allows for suitable comparison of the effects of the three chemokines *in vivo*, with at the very least being able to use each chemokine as a control for the other.

*In Vivo Localization of Chemokine-Producing C17.2 Cell Lines.* In order to further define the *in vivo* characteristics of the system, we sought to delineate the migratory distribution of the transduced cells in the CNS of the treated mice at the three time points studied. To this end two histological techniques were employed with varying degrees of success. First, *in situ* hybridization was performed on sagittal sections of all the treatment groups for each of the three chemokines, CCL2, CCL7, and CCL12 (Figure 6). Sections from each of the three chemokine-expressing groups were then scored for the presence of CCL-positive cells in each of the olfactory bulb, cortex/hippocampus, thalamus/midbrain, colliculus, cerebellum, brain stem, and meningial regions of the CNS (Table 1). Due to variations in sensitivity to detection of gene expression between samples, and the analysis of sagittal sections with varying depth from the mid-sagittal region, these results were used only to identify general trends in cell migration, rather
Table 1. Localized distribution of transduced C17.2 neural stem cells in the central nervous system of treated neonatal Inbred Rocky Mountain White mice. All mice were intracranially inoculated through the lateral cerebral ventricles with $10^5$ cells within 48 hours after birth. Whole brain samples were removed at the indicated times post-inoculation, sagittally sectioned, and fixed in 3.7% neutral buffered formalin. *In situ* hybridization analysis for mRNA of each of the three CCLs was conducted on the six mice used in each group. The data presented represents scoring of each of the seven general areas of the brain for the presence of positively stained cells as seen following *in situ* hybridization. The presence of no positively stained cells is represented as 0, 1-10 cells is represented as *, 11-50 cells as **, and 51+ cells as ***.

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than quantitative assessment of cell location. All treatment groups demonstrated similar patterns of in situ-positive cell presence in the regions examined. At day 7 p.i. the cells were most consistently observed in the meninges. By day 14 p.i. greater numbers of chemokine-producing cells were visible in the major regions of the brain, indicating further penetration of the cells into the tissue. Lastly, at day 21 p.i. the vast majority of the transduced cells were found in the interior regions of the brain rather than in the meninges. The most consistent region to which the greatest number of cells eventually trafficked was the thalamus/midbrain region, which is also consistent with this area’s proximity to the lateral ventricles.

Since the pSFF C17.2 control line obviously could not be detected via in situ hybridization for CCL production, these sections were examined by immunohistochemical analysis for the presence of β-galactosidase, the marker protein produced by the lacZ gene included in the C17.2 NSC line. Again, staining was

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Figure 6. Transduced C17.2 neural stem cells in the CNS of inoculated mice. Panels 6A through 6F show positive staining of chemokine-producing transduced C17.2 NSCs by \textit{in situ} hybridization. Meningial localization of CCL12 C17.2 cells at day 7 p.i. (6A, 20X magnification). CCL7 C17.2 cells in the olfactory bulb at day 21 p.i. (6B (4X magnification) and 6D (10X magnification)). CCL12 C17.2 cells in the brain stem at day 14 p.i. (6C, 10X magnification). CCL2 C17.2 cells in the thalamus at day 21 p.i. (6E, 4X magnification). CCL12 C17.2 cells in the thalamus at day 14 p.i. (6F, 40X magnification). Panels 6G through 6I show \(\beta\)-galactosidase-positive staining by immunohistochemistry. pSFF C17.2 cells in the olfactory bulb at day 21 p.i. (6G, 20X magnification). pSFF C17.2 cells in the thalamus at day 14 p.i. (6H (10X magnification) and 6I (40X magnification)).

inconsistent, but for those sections that were positive it was clear that the cells tracked to similar regions of the CNS as the chemokine-producing cells (Figure 6G-I). As increased mRNA expression of the CCL of interest was detected in all tissue samples (Figure 5), the lack of C17.2 detection by \textit{in situ} hybridization or immunohistochemistry for some
**Figure 7.** *In situ* hybridization for CCL expression in the thalamus/mid-brain regions of inoculated mice. 7A, 7D, and 7G are samples collected from CCL2 C17.2-inoculated mice. 7B, 7E, and 7H are samples collected from CCL7 C17.2-inoculated mice. 7C, 7F, and 7I are samples collected from CCL12 C17.2-inoculated mice. Samples in 7A through 7C were collected at day 21 p.i. and stained using probes specific for CCL2. Samples in 7D through 7F were collected at day 21 p.i. and stained using probes specific for CCL7. Samples in 7G through 7I were collected at day 14 p.i. and stained using probes specific for CCL12. Positively stained sections were seen in CCL C17.2-inoculated mice only when subjected to staining with the appropriately corresponding *in situ* probe. All pictures were taken at a lens magnification of 4X.

tissue samples appears to be the result of variations in tissue handling, processing, or slide preparation, rather than an actual deficit of chemokine-producing cells.

Additionally, as can be seen in Figure 7, the CCL mRNA detected by the *in situ* hybridization was specific to the appropriate experimental group. Each treatment group was stained using all three of the CCL-specific probes, but no positive staining was
observed where it was not expected, showing that the CCL over-expression was due to the specific transduced cells that were inoculated, and not to a general immune reaction to the presence of the cells.

**Glial Activation and Inflammatory Infiltration**

**CNS mRNA Expression of Molecular Markers for Microglia/Macrophage and Astrocyte Activation and Infiltration.** CCL2, CCL7, and CCL12 have all been associated with neurological diseases of which glial activation is a common characteristic (Conant et al., 1998; McManus et al., 1998; Simpson et al., 1998; Van, V et al., 1999; Xia et al., 1998). Additionally, these three chemokines have been well-characterized as being chemotactic for monocytic cells (Fuentes et al., 1995; Gonzalo et al., 1998; Gunn et al., 1997; Sarafi et al., 1997; Sozzani et al., 1994; Sozzani et al., 1995). As such, one of the purposes of this study, beyond characterizing the system, was to examine the effects of individual CCL over-expression on CNS glial activation and/or macrophage infiltration. To this end, molecular analysis was performed on RNA isolates from each group and each time point by quantitative real-time PCR. mRNA expression of the astrocyte activation marker Glial Fibrillary Acidic Protein (GFAP) was used as a means of determining relative astrocyte activation, while mRNA expression of the microglia/macrophage activation marker F4/80 was used to measure relative microglia activation and macrophage recruitment. F4/80 mRNA expression remained at a consistent level across all three time points for all treatment groups, with the exception of the CCL7 C17.2 group (Figure 8A). Here there was a decrease in expression to a significant level at both days 14 and 21 p.i. relative to the pSFF C17.2 control. GFAP mRNA expression analysis gave similar results with the CCL7 C17.2 group showing a
Figure 8. mRNA expression of glial activation markers in the central nervous system of Inbred Rocky Mountain White mice treated with one of four transduced C17.2 neural stem cell lines. All mice were treated, and RNA isolated, as previously described in Figure 5. mRNA expression for the macrophage/microglia activation marker F4/80 (8A), and the astrocyte activation marker GFAP (8B) were analyzed by quantitative real-time PCR. Variation within and between samples was normalized by presentation relative to the housekeeping gene β-actin. Two-way ANOVA and Bonferroni post-test statistical analyses were performed on all data shown, and where indicated (*) statistical significance was seen with P<0.05 with respect to values observed for the pSFF C17.2 control group.

A slight, but significant decrease at day 14 p.i. and both the CCL2 C17.2 and CCL12 C17.2 groups showing a slight decrease in expression relative to the pSFF C17.2 control groups at day 21 p.i. (Figure 8B). Unexpectedly, the group inoculated with untreated C17.2 cells showed similar levels of mRNA expression of both glial activation markers when compared to all other treatment groups, suggesting that the presence of recombinant viruses played no part in inducing the expression of these genes. Additionally, the relatively lower basal levels of expression of both molecular markers seen in the untreated group of mice (not shown) suggests that levels observed in the treatment groups, controls included, may be due to a response to the invasive nature of the inoculation process, and not to the presence of the infectious recombinants or the effects of chemokine over-expression.

Immunohistochemical Analysis of Glial Activation and Inflammatory Infiltration

In order to complement the molecular data obtained for glial activation, and to determine
Figure 9. Glial activation in the thalamus/mid-brain region of CCL C17.2-treated mice. 9A is from day 21 p.i. of a mouse inoculated with CCL2 C17.2-expressing cells. The section was dual-stained for CCL2 mRNA via *in situ* hybridization (red staining), and reactive astrocytes via immunohistochemistry for GFAP expression (brown staining). 9B is from day 14 p.i. of a mouse inoculated with CCL12 C17.2-expressing cells. The section was dual-stained for CCL12 mRNA via *in situ* hybridization (red staining), and reactive microglia/macrophages via immunohistochemistry for Iba1 expression (brown staining). Both pictures were taken at a lens magnification of 40X.

The proximity of activated glial cells to the CCL-producing stem cells, immunohistochemical analysis was performed on fixed sagittal sections taken from the treated mice. Anti-GFAP antibodies were used to assess astrocyte activation, and anti-Iba1 antibodies were used to detect microglia/macrophage activation and recruitment. Our histological observations supported the molecular results in that there was no visually noticeable difference in the overall number and distribution of activated glial cells between the CCL and pSFF or untreated C17.2 treatment groups (not shown). Additionally, as demonstrated in Figure 9, there was no visible evidence of reactive glia staining positive for any of the three CCLs being examined, showing that the significant up-regulation of each of the CCLs was not due to glial production.

Immunohistochemical analysis using anti-CD3 antibodies was also performed to determine the extent, if any, of lymphocytic infiltration in the CNS due to the over-
expression of the three CCL chemokines. No visually significant CD3-positive lymphocyte infiltration was present in any of the treatment groups (not shown).

**Gross Pathology**

In order to fully assess any histopathological effects of the over-expression of CCL2, CCL7, or CCL12 in the CNS, H&E stained tissue sections were examined for each sample. Interestingly, the presence of spongiform degeneration was observed in the thalamus/mid-brain section of a large number of CNS samples from the CCL12 C17.2 treatment group (Figure 10). In a majority of these samples the pathology was evident by day 14 p.i., and was also present in the day 21 samples of the same group. The presence of both the amphotropic and ecotropic recombinant viruses in the CCL12 C17.2 cells confounds these observations, especially given prior evidence that these viruses are associated with such histopathology (Munk et al., 1997). However, all other groups inoculated with transduced cell lines were also contaminated with recombinant virus, including the pSFF C17.2 group with both varieties, and this effect was not seen with any consistency. Consequently, these results suggest the possible involvement of CCL12 in spongiform degeneration, if not on its own, then at least in the presence of a retrovirus infection.

**Fr54 Retrovirus Infection Concomitant with CCL C17.2 Inoculation**

It is currently unclear what the precise roles of CCL2, CCL7, or CCL12 are, if any, in the development of neurological disease. It is likely that CCL over-expression alone is not directly responsible for clinical disease during retrovirus infection, and the possibility remains that some aspect of the viral replication cycle is necessary in conjunction with immune factor expression to induce disease. As such, we employed the
Figure 10. Spongiform degeneration in the CNS of mice inoculated with CCL12 C17.2 neural stem cells. The thalamus/mid-brain region of H&E stained tissue sections at day 14 p.i. (10A (20X magnification)) and day 21 p.i. (10B (20X magnification)). 10C (10X magnification) shows the colliculus at day 21 p.i. in a tissue section dual stained for CCL12 mRNA by *in situ* hybridization (red staining), and GFAP by immunohistochemistry (brown staining).

use of the non-neurovirulent retrovirus Fr54 infection in conjunction with inoculation with the CCL-expressing C17.2 cells to observe for the development of signs of clinical neurological disease. Fr54 viral stocks were generated in culture and each treatment group was intraperitoneally infected with 100 µl of virus stock, while simultaneously being inoculated with the CCL C17.2 cells as described previously. Each group was observed for 21 days post-infection with no signs of disease ever developing.
Discussion

The first objective of the present study was to establish the *in vivo* CNS expression of CCL chemokines via retroviral-transduced C17.2 neural stem cells. This was accomplished using pSFF vector virions containing the individual CCL genes that were generated in ψ2/PA317 retroviral packaging cell co-cultures. Real-time PCR analysis of RNA isolates from each cell line revealed some variation in the levels of expression of CCL mRNA among the respective chemokine-producing cell lines; however the results overall revealed analogous patterns of expression when compared to the other lines tested for each specific CCL.

Retroviral *gag* expression in the transduced cell lines was intended to be used as a relative measure of gene transfer at both mRNA and protein levels. However, recombinant retrovirus contamination in the transduced cultures invalidated these results, as this gene could also be transferred by the recombinants. As such, it became evident that the later inconsistencies in the percentages of CCL-positive cells observed in the flow cytometry data could have been due, in part, to disruptive selection in the transduced populations caused by these viruses. In all, the presence of the recombinants prevented an accurate measure of the rate of transduction.

While the *in vitro* data obtained was partially tainted by the presence of contaminating retroviruses, the potential effects of the recombinant contamination were not evident in the *in vivo* data that was generated by this study. CCL2, CCL7, and CCL12 mRNA were all expressed at significant and consistently higher levels in their respective treatment groups as compared to the controls. Additionally, CCL mRNA expression, when adjusted for basal levels, was found to be quite consistent for each of
the three CCL C17.2-treated groups. These observations give support to the idea that this system provides a viable means to study different CNS chemokines being produced at consistently high levels *in vivo*, which would allow for reliable cross-comparison of any effects seen.

One of the unknowns that had to be addressed with this system was the migratory patterns of the chemokine-expressing C17.2 cells to different regions of the brain, and the potential effects on the distribution of chemokine production. In the current study, as seen through *in situ* hybridization analysis, there was clear evidence of wide dissemination of the desired gene product. While the inconsistencies in positive staining between samples prevented any quantitative assessment of C17.2 migration, this may have resulted from the use of sections with different spatial relationships from the mid-sagittal point, or variations in tissue processing. Additionally, the CCL mRNA expression observed in all samples as determined by real-time PCR analysis verifies the presence of the CCL-producing cells, and supports tissue processing as a cause for the inconsistencies seen. Nevertheless, chemokine-expressing cells for all three CCL groups were present in every major region of the brain, both interior and exterior. Furthermore, no difference was observed in the migration pattern of the pSFF C17.2 control cells in comparison with the CCL-producing C17.2 cells. This data indicates that there was most likely no auto-stimulatory homing effect of the chemokines on the C17.2 cells. Though *in vitro* studies demonstrated that CCL2 could induce the migration of neural progenitor cells, the expression of the chemokines by the C17.2 cells themselves may not generate the necessary directional gradient, or the constant exposure in culture could have desensitized the transduced cells to the chemotactic effects of the chemokines (Widera et
al., 2004). Nonetheless, the migratory nature of the transduced C17.2 system could provide a more accurate representation of the spatial distribution of activated, chemokine-producing glia seen with neurological conditions, rather than asserting a more concentrated effect such as with direct injection of the chemokine (Perrin et al., 2005).

Since one of the most consistently reported functions of CCL2, CCL7, and CCL12 is the ability to chemoattract cells of monocytic lineage, we expected to see an increase in the expression of molecular markers of activation for these cells in conjunction with the over-expression of the CCL chemokines *in vivo* (Fuentes et al., 1995; Gonzalo et al., 1998; Gunn et al., 1997; Sarafi et al., 1997; Sozzani et al., 1994; Sozzani et al., 1995). Additionally, as astrocyte activation often correlates with the presence of increased production of pro-inflammatory chemokines during the course of disease, we also investigated the potential effect of the CCL over-expression on the population of these cells in the CNS (Persidsky et al., 1999; Peterson et al., 2004; Van, V et al., 1999). Somewhat surprisingly, our results gave no evidence of significant glial activation or monocytic infiltration as a result of CCL over-expression, either by mRNA analysis, or by histological examination. These observations reveal two important facts. First, they suggest that even though there was the presence of recombinant retroviruses, this infection had no obvious influence on the *in vivo* effects of the system. This is evident by the similar levels of both GFAP and F4/80 mRNA expression seen in all groups that were inoculated with C17.2 cells, including the untreated C17.2 cell line which possessed no detected contaminating infection. Considering the apparent absence of recombinant influence *in vivo* on glial activation, this data can be further interpreted to say that the over-expression of CCL2, CCL7, or CCL12 alone did not have any
significant impact on glial activation or monocytic infiltration in the CNS. This is in agreement with previous CCL2 transgenic studies that showed the chemokine had no major effects until the CNS was stimulated with a pathogenic medium, suggesting more of an immune priming function (Huang et al., 2002). This concept can also be extended for both CCL7 and CCL12 now as there previously was no direct study of the effects of over-expression of these chemokines in the CNS. When considering the possible priming effect though, it is interesting that even in the presence of the infectious recombinants there was no observed immune activation. This suggests the possibility that these chemokines lack the ability to assert a detectable unilateral immune response, and that they potentially require the presence of some other soluble immune factor, or possibly a more pathogenic medium, to assert their full influence.

Another interesting, and initially unexpected effect observed, was the consistent presence of spongiform degeneration in the thalamus/mid-brain region of the CCL12 C17.2-treated mice. While it has been reported that the presence of amphotropic recombinant retroviruses have been associated with this pathological phenomenon, the absence of this effect in the other treatment groups where recombinant virus was also present suggests the possible involvement of CCL12 in initiation or exacerbation of spongiform degeneration (Munk et al., 1997). Lending support to this analysis was the close proximity of the spongiform lesions to the main region of C17.2 migration. Since the spongiform mechanism is not fully understood, this data, while preliminary, presents the possibility of CCL12 involvement, potentially in jump-starting some signaling cascade that leads to cellular vacuolization. As this was an incidental finding, and not the
focus of this study, further investigation will be needed to establish any kind of conclusive role for CCL12 in spongiform degeneration.

While there are clearly improvements to be made, and obstacles to overcome in the continued development of this system, this study represents a positive first step in providing a reliable model for examining individual chemokine over-expression in the CNS. The most glaring concern is the confounding presence of two types of recombinant retroviruses. Upon further investigation into the matter, it has become evident that recombinant production is an inherent characteristic of the $\psi$2/PA317 retroviral packaging cell co-culture method for generating virions for transduction. It is a possibility that the use of this co-culture system in conjunction with the pSFF vector specifically increased the likelihood of recombination. Since pSFF still maintains partial envelope sequences, there could be regions of homology that exist within the vector with respect to the envelope sequences found in the packaging cells that would promote recombination. This phenomenon could also be a result of potentially homologous regions existing in pSFF with regard to the altered $\psi$ sequences found in the packaging cell lines. Additionally, based on the evidence provided via the real-time PCR detection of the ecotropic and amphotropic envelope sequences in vitro we can not definitively conclude that there was a true replication competent recombinant virus present in vivo, as it remains a possibility that only the envelope sequences were transferred to the pSFF virions, and not the ability to actually replicate. We are currently addressing these issues by examining the feasibility of a variety of other packaging cell lines in conjunction with the pSFF retroviral vector. These new cell lines maintain the retroviral envelope and packaging sequences on separate plasmids, and therefore further reduce the potential for
recombination. Additionally, since we have now developed effective means of detecting the presence of, at the very least, recombinant envelope expression, regular testing of transduced C17.2 cultures for the presence of contaminating recombinant virus will help reduce the possibility of confounding variables in the future.

While the reduced capacity for recombination would obviously allow for a more complete assessment of the validity of this system, there are additional factors that need to be considered when making further adjustments. For one, an accurate quantification of both *in vitro* and *in vivo* CCL protein production should be undertaken via ELISA or possibly Bio-Plex assay in order to complement the mRNA expression analysis, and help to further define the function of the system. Additionally, the effects of the actual inoculation procedure need to be taken into account with regard to any effects observed. An additional control group could potentially be added to allow consideration of any immune response that might result from the needle puncture required for delivery of the CCL-expressing cells. A dual-staining technique making use of the C17.2 expression of β-galactosidase must also be optimized that would give definitive evidence, as opposed to the indirect evidence provided in this study, that the CCL-expressing cells observed *in vivo* are truly the transduced C17.2 cells that were used in the inoculation.

Once the missing links in this system have been dealt with in a satisfactory manner, however, there are a number of potential applications beyond what was examined here. In its usefulness as a model for accurate examination of the isolated *in vivo* effects of individual CNS chemokine over-expression, other factors could be examined. A more in-depth analysis of the effects on resident glia and neuronal function should be attempted, possibly focusing on more localized effects in the areas directly
surrounding the chemokine-producing cells. Additionally, in continuing with the idea that these factors act as primers for the immune response, there could be a subsequent pathogenic or toxic stimulation, perhaps pertussis toxin, LPS, or a more pathogenic virus, in order to assess either the exaggerated immune response that may follow, or potentially the protective effects that have been proposed for these chemokines. This system should provide the opportunity for more studies that will provide a reasonable examination of immune factor over-expression in the CNS in a manner more consistent with what is seen during the course of naturally occurring neurological conditions.
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63


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Vita

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