6-2013

Expression of the Chemokine Receptor, CXCR4, and Its Ligand, SDF-1, Are Increased in Purkinje Cells of the Multiple System Atrophy Cerebellum

Megan Welter

Western Michigan University, mwelter1@emich.edu

Follow this and additional works at: http://scholarworks.wmich.edu/masters_theses

Part of the Genetic Processes Commons, Nervous System Diseases Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation


http://scholarworks.wmich.edu/masters_theses/168
EXPRESSION OF THE CHEMOKINE RECEPTOR, CXCR4, AND ITS LIGAND, SDF-1, ARE INCREASED IN PURKINJE CELLS OF THE MULTIPLE SYSTEM ATROPHY CEREBELLUM

by

Megan Welter

A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science
Department of Biological Sciences
Western Michigan University
May 2013

Thesis Committee:

Charles F. Ide, Ph.D., Chair
John M. Spitsbergen, Ph.D.
Cindy Linn, Ph.D.
Multiple System Atrophy (MSA) is a sporadic, neurodegenerative disease that consists of three conditions: autonomic dysfunction, Parkinsonism and cerebellar ataxia. Our lab conducted an Affymetrix global gene expression analysis using pons tissue of MSA patients to determine genes that are differentially expressed when compared to non-MSA controls. This study identified upregulated genes, including the C-X-C chemokine receptor type 4, CXCR4, to which stromal cell-derived factor-1 (SDF-1) is the natural ligand. The CXCR4/SDF-1 signaling pair has been shown to play multiple roles in the brain, such as inducing neuronal apoptosis and promoting leukocyte recruitment during inflammation. The MSA cerebellum presents a significant decrease in Purkinje cells, which are responsible for coordinating motor movement; MSA patients suffer from cerebellar ataxia. The study described in this thesis is the first to quantitatively demonstrate that both CXCR4 and SDF-1 protein levels are significantly upregulated in MSA Purkinje cells compared to controls. Furthermore, the apoptotic protease Caspase 3 is also expressed at a significantly higher level in Purkinje cells of MSA patients compared to controls. These findings suggest that CXCR4/SDF-1 are associated with neuronal apoptosis. Intervention of this pair’s signaling cascade has the potential as being a therapeutic target in MSA and other diseases affected by neuronal loss.
ACKNOWLEDGMENTS

I would like to begin by expressing my gratitude to Dr. Charles Ide for being an excellent mentor. His expertise and passion for finding a cure for Multiple System Atrophy has made this project possible. I am thankful for his encouragement not only on this thesis, but also on my goal of pursuing a career as a physician assistant. I can undoubtedly say that he has helped me develop myself professionally by implementing high standards of work. I would also like to thank the members of my committee, Dr. John Spitsbergen and Dr. Cindy Linn for their advice and input on this project. Additionally, I am grateful to Dr. Bharti Katbamna and Derrick Hilton for their technical assistance.

Furthermore, I would like to thank my parents, Lorrie and Steve Welter, for always supporting me during this journey, as this would not have been possible without them. I would like to acknowledge the remaining family members for their encouragement, especially my grandparents, Shirley and Jerome Barton. Finally, thank you to all of my friends who have always been so optimistic, notably Taylor Ruder, Kim Richardson, Corinne Berger and Brooklyn. Thank you all for dealing with me during the stressful times, I know it wasn’t always easy.

Megan Welter
# TABLE OF CONTENTS

**ACKNOWLEDGMENTS** ........................................................................................................ ii 

**LIST OF TABLES** ........................................................................................................ vii 

**LIST OF FIGURES** ......................................................................................................... viii 

**INTRODUCTION** ................................................................................................................. 1  

Multiple System Atrophy ........................................................................................................ 1 

Cerebellum Anatomy and Background .................................................................................. 2 

Purkinje Cells of the Cerebellum ............................................................................................ 4 

Pathophysiology of Multiple System Atrophy ....................................................................... 5 

CXCR4 ..................................................................................................................................... 6  

SDF-1 ..................................................................................................................................... 6 

CXCR4/SDF-1 Function as a Pair in the Mature Brain ............................................................. 7 

CXCR4/SDF-1 Involvement During Inflammation and Disease .............................................. 9 

CXCR4/SDF-1 Expression in Parkinson’s Disease ................................................................... 9
Table of Contents – Continued

CXCR4/SDF-1 Expression Affecting Neurons .................................................. 10

CXCR4/SDF-1 Activating Apoptosis: Caspase 3 .............................................. 11

AIMS OF THE CURRENT STUDY ....................................................................... 13

MATERIALS AND METHODS ........................................................................... 14

Tissue Collection .............................................................................................. 14

Antibodies .......................................................................................................... 14

Immunohistochemistry ....................................................................................... 15

Image Collection for Analysis ........................................................................... 16

Image Analysis .................................................................................................. 17

Statistical Tests .................................................................................................. 20

RESULTS ........................................................................................................... 22

Patient Demographics ....................................................................................... 22

Protein Expression of the Chemokine SDF-1 ............................................... 23

Protein Expression of the Chemokine Receptor CXCR4 ............................... 27
# Table of Contents – Continued

Caspase 3 Expression in Purkinje Cells........................................................... 29

Circularity of Purkinje Cells............................................................................ 33

Area of Purkinje Cells..................................................................................... 35

DISCUSSION........................................................................................................... 37

Chemokines are Implicated in Neurodegeneration ........................................ 37

Neuronal Degeneration ................................................................................... 37

CXCR4/SDF-1 in Parkinson’s Disease ............................................................ 38

Purkinje Cells From MSA Cerebella Characterized in the Current Study ...... 39

CXCR4 and SDF-1 Expression in Multiple System Atrophy ......................... 39

Caspase 3 Expression in Multiple System Atrophy ........................................ 40

Purkinje Cell Circularity .................................................................................. 40

Purkinje Cell Area.............................................................................................. 41

CXCR4/SDF-1 as Neurotoxic Molecules ......................................................... 41

Involvement in Multiple Sclerosis ................................................................... 42

CXCR4 in Human Immunodeficiency Virus .................................................... 44
Table of Contents – Continued

Future Analyses ........................................................................................ ........ 44

Conclusion ........................................................................................................ 44

BIBLIOGRAPHY ................................................................................................ 46
LIST OF TABLES

1. Patient Demographics from New York Brain Bank at Columbia University ........ 23
LIST OF FIGURES

1. Complete Folium of the Cerebellum ...................................................... 17
2. Red Pixel Measurement From CXCR4 Dual Label .................................... 20
3. Mean Integrated Densities of SDF-1 Immunohistochemistry ....................... 25
4. SDF-1 Immunohistochemistry in the Cerebellum ........................................ 26
5. Mean Integrated Densities of CXCR4 Immunohistochemistry ..................... 28
6. CXCR4 Immunohistochemistry in the Cerebellum ...................................... 29
7. Mean Integrated Densities of Caspase 3 Immunohistochemistry .................... 31
8. Caspase 3 Immunohistochemistry in the Cerebellum .................................. 32
9. Purkinje Cell Mean Circularity Measurement ........................................... 34
10. Purkinje Cell Circularity ........................................................................ 35
11. Purkinje Cell Mean Area Measurement ..................................................... 36
INTRODUCTION

Multiple System Atrophy

Multiple System Atrophy (MSA) is a sporadic neurodegenerative disease that is characterized by a combination of autonomic dysfunction, parkinsonism, and cerebellar ataxia (Kaufmann et al., 2003; Wenning et al., 2004). Other characteristics seen in MSA include dysautonomia, erectile dysfunction, urinary incontinence, and constipation (Wenning et al., 2004; Wenning et al., 1994). This disease pathologically presents gliosis, cellular death, and the presence of protein aggregates termed glial cytoplasmic inclusions (Wenning et al., 2004). Interestingly, cognitive impairment is not seen in MSA patients (Kaufmann et al., 2003; Wenning et al., 2004). MSA affects both men and women and has an average age onset in the 50's (Kaufmann et al., 2003; Stefanova et al., 2005; Schrag et al., 1999). There is rapid progression of the disease with a mean survival rate of 9 years (Kaufmann et al., 2003; Stefanova et al., 2005; Schrag et al., 1999). Postmortem pathological confirmation is necessary for a definite diagnosis of MSA (Kaufmann et al., 2003; Lin et al., 2004; Pountney et al., 2004). During the early stages of MSA, it is difficult to differentiate from other neurodegenerative diseases such as Parkinson's disease (PD) which can lead to a misdiagnosis (Kaufmann et al., 2003; Stefanova et al., 2005; Schrag et al., 1999). The prevalence of MSA is estimated to be 2-15 per 100,000, although recently it has been suggested that this number is actually higher, but is reported this way because of the previously mentioned difficulty of distinguishing MSA from other disorders (Kaufmann et al., 2003; Stefanova et al., 2005; Schrag et al., 1999). Studies have investigated MSA for genetic alterations that are responsible for autosomal dominant ataxias (SCA-1 and SCA-3), but no changes in these genes were found.
(Castellani 1998). From what is known, MSA is a sporadic disease and studies strongly suggest that MSA is acquired in a way that is separate from other central nervous system diseases that have a known genetic cause (Castellani 1998). Currently, MSA is a terminal disease without a cure.

There are two major forms of MSA: striato-nigral MSA (MSA-SND) and olivopontocerebellar MSA (MSA-OPCA), with autonomic dysfunction occurring in both types (Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997; Kaufmann et al., 2003). In MSA-SND, degeneration occurs primarily in the striatum and substantia nigra, while the brainstem and cerebellum also undergo degeneration in MSA-OPCA (Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997). There is much overlap between the two types and all MSA patients display degeneration in the pons (Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997). Generally, MSA-SND patients are characterized as having Parkinson-like symptoms, while patients with MSA-OPCA typically present a more advanced cerebellar ataxia (Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997).

Cerebellum Anatomy and Background

MSA-OPCA is the form of interest for the current study in which degeneration is present in the brainstem and cerebellum. The cerebellum is located below the cerebrum and just above the brain stem. Throughout the years, the cerebellum has always been categorized as its own distinct subdivision of the brain (Glickstein et al., 2009). Anatomically, the cerebellum is a structure that is highly foliated and is connected to the rest of the brain by three column-like structures called cerebellar peduncles (Glickstein et
When looking at the human brain from a superior view, the cerebellum is not visible because it is covered by the occipital lobe of the cerebral cortex (Glickstein et al., 2009). The cerebellum is well known for its involvement in reflex adaptation and most notably for its role in the process of fine and sequential motor movements (Glickstein et al., 2009). Since this current study focuses specifically on MSA-OPCA, and therefore, cerebellar ataxia, the cerebellum’s function of controlling motor movements is of particular interest.

Despite its physiological complexity, the cerebellum is composed of only 3 layers: granule cell (GC) layer, Purkinje cell (PC) layer, and molecular layer (Sillitoe et al., 2007). Directly above the white matter core of the cerebellum is the granule cell layer and it is composed mostly of small granule cells which are the most abundant neuronal type in the brain (Sillitoe et al., 2007). The GC layer also contains other cell types such as Golgi cells, Lugaro cell interneurons, and unipolar Brush cells (Mugnaini et al., 1997). The neighboring layer is the Purkinje cell layer and it is composed of large Purkinje cell bodies and smaller Bergmann glia cells (Voogd & Glickstein 1998). The final and most superficial layer is the molecular layer which contains components such as GC axons, PC dendrites, Bergmann glia fibers, and basket and stellate cell interneurons (Voogd & Glickstein 1998).

There are two types of afferent structures involved in the circuitry of the cerebellum, which are called mossy fibers and climbing fibers (Sillitoe et al., 2007). Mossy fibers are specialized projections that supply the cerebellum with input from numerous regions of the brain and spinal cord (Sillitoe et al., 2007). Climbing fibers are also specialized projections that provide the cerebellum with input, but only from a single
area of the brain stem called the inferior olive (Sillitoe et al., 2007). Each climbing fiber in the cerebellum interacts with the dendrites of one Purkinje cell (Sillitoe et al., 2007). Mossy fibers have their termination point in the GC layer, while climbing fibers have their termination in the molecular layer (Sillitoe et al., 2007). Ultimately, information flows into the cerebellum when mossy fibers synapse on granule cells, and climbing fibers synapse on Purkinje cells (Sillitoe et al., 2007). Interestingly, all afferent pathways into the cerebellum ultimately signal the Purkinje cells (Purves et al., 2001). Parallel fibers, which are the axons of granule cells, synapse on a specialized area of the Purkinje cell dendritic tree (Purves et al., 2001). The parallel fibers and climbing fibers both excite Purkinje cells (Purves et al., 2001).

**Purkinje Cells of the Cerebellum**

Purkinje cells of the cerebellum are found in the Purkinje cell layer, and have a noticeably large soma (Sillitoe et al., 2007). Purkinje cells are inhibitory cells, and they are of great importance because their axons provide the only output from the cerebellar cortex (Glickstein et al., 2009). Thus, Purkinje cells are a necessary component of the motor system of the human body that is responsible for muscle tone and movement (Herndon 1963). Each Purkinje cell receives its input from the inferior olive via climbing fibers that synapse on the Purkinje cell dendrites and from other brain areas through parallel fibers that also innervate Purkinje cells (Purves et al., 2001). Purkinje cells have gained much attention for their degeneration in many conditions such as ischemia, mitochondrial disorders, Alzheimer's disease, Huntington's disease, autism, and alcoholism (Sarna & Hawkes 2003). Studies have confirmed that there is an evident loss of Purkinje cells in MSA (Spokes et al., 1979; Kume et al., 1991; Wenning et al., 1996).
Purkinje cell degeneration phenotypically presents as ataxia and tremor (Sarna & Hawkes 2003), which is the common symptom observed in MSA-OPCA (Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997). Although Purkinje cell degeneration is present in normal aging to a certain degree, a pronounced loss of Purkinje cells is not normal (Sarna & Hawkes 2003). It is thought that in the adult human, oxidative stress plays a role in the normal Purkinje cell death that accompanies aging (Bickford et al., 2000), yet the cause of death of Purkinje cells in MSA is unknown. It is important to understand why the Purkinje cells are undergoing death because they are responsible for coordinating motor movement.

Pathophysiology of Multiple System Atrophy

MSA has a hallmark of specific oligodendroglial inclusions termed glial cytoplasmic inclusions (GCIs) that contain aggregates of a protein called α-synuclein (SNCA) (Castellani 1998). Oligodendrocytes are responsible for producing myelin, and myelin in MSA undergoes extensive degeneration (Castellani 1998; Matsuo et al., 1998). Interestingly, aggregates containing α-synuclein are observed in other neurodegenerative diseases such as Parkinson’s disease, dementia with Lewy body (DLB) and Lewy Body Variant of Alzheimer’s disease (LBVAD) (Lundvig et al., 2005). These diseases, grouped together with MSA, have been termed synucleinopathies (Lundvig et al., 2005). However, these aggregates, seen in the previously mentioned diseases, are not found in oligodendrocytes as in MSA (Castellani 1998). Instead, aggregations of α-synuclein are typically found in neurons and are termed neuronal cytoplasmic inclusions (NCIs) or Lewy bodies (Lundvig et al., 2005). Although α-synuclein aggregates are commonly seen in these diseases, the definite role of α-synuclein in the initiation and progression of
MSA is still under investigation (Langerveld et al., 2007). Upon investigation of the distribution of GCIs, it was found that GCIs tend to aggregate in areas of the brain that are undergoing degeneration including but not limited to the putamen, spinal cord intermediate gray matter, middle cerebellar peduncle, and ponto-cerebellar tracts (Castellani 1998; Walburn 2010).

**CXCR4**

CXCR4, also called fusin, is a chemokine receptor for the Cys-X-Cys class of chemokines (Feng et al., 1996; Hesselgesser et al., 1998). Structurally, this cell surface receptor is a seven transmembrane domain that is linked to G proteins (Bajetto et al., 1999; Hesselgesser et al., 1998). Upon activation, CXCR4 mediates calcium flux (Bokoch 1995; Ari & Charo 1996). CXCR4 has been observed as being constitutively expressed in astrocytes, microglia, neurons, and granule cells (Banisadr et al., 2002; Ragozzino 2002). Of particular interest for the current study, neuronal expression of CXCR4 has been found in the following brain areas: cerebral cortex, substantia innominata, globus pallidus, caudate putamen, substantia nigra, supraoptic/paraventricular hypothalamic nuclei, and ventromedial thalamic nucleus (Banisadr et al., 2002). Focusing specifically on neurons, CXCR4 protein expression is constitutively expressed in Purkinje, dopaminergic, GABAergic, and cholinergic neurons (Ragozzino 2002; Banisadr et al., 2002; Trecki et al., 2010).

**SDF-1**

Chemokines are small secreted proteins that have chemoattractant properties, specifically for immune cells, and they also bind to chemokine receptors to initiate a signaling cascade (Luster 1998; Luther & Cyster 2001). Chemokines are categorized into
four subfamilies by classification of the spacing of their first two cysteine residues: C, CC, CXC, and CX3C (Jiang et al., 2012). Stromal cell-derived factor 1 (SDF-1), also known as chemokine ligand 12 (CXCL12), is the sole ligand for CXCR4 under normal conditions (Jiang et al., 2012; Bajetto et al., 1999). It has been found that most chemokines have the ability to bind to several chemokine receptors and likewise, most chemokine receptors are able to recognize several chemokines, but SDF-1 is unique in that it only interacts with CXCR4 (Bacon & Harrison 2000; Cartier et al., 2005). However, recent studies have shown that there is another receptor for SDF-1 in T lymphocytes and it is called CXCR7, but currently there is no evidence for the presence of this receptor in the normal brain (Balabanian et al., 2005). The SDF-1 gene undergoes alternative mRNA splicing which creates three isoforms: SDF-1α, SDF-1β, and SDF-1γ, although most studies tend to focus on SDF-1α (Gleichmann et al., 2000; Pillarisetti & Gupta 2001; Stumm et al., 2002). SDF-1α protein is constitutively expressed in the human adult brain in neurons, astrocytes, and microglia (Stumm et al., 2002; Banisadr et al., 2003). Studies have shown that SDF-1α is produced by numerous cell types, including neuroglia and bone marrow stromal cells (Bleul et al., 1996; Oberlin et al., 1996). The antibody that was used in this study detects all three forms of SDF-1 (α, β, γ), therefore, for the remainder of this paper I will only refer to this protein as SDF-1 (total SDF-1).

**CXCR4/SDF-1 Function as a Pair in the Mature Brain**

Chemokines are notoriously known for their role during inflammatory processes, such as a host defense response, in which they activate and cause directed migration of leukocytes (Lazarini et al., 2003). SDF-1 is involved in many important processes in both
the developing and the mature central nervous system (Lazarini et al., 2003). For example, SDF-1 is involved in the regulation of cell growth, migration of hematopoietic stem cells, and it plays an important role in brain development (Lazarini et al., 2003). Specifically, CXCR4/SDF-1 mediate neuronal migration during formation of the granule-cell layer of the cerebellum (Ma et al. 1998; Zou et al. 1998; McGrath et al. 1999). Similarly with brain development, knockout murine models of the CXCR4 gene result in fetal death (Bajetto et al., 1999). Blocking the activation of either the CXCR4 or the SDF-1 gene results in cells in the external granular layer (EGL) to prematurely migrate into the cerebellum (Ma et al., 1998; Zou et al., 1998). The expression of CXCR4/SDF-1 is absolutely necessary for the development of the dentate gyrus, cerebellum, and neocortex (Cartier et al., 2005).

CXCR4/SDF-1 also plays an important role in central nervous system processes such as neurogenesis, axonal growth, pain, and neurotransmission (Tran & Miller 2005; Lieberam et al., 2005; Szabo et al., 2002; Limatola et al., 2000).

In inflammatory responses, SDF-1 is a strong chemoattractant for cells such as lymphocytes, monocytes, and CD34+ hematopoietic progenitor cells (Kim & Broxmeyer, 1998). Interestingly, when SDF-1 is expressed at high levels, it leads to selective repulsion of certain types of T cells (Poznansky et al., 2000). On the other hand, low levels of SDF-1 result in activation of leukocytes and release of leukocytes from capillaries (Peled et al., 1999). The activation of CXCR4 by SDF-1 leads to many important processes including the aided migration and proliferation of cerebellar granule cells, chemoattraction of microglia, and activation of the production and release of cytokines and glutamate from astrocytes (Lazarini et al., 2003). Additionally, SDF-1
activation of CXCR4 provokes postsynaptic currents in Purkinje cells, as well as triggers the migration of cortical neuron progenitors (Lazarini et al., 2003).

**CXCR4/SDF-1 Involvement During Inflammation and Disease**

The chemokine receptor and ligand pair CXCR4/SDF-1 has gained attention for its involvement in many diseases. Studies have shown that the signaling system CXCR4/SDF-1 plays a role in the pathogenesis of tumors (Abi-Younes et al., 2000; Buckley et al., 2000; Gonzalo et al., 2000; Lukacs et al., 2002). SDF-1 is up-regulated during the inflammatory processes in diseases such as allergic airway diseases, rheumatoid arthritis, and artherosclerosis (Abi-Younes et al., 2000; Buckley et al., 2000; Gonzalo et al., 2000; Lukacs et al., 2002). CXCR4/SDF-1 have been shown to be overexpressed in inflammatory processes in several brain diseases such as spinocerebellar ataxia type 3 and experimental allergic encephalitis (EAE) (Lazarini et al. 2003). Local chemokine release is common in neurodegenerative and neuroinflammatory diseases such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and human immunodefiency virus (HIV)-associated dementia (Streit et al., 2001; Vila et al., 2001; Lee et al., 2002; McGeer & McGeer 2004; Cartier et al., 2005). Specifically, CXCR4 can act as a receptor for the human immunodefiency virus (HIV-1) glycoprotein gp120 (Lapham et al., 1996; Berson et al., 1996; Feng et al., 1996). Gp120 has the ability to signal through CXCR4, therefore this chemokine receptor is of great importance in HIV (Davis et al., 1997).

**CXCR4/SDF-1 Expression in Parkinson’s Disease**

As previously mentioned, MSA is often misdiagnosed in the beginning stages, commonly as Parkinson’s disease (PD) (Langerveld et al., 2007). Similar to MSA, the
role of chemokines as inflammatory molecules in Parkinson’s disease is without a doubt very important, but is also not fully understood (Shimoji et al., 2009). Since MSA has many similarities with Parkinson’s disease, it is very interesting that both CXCR4 and SDF-1 protein expression are up-regulated in PD patients compared to controls (Shimoji et al., 2009). An Affymetrix DNA microarray study on pons tissue revealed that mRNA levels of CXCR4 are up-regulated 2.157 fold in MSA compared to controls (Langerveld et al., 2007). Studies have suggested that CXCR4/SDF-1 play a role in the loss of dopamine (DA) neurons in PD, yet no studies to date have investigated the cause of Purkinje cell death in MSA (Shimoji et al., 2009). Interestingly, other studies performed on rodent brains, or in vitro, have shown that the activation of CXCR4 induces neuronal apoptosis (Hesselgesser et al., 1998; Meucci et al., 1998; Kaul & Lipton 1999; Zheng et al., 1999; Bachis & Mocchetti 2004). It has been suggested that the activation of CXCR4 by SDF-1 causes neuronal death in both HIV encephalitis and Parkinson’s disease (Shimoji et al., 2009). Along with PD, CXCR4/SDF-1 signaling has been suggested to play a role in inflammatory processes and neurodegeneration in a variety of diseases such as rheumatic arthritis, atherosclerosis, and spinocerebellar ataxia (Burman et al., 2005; Abi-Younes et al., 2002; Evert et al., 2001).

**CXCR4/SDF-1 Expression Affecting Neurons**

CXCR4 has been shown to be expressed on neurons and neuroglia (He et al., 1997; Horuk et al., 1997; Lavi et al., 1997; Ghorpade et al., 1998; Hesselgesser et al., 1998; Vallat et al., 1998; Zheng et al., 1998; Zou et al., 1998). Interestingly, it has been shown that if SDF-1 is produced in excess, it has the ability to affect neural function through CXCR4 signaling (Zheng et al., 1999). Similarly, there is evidence from in vitro
studies that SDF-1 is neurotoxic (Kaul & Lipton 1999; Bachis & Mocchetti 2004).
CXCR4/SDF-1 have been shown to be neurotoxic to neuronal populations either by a
direct signaling mechanism or an indirect mechanism such as activating microglia to
release neurotoxins (Hesselgesser et al., 1998; Meucci et al., 1998; Kaul & Lipton 1999;
Bezzi et al., 2001). When the neuronal cell line hNT is exposed to either SDF-1 or HIV
gp120, which both play a similar role in activating CXCR4, apoptosis is observed
(Hesselgesser et al., 1998). However, the mechanism that is activated to cause this
neuronal death is currently unknown (Zheng et al., 1999). A study by Zheng and
colleagues investigated the mechanisms for CXCR4-mediated apoptosis and the signaling
pathway for GTP binding protein, since these pathways have the ability to alter neuronal
function (Zheng et al., 1999).

Although the binding of SDF-1 to CXCR4 has the ability to cause cell death in
neurons, cell survival is seen in cell types such as CD4 T cells (Vlahakis et al., 2002). It
is not known why the activation of CXCR4 by SDF-1 does not cause cell death in CD4 T
cells, yet does result in cell death in neurons (Vlahakis et al., 2002). When CXCR4/SDF-
1 activate Akt, it leads to an anti-apoptotic fate for the cell, such as that seen in the CD4
T cells (Vlahakis et al., 2002). On the other hand, when CXCR4/SDF-1 activate MAPK
(p38), the affected cell will become pro-apoptotic, such as that seen in neurons (Vlahakis
et al., 2002). It has also been shown that by inhibiting p38 MAPK, neuronal apoptosis
caused by SDF-1 is reduced (Kaul & Lipton 1999).

**CXCR4/SDF-1 Activating Apoptosis: Caspase 3**

Apoptosis involves a signaling cascade with regulatory genes (Yonguc et al.,
2012). One of the most effective and therefore well known apoptotic regulatory genes
includes those of the cysteiny l aspartate specific protease (caspase) family (Yonguc et al., 2012). Caspases are cysteine proteases that play a central role in apoptotic cell death. Since CXCR4/SDF-1 have been shown in numerous studies to cause neuronal injury, it is of interest to identify the protein that is activated to ultimately carry out cell death. A study investigated the Caspase pathway for neuronal apoptosis and found that when human neurons are treated with SDF-1, Caspase 3 protein was observed (Zheng et al., 1999). Caspase 3 is a protease that is responsible for neuronal apoptosis (Mogi et al., 2000; Tatton 2000). Specifically, the protein observed was the 17 kDa form of Caspase 3 (Zheng et al., 1999). Caspase 3 has a precursor form which measures 32 kDa and once it becomes activated, it has two subunits which are 17 kDa and 11 kDa, all of which can be identified by Western blot (Zheng et al., 1999). The levels of Caspase 3 that were measured in the SDF-1 treated neurons increased 2.5-fold compared to controls (Zheng et al., 1999). Interestingly, activated Caspase 3 is also observed in post-mortem brains of Parkinson’s disease patients (Mogi et al., 2000; Tatton 2000). In the rat striatum, stimuli that have the ability to activate CXCR4 have been shown to activate Caspase 3, therefore inducing cell loss (Bachis et al., 2006; Nosheny et al., 2006).
AIMS OF THE CURRENT STUDY

The aim of this study is to determine if the chemokine and chemokine receptor SDF-1 and CXCR4 are regulated differently in the Purkinje cells of the Multiple System Atrophy cerebellum. Since both proteins are constitutively expressed in the mature brain, my goal is to determine if they are being regulated differently in MSA Purkinje cells compared to control Purkinje cells. Since MSA is very similar to Parkinson’s disease and studies have shown that both proteins are up-regulated in PD, my hypothesis is that both proteins will also be up-regulated in the MSA cerebellum. CXCR4/SDF-1 have been associated with neuronal death in PD and other diseases, therefore I hypothesize that this pair is also associated with neuronal death in MSA, specifically in the Purkinje cells. Furthermore, I hypothesize that Caspase 3 protein levels will be increased in Purkinje cells in MSA, as it has been shown to be increased in post-mortem brains of PD. The work presented here tested the hypothesis that CXCR4, SDF-1, and Caspase 3 are all up-regulated in the Purkinje cells of MSA cerebellum compared to the Purkinje cells of control cerebellum, suggesting that the CXCR4/SDF-1 signaling system is involved in Purkinje cell death in MSA.
MATERIALS AND METHODS

Tissue Collection

Formalin-fixed paraffin-embedded cerebellar tissue sections from MSA and control patients, measuring 10 µm thick, were obtained from the New York Brain Bank (NYBB) at Columbia University. Post-mortem neuropathological analysis was performed at NYBB to deliver a definite diagnosis of MSA. Control tissue was determined as having no known neuropathology. Cerebellar sections were obtained from six patients diagnosed with MSA and six control patients with no known neurological diagnosis.

Antibodies

CXCR4 protein was detected using a goat anti-human CXCR4 polyclonal antibody at a dilution of 1:150. This antibody detects N terminal amino acids 17-43 of human CXCR4 purchased from Abcam, Cambridge, MA. The secondary antibody used in conjunction with CXCR4 was a biotin conjugated donkey anti-goat IgG purchased from Abcam, Cambridge, MA.

SDF-1 protein was detected using a rabbit anti-human SDF-1 polyclonal antibody at a dilution of 1:25. This antibody detects total human SDF-1 protein, therefore all three SDF-1 isoforms were recognized: α, β, and γ. SDF-1 antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The secondary antibody used in conjunction with SDF-1 was a biotin conjugated goat anti-rabbit IgG purchased from Santa Cruz Biotechnology, Santa Cruz, CA.
Caspase 3 protein was detected using a rabbit anti-Caspase 3 polyclonal antibody at a dilution of 1:100. This antibody specifically detects the 17 kDa subunit of activated Caspase 3 purchased from Sigma-Aldrich, St. Louis, MO. The secondary antibody used in conjunction with Caspase 3 was a biotin conjugated goat anti-rabbit IgG purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Immunohistochemistry

Brain sections fixed on glass slides were deparaffinized and rehydrated with a series of xylenes and ethanols. Sections were steamed in a 1mM sodium citrate buffer (pH 6.0) for 40 minutes, allowed to cool at room temperature for 20 minutes, and then quenched in 3% hydrogen peroxide for 5 minutes in order to block endogenous peroxide activity. Two 5 minute TBS washes followed the quench. In order to block non-specific staining, 3% normal goat serum (NGS) for SDF-1 and Caspase 3 antibody, or 3% normal donkey serum (NDS) for CXCR4 antibody was applied for one hour at room temperature. Brain sections were then incubated with one of the following primary antibodies: anti-SDF-1 (1:25, made in rabbit), anti-CXCR4 (1:150, made in goat), or anti-Caspase 3 (1:50, made in rabbit) at 4°C overnight. Following the overnight incubation with the primary antibody, a series of three 5 minute TBS washes was followed by application of a biotinylated secondary antibody (goat anti-rabbit for SDF-1 and Caspase 3, donkey anti-goat for CXCR4) at a dilution of 1:100. All TBS washes were performed as a series of three for 5 minutes each, unless stated otherwise. After 30 minutes of incubation for the secondary antibody, sections were washed in a series of TBS washes followed by a one hour application of Elite Vectastain avidin and biotinylated horseradish peroxidase macromolecular complex (ABC complex) (Vector Laboratories, Burlingame, CA).
Sections were then washed in TBS and antibody staining was visualized with 3’3’
diaminobenzidine (DAB). The brain sections were then dehydrated in a series of
ethanols, cleared with a series of xylenes, and cover slipped using a mounting medium
(Richard-Allan Scientific). Sections labeled as negative controls were processed
simultaneously with the sections that were processed for the primary antibody. These
negative controls underwent the exact same process as those sections that were processed
for the primary antibody, except that the application of the primary antibody was omitted.
The secondary antibody, however, was applied to the negative controls. This was done to
ensure that the staining observed was not non-specific staining due to an unwanted
reaction from a reagent used during the process. The negative controls for SDF-1,
CXCR4, and Caspase 3 all showed no staining; therefore it can confidently be stated that
sections treated with primary antibody were specifically stained.

Image Collection for Analysis

Brain sections from both MSA and control patients were visualized and
photographed using Spot Cooled Color Digital Camera system (Diagnostic Instruments,
Inc, Sterling Heights, MI) attached to a Nikon Eclipse E600 Microscope. Images were
quantitatively analyzed for protein expression of SDF-1, CXCR4, and Caspase 3. To
ensure that differential background intensities would not affect the analysis, all images
were photographed using the same digital gain settings. Twenty Purkinje cells were
photographed from both MSA and control cerebellar sections. To ensure randomness, ten
images were captured left to right from the top folium and ten images right to left from
the bottom folium. In the event that the section was fixed on the slide at a different
orientation, ten images were captured left to right from the far left folium and ten images
right to left from the far right folium. To furthermore ensure randomness during image collection, only every third Purkinje cell was photographed for use in analysis.

Purkinje cells were photographed from the Purkinje cell layer of cerebellar folia. Figure 1 illustrates one complete foliar section captured at a 4X objective. The arrows identify one complete folium. The tissue that was fixed on slides for these experiments contained multiple folia. Images were captured from folia that appeared very similar to figure 1, but at a 40X objective rather than 4X.

*Figure 1: Complete Folium of the Cerebellum. A photograph at 4X objective showing a complete foliar section in the cerebellum. The arrows are within one folium. (Scale bar = 100 µm.)*

*Image Analysis*

Purkinje cells were quantitatively analyzed for protein levels using the ImageJ program (nih.gov) as described in the ImageJ tutorial pages. Images were calibrated to 10 micrometers using a scale bar for a 40X objective photograph to equal 3.8 pixels per 1 micrometer. The image was then quantitatively analyzed using the color deconvolution
tool. The color deconvolution tool uses red, green, and blue (RGB) vectors, which are predetermined values for red and brown color, which differentiate between red (AP) and brown (DAB) colors. Once the color deconvolution tool is selected, the ImageJ program automatically generates the following three separate images from the original image: one with only brown color (DAB), one with only red color (AP), and one with both brown and red colors (double label). Since all of the proteins analyzed in this study were only labeled with DAB, only the newly generated image with just brown color was measured. Therefore, all of the measurements for this study came from the image that was separated to contain only brown color. The other two generated images with red color (AP) and brown/red color (double label) were closed. The image was then converted to a black and white image and the threshold was adjusted. The Purkinje cell was carefully traced using the free hand drawing tool and was measured for values including area, mean gray value, integrated density, and circularity. The measurement of integrated density is the value that was used to measure the amount of antibody stain present in the sections. Integrated density is the product of area and mean gray value of the selected area. The area is defined as the area of selection in calibrated units, which are square micrometers, since the images were spatially calibrated before analyzing. The mean gray value is the sum of the gray values of all the pixels in the selection divided by the number of pixels.

The CXCR4 that was measured for this thesis was a double labeled IHC (with CXCR4 being labeled with DAB), but the other antibody was not measured, because the alkaline phosphatase based staining (red color) did not work. Although the AP did not work in the double labeled CXCR4 IHC stain, it was decided to analyze the CXCR4 protein because MSA cerebellar sections are not available in excess. Two measures were
taken in order to ensure that only the CXCR4 was being measured; first, CXCR4 protein levels were only measured using the brown image obtained from the color deconvolution. As described earlier, the color deconvolution method was used to separate the brown and red pixels in the images. The color deconvolution method generates three different images: one with only brown pixels (DAB), one with only red pixels (AP), and one with both pixels (double labeled). The CXCR4 antibody measured for this study was labeled with DAB, which is brown color. Therefore, once the color deconvolution was performed on this double labeled IHC set, the only image that was analyzed for integrated density was the one containing only brown pixels. This ensures that only the CXCR4 antibody labeled with DAB was measured. Secondly, a histogram was created using the program Photoshop, in which single and doubled labeled Purkinje cells were measured for the presence of red by the number of pixels with a bright value (Fig. 2). Purkinje cells were measured for red pixels from the following three separate, previously performed IHC stains: a single labeled DAB only image, a single labeled AP only image, and the doubled labeled image from which the CXCR4 was measured. The single labeled images (DAB only and AP only) were created at an earlier time. The arrows in Figure 2 identify the measurement of red pixels for the three different IHC stains. Image A is the red pixel measurement from an AP only image, image B is the red pixel measurement from a DAB only image, and image C is the red pixel measurement from the double labeled image from which CXCR4 was analyzed. The red measurement of AP (Image A) appears to the far right of the histogram, compared to the red measurements localized to the middle of the histogram for the measurements of DAB (Image B) and the double labeled image (Image C). Therefore, images B and C are very similar, showing no measurement of red
pixels, as seen in image A. These histograms along with only measuring the brown color from the color deconvolution method indicates that there was no red color being analyzed in the double labeled CXCR4 IHC stain. This process was only performed for the CXCR4 antibody, as the other two antibodies (SDF-1 and Caspase 3) were single labeled in DAB.

Figure 2: Red Pixel Measurement From CXCR4 Dual Label. "A" shows the measurement of red pixels from an AP only image. "B" shows the measurement of red pixels from a DAB only image. "C" is the measurement of red pixels from the double labeled image that CXCR4 was analyzed from. Arrows identify the measurement of red pixels. "B" and "C" are very similar, while "A" isn't similar because it is the measurement of AP alone. The histogram shows that the double labeled (AP & DAB) image from which CXCR4 was analyzed from is not different from the DAB only image, therefore, no red was present in the stain that would interfere with the results.

Statistical Tests

Statistics were run using SAS Institute © Statview 5.0. An unpaired T-test was performed to detect differences in integrated densities between MSA and controls for the measurements of SDF-1, CXCR4, and Caspase 3. An unpaired T-test was also performed
to determine the difference between the mean circularity and the mean area of Purkinje
cells in MSA versus control. A p-value of $p \leq 0.05$ was considered significant.
RESULTS

Patient Demographics

Table 1 lists the patients, both MSA and controls, from which cerebellar sections were used for this study. The patients included in this study have a random, diverse background as far as age and gender. Age ranges from 44-78 years and both males and females were involved, which shows that choosing brain sections for this study was a random process. A diagnosis of MSA is defined as “striato-nigral-olivo-ponto-cerebellar atrophy with glial cytoplasmic inclusions”. A diagnosis of control is defined as “no diagnostic abnormality recognized”. Cold PMI describes the post mortem interval calculated from the patients reported time of death to the time the patient was brought into the cold room. Frozen PMI describes the post mortem interval calculated from the patients reported time of death to the mean time the brain was processed; brain areas used for histopathology were not frozen, but were fixed in formalin. The term NaN is used for “not a number”, for example, the calculation failed because the time of death or the time when the patient was brought into the cold room was not reported.
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Age</th>
<th>Gender</th>
<th>Cold PMI</th>
<th>Frozen PMI</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>330</td>
<td>73</td>
<td>Female</td>
<td>3:35</td>
<td>13:15</td>
<td>MSA</td>
</tr>
<tr>
<td>4447</td>
<td>65</td>
<td>Female</td>
<td>NaN</td>
<td>NaN</td>
<td>MSA</td>
</tr>
<tr>
<td>4485</td>
<td>70</td>
<td>Female</td>
<td>1:05</td>
<td>21:25</td>
<td>MSA</td>
</tr>
<tr>
<td>4640</td>
<td>54</td>
<td>Male</td>
<td>1:00</td>
<td>6:10</td>
<td>MSA</td>
</tr>
<tr>
<td>4261</td>
<td>61</td>
<td>Male</td>
<td>2:45</td>
<td>33:50</td>
<td>MSA</td>
</tr>
<tr>
<td>3873</td>
<td>61</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>MSA</td>
</tr>
<tr>
<td>338</td>
<td>67</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>MSA</td>
</tr>
<tr>
<td>3569</td>
<td>NaN</td>
<td>No Data</td>
<td>NaN</td>
<td>NaN</td>
<td>MSA</td>
</tr>
<tr>
<td>29</td>
<td>68</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>MSA</td>
</tr>
<tr>
<td>4523</td>
<td>62</td>
<td>Female</td>
<td>4:33</td>
<td>22:38</td>
<td>Control</td>
</tr>
<tr>
<td>99</td>
<td>44</td>
<td>Male</td>
<td>NaN</td>
<td>49:15</td>
<td>Control</td>
</tr>
<tr>
<td>4233</td>
<td>74</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>Control</td>
</tr>
<tr>
<td>4259</td>
<td>49</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>Control</td>
</tr>
<tr>
<td>638</td>
<td>78</td>
<td>Male</td>
<td>NaN</td>
<td>NaN</td>
<td>Control</td>
</tr>
<tr>
<td>4534</td>
<td>60</td>
<td>Male</td>
<td>NaN</td>
<td>7:07</td>
<td>Control</td>
</tr>
</tbody>
</table>

*Table 1: Patient Demographics from New York Brain Bank at Columbia University. Demographics of the patients from the New York Brain Bank at Columbia University used for this study, both MSA and control.*

**Protein Expression of the Chemokine SDF-1**

In order to test the hypothesis that SDF-1 protein levels are up-regulated in MSA Purkinje cells compared to control Purkinje cells, immunohistochemistry (IHC) was performed on cerebellar sections obtained from the New York Brain Bank, using a goat
anti-human SDF-1 antibody. Brain sections of both MSA and control underwent the IHC method described in detail in the methods section, including a negative control. The Purkinje cells in the images were carefully outlined and statistically analyzed using color deconvolution, specifically using only the brown (DAB) image, in the ImageJ program. Analysis revealed that MSA patients have significantly higher levels of the protein SDF-1 in the Purkinje cells compared to control Purkinje cells ($p = 0.0195$) (Figure 3). Integrated density is the measurement unit that quantifies the amount of antibody stain present in the tissue. Figure 3 is a bar graph representing the SDF-1 mean integrated densities (area multiplied by mean gray value) for both MSA and control, with the mean integrated density measurement on the Y axis and patient group on the X axis. SDF-1 integrated density means are as follows; MSA=$7.93 \times 10^5$ integrated density units, control=$0.074 \times 10^5$ integrated density units. These measurements were obtained from six MSA patients and six control patients.
Figure 3: Mean Integrated Densities of SDF-1 Immunohistochemistry. Mean integrated densities of SDF-1 protein in the Purkinje cells of MSA and control patients; MSA mean of $7.93 \times 10^5$ integrated density units compared to control mean of $0.074 \times 10^5$ integrated density units. ($p = 0.0195; N = 6$. Error bars = S.E.)

Images from SDF-1 immunohistochemistry appear in Figure 4, including a negative control (A), an MSA patient (B), and a control patient (C). The distribution of SDF-1 appears on the Purkinje cell surface or in the cytoplasm, but not specifically localized in the nucleus or at one end of the cell. The arrows point to Purkinje cells (PC) in both MSA and control. The Purkinje cells of the MSA patients are a darker brown color than the control Purkinje cells showing that SDF-1 protein expression is significantly higher in the Purkinje cells of MSA compared to control (Figure 3). Image A, the negative control, is completely clean of any staining, which demonstrates that the staining in images B and C are specific to the protein SDF-1.
**Figure 4:** SDF-1 Immunohistochemistry in the Cerebellum. Arrows point to Purkinje cells (PC). Image A is a photo from the cerebellar section in which the primary antibody (SDF-1) was not applied (negative control). Image B illustrates SDF-1 expression in an MSA patient, while image C shows SDF-1 expression in a control patient. (Scale bar = 20 µm.)

Thus, image analysis carried out on Purkinje cells stained via IHC methods shows expression of the chemokine SDF-1 is significantly up-regulated in MSA cerebellar folia compared to controls (Figures 3 and 4).
Protein Expression of the Chemokine Receptor CXCR4

To test the hypothesis that MSA Purkinje cells express the chemokine receptor, CXCR4, at a significantly higher level than measured in control Purkinje cells, expression of CXCR4 was analyzed in both MSA and control cerebellar tissue by performing immunohistochemistry (IHC) on cerebellar sections obtained from the New York Brain Bank using a donkey anti-human CXCR4 antibody. Brain sections of both MSA and control underwent the IHC method described in detail in the methods section, including a negative control. The Purkinje cells in the images were carefully outlined and statistically analyzed using color deconvolution, specifically using only the brown (DAB) image, in the ImageJ program. Statistical analysis revealed that the expression of CXCR4 is significantly higher in MSA Purkinje cells compared to control Purkinje cells (p < 0.0001) (Figure 5). Integrated density is the measurement unit that quantifies the amount of antibody stain present in the tissue. Figure 5 is a bar graph representing the CXCR4 mean integrated densities (area multiplied by mean gray value) for both MSA and control, with the mean integrated density measurement on the Y axis and patient group on the X axis. CXCR4 mean integrated densities are as follows; MSA=16.34 x 10^5 integrated density units, control=2.96 x 10^5 integrated density units. These measurements were obtained from six MSA patients and six control patients.
**Figure 5:** Mean Integrated Densities of CXCR4 Immunohistochemistry. Mean integrated densities of CXCR4 protein in the Purkinje cells of MSA and control patients; MSA mean of $16.34 \times 10^5$ integrated density units compared to control mean of $2.96 \times 10^5$ integrated density units. ($p < 0.0001; N = 6$. Error bars = S.E.)

Figure 6 shows images from CXCR4 immunohistochemistry, which include a negative control (A), an MSA patient (B), and a control patient (C). The distribution of CXCR4 does not appear exclusively in the nucleus or localized to one end of the cell (CXCR4 is a cell surface receptor). The arrows point to Purkinje cells (PC). Purkinje cells of MSA patients are a darker brown color than control Purkinje cells, which demonstrates that CXCR4 protein expression is higher in Purkinje cells of MSA compared to control. Image A, the negative control, is completely clean of any staining, which demonstrates that the staining in images B and C are specific to the protein CXCR4.
Figure 6: CXCR4 Immunohistochemistry in the Cerebellum. Arrows point to Purkinje cells (PC). Image A is a photo from the cerebellar section in which the primary antibody (CXCR4) was not applied (negative control). Image B illustrates CXCR4 expression in an MSA patient, while image C is CXCR4 expression in a control patient. (Scale bar = 20 µm.)

Therefore, image analysis carried out on Purkinje cells stained via IHC methods shows expression of the chemokine receptor CXCR4 is significantly up-regulated in MSA cerebellar folia compared to controls (Figures 5 and 6).

Caspase 3 Expression in Purkinje Cells

In order to test the hypothesis that Caspase 3 protein levels are up-regulated in MSA Purkinje cells compared to control Purkinje cells, immunohistochemistry (IHC)
was performed on cerebellar sections obtained from the New York Brain Bank using a goat anti-human Caspase 3 antibody. Brain sections of both MSA and control underwent the IHC method described in detail in the methods section, including a negative control. The Purkinje cells in the photographs were then carefully outlined and statistically analyzed using color deconvolution, specifically using only the brown (DAB) image, in the ImageJ program. Statistical analysis revealed that Caspase 3 expression is significantly higher in MSA Purkinje cells versus control Purkinje cells ($p < 0.0001$) (Figure 7). Integrated density is the measurement unit that quantifies the amount of antibody stain present in the tissue. Figure 7 is a bar graph representing the SDF-1 mean integrated densities (area multiplied by mean gray value) for both MSA and control, with the mean integrated density measurement on the Y axis and patient group on the X axis. Caspase 3 integrated density means are as follows; MSA=1.53 x 10$^5$ integrated density units, control= 1.53 x 10$^8$ integrated density units. These measurements were obtained from six MSA patients and six control patients.
Figure 7: Mean Integrated Densities of Caspase 3 Immunohistochemistry. Mean integrated densities of Caspase 3 protein in the Purkinje cells of MSA and control patients; MSA mean of $15.38 \times 10^5$ integrated density units compared to control mean of $1.53 \times 10^5$ integrated density units. ($p < 0.0001; N = 6$. Error bars = S.E.)

Images from Caspase 3 immunohistochemistry appear in Figure 8, including a negative control (A), an MSA patient (B), and a control patient (C). The distribution of Caspase 3 does not appear exclusively in the nucleus or localized to one end of the cell, but instead in the cytoplasm. The arrows point to Purkinje cells (PC) in both MSA and control. The Purkinje cells of the MSA patients are a darker brown color than the control Purkinje cells showing that Caspase 3 protein expression is significantly higher in the Purkinje cells of MSA compared to control (Figure 8). Image A, the negative control, is completely clean of any staining, which demonstrates that the staining in images B and C are specific to the protein Caspase 3.
Figure 8: Caspase 3 Immunohistochemistry in the Cerebellum. Arrows point to Purkinje cells (PC). Image A is a photo from the cerebellar section in which the primary antibody (Caspase 3) was not applied (negative control). Image B illustrates Caspase 3 expression is dark brown in color in an MSA section, while image C shows Caspase 3 expression is a lighter color in a control section. (Scale bar = 20 µm.)

Image analysis carried out on Purkinje cells stained via IHC methods shows expression of the pro-apoptotic protein, Caspase 3, is significantly up-regulated in MSA cerebellar folia compared to controls (Figures 7 and 8).
Circularity of Purkinje Cells

To further investigate the hypothesis that MSA Purkinje cells are undergoing apoptosis, MSA and controls Purkinje cells were analyzed for circularity.Circularity of Purkinje cells is of importance because cells that are experiencing cellular stress and/or apoptosis are irregularly shaped. This was performed by using the free hand drawing tool to carefully trace each Purkinje cell and having the ImageJ program measure its circularity. Circularity of the Purkinje cell was evaluated using the value of “1” as being a perfect circle, therefore, as measurements become further from “1”, they become less circular. Upon statistical analysis, Purkinje cells from controls are significantly more circular than those from MSA individuals, suggesting that MSA Purkinje cells are under stress and experiencing apoptosis (p=0.0033) (Figure 9). Using a value of “1” as being a perfect circle, circularity measurements are as follows; control mean of 0.688, MSA mean of 0.594. Figure 9 is a bar graph representing the mean circularity measurements for both MSA and control, with the mean circularity measurement on the Y axis and patient group on the X axis. These measurements were obtained from six MSA patients and six control patients.
Figure 9: Purkinje Cell Mean Circularity Measurement. Control Purkinje cells are more circular than those of MSA patients. Using a value of “1” as a perfect circle, circularity measurements are as follows; control mean of 0.688, MSA mean of 0.594. (p=0.0033; N = 6. Error bars = S.E.)

Purkinje cells of MSA patients have an elongated shape when compared to Purkinje cells of control patients, which have more of a circular shape (Figure 10). Image A shows a Purkinje cell from an MSA patient while image B shows Purkinje cells of control tissue. The arrows point to Purkinje cells (PC). The elongated shape seen in image A is of importance because studies have shown that the morphology of the cellular membrane changes during the execution phase of apoptosis. MSA patients having elongated cells further supports the hypothesis that the Purkinje cells in MSA are undergoing apoptosis.
Figure 10: Purkinje Cell Circularity. Image A shows an elongated Purkinje cell from MSA cerebellar tissue, while image B shows a circular control Purkinje cell. The arrows point to Purkinje cells (PC). (Scale bar = 20 µm.)

Statistical analysis carried out on Purkinje cells shows that control Purkinje cells are significantly more circular than MSA Purkinje cells, using a value of “1” as a perfect circle (Figures 9 and 10).

Area of Purkinje Cells

To ensure that the result showing MSA Purkinje cells to have significantly higher protein expression (SDF-1, CXCR4, Caspase 3) was not affected by MSA cells possibly having a larger cellular area, Purkinje cells of MSA patients and control patients were measured for area. This was performed by using the free hand drawing tool to carefully trace each Purkinje cell and selecting area measurement in the ImageJ program. Statistical analysis showed that control Purkinje cells have significantly larger area than MSA Purkinje cells (p=0.0116) (Figure 11). As previously mentioned, integrated density is the product of the area and the mean gray value. Therefore, MSA Purkinje cells having a smaller area means that they have an extremely high mean gray value. Figure 11 is a
bar graph representing the mean area of Purkinje cells for both control and MSA, with the following area measurements; control mean of 9,547 µm², MSA mean of 7,250 µm². The measurement of mean area is labeled on the Y axis and patient group is labeled on the X axis. These measurements were obtained from six MSA patients and six control patients.

*Figure 11*: Purkinje Cell Mean Area Measurement. Control Purkinje cells have a larger cellular area than those of MSA patients; control mean of 9,547 µm², MSA mean of 7,250 µm². (p=0.0116; N = 6. Error bars = S.E.)

Thus, statistical analysis carried out on Purkinje cells shows that control Purkinje cells have a significantly larger area than MSA Purkinje cells, meaning that MSA Purkinje cells have an extremely high mean gray value (Figure 11).
DISCUSSION

Chemokines are Implicated in Neurodegeneration

Chemokines and chemokine receptors are involved in the pathogenesis of numerous neurological disorders, including Alzheimer’s disease, multiple sclerosis, cerebral ischemia, and HIV associated dementia (Cartier et al., 2005). Interestingly, the pathology of these conditions is associated with the over-expression of certain chemokine receptors in particular brain areas (Cartier et al., 2005). Chemokines have been implicated as important inflammatory molecules in Parkinson’s disease, however, their role is not fully understood (Shimoji et al., 2009). This is similar to MSA, since the role of chemokines in this disease is under investigation.

Furthermore, there is emerging evidence that chemokine receptors are involved in neuronal death and therefore, neurodegenerative diseases (Cartier et al., 2005). Several neurological disorders present with neuronal injury and death (Cartier et al., 2005). For example, damaged neurons are observed in Alzheimer’s disease and neuronal loss is seen in multiple sclerosis (Akiyama et al., 2000; Kutzelnigg et al., 2007). The neurological disorder HIV-1 encephalitis (HIVE) also presents with loss of neuronal subpopulations (Glass et al., 1993; Koenig et al., 1986).

Neuronal Degeneration

Neuronal loss is of importance because it results in behavioral pathology such as ataxia, which is loss of motor coordination (Sarna & Hawkes 2003). In another example, slowing of motor movements, or Parkinsonian motor abnormalities, is observed in Parkinson’s disease, MSA, and HIV-1 encephalitis (Glass et al., 1993; Koenig et al.,
MSA involves extensive loss of a particular population of neurons called Purkinje cells, which are located in the cerebellum, and are responsible for coordinating muscle movements (Spokes et al., 1979; Kume et al., 1991; Wenning et al., 1996; Herndon 1963). Degeneration of Purkinje cells phenotypically presents as ataxia and tremor, yet the cause of Purkinje cell death in MSA is unknown (Sarna & Hawkes 2003; Wenning et al., 2004; Ozawa et al., 2004; Stefanova et al., 2005; Wenning et al., 1997).

**CXCR4/SDF-1 in Parkinson’s Disease**

The present study drew upon available information regarding neuronal death in Parkinson’s disease, a neurodegenerative disease that is characterized by tremors, similar to MSA. Studies performed on post-mortem Parkinson’s disease (PD) brains and mouse models of PD (MPTP-treated mice) investigated the role of the signaling pair, CXCR4 and SDF-1, in neuronal death. Parkinson’s disease patients express both CXCR4 and SDF-1 at higher levels compared to controls (Shimoji et al., 2009). Activation of CXCR4 by SDF-1 has been suggested to cause neuronal death, specifically dopamine neurons, in Parkinson’s disease (Shimoji et al., 2009). CXCR4/SDF-1 signaling has also been shown to cause neuronal death in HIV encephalitis (Shimoji et al., 2009). Furthermore, studies have shown that post-mortem PD brains exhibit activated Caspase 3, which is the protease expressed during the execution phase of neuronal apoptosis (Mogi et al., 2000; Tatton 2000). This information, together with an Affymetrix DNA microarray on MSA pons tissue revealing up-regulation of CXCR4 mRNA, directed the current study to investigate protein levels of CXCR4, SDF-1, and Caspase 3 in MSA Purkinje cells compared to control Purkinje cells.
Purkinje Cells from MSA Cerebella Characterized in the Current Study

Multiple System Atrophy is a neurodegenerative disease with little information available regarding specific proteins involved in regulating cell death in diseased, dysfunctional brain tissues. However, this thesis shows for the first time that the levels of three proteins involved in neuronal apoptosis, i.e., SDF-1, CXCR4, and Caspase 3, are all significantly up-regulated in MSA Purkinje cells compared to control Purkinje cells. Furthermore, the fact that MSA Purkinje cells are significantly less circular than control Purkinje cells, supports the idea that MSA Purkinje cells are undergoing cellular stress and/or apoptosis. In addition, the fact that control Purkinje cells have a significantly larger area than MSA Purkinje cells, confirms image analysis results indicating that MSA Purkinje cells have significantly more antibody stain for the three proteins measured in the study.

CXCR4 and SDF-1 Expression in Multiple System Atrophy

Although it is unclear why Purkinje cells in MSA degenerate, this study suggests for the first time that CXCR4/SDF-1 may play a role in neuronal death in MSA, as has been shown in Parkinson’s disease (PD) (Shimoji et al., 2009). SDF-1 is a chemokine that has chemoattractant properties, specifically for immune cells, and it also binds to the chemokine receptor, CXCR4, to initiate a signaling cascade (Luster 1998; Luther & Cyster 2001). The research presented in this thesis is the first to quantitatively demonstrate that these two proteins are significantly up-regulated in the Purkinje cells of MSA patients compared to control Purkinje cells (SDF-1, p=0.0195; CXCR4, p<0.0001). It is interesting that both proteins are up-regulated in MSA, and PD, since MSA has so
many similarities to PD, such as behavioral symptoms, neuronal loss and the presence of aggregates containing misfolded alpha-synuclein.

_Caspase 3 Expression in Multiple System Atrophy_

Caspase 3 protein is involved in execution of apoptosis (Mogi et al., 2000; Tatton 2000). During induction of apoptosis, Caspase 3 becomes cleaved into two activated subunits, 11 kDa and 17kDa (Zheng et al., 1999). In a study performed by Zheng and colleagues, neurons treated with SDF-1 increased levels of Caspase 3 when compared to controls (Zheng et al., 1999). Thus, it was important to investigate Caspase 3 levels in MSA Purkinje cells, since our earlier experiments showed that MSA Purkinje cells express SDF-1 at higher levels than controls (Zheng et al., 1999).

The activated 17 kDa subunit of Caspase 3 was investigated in this study to determine if Purkinje cells are experiencing more apoptosis driven by activation of the Caspase pathway, possibly related to CXCR4/SDF-1 signaling. Caspase 3 levels are also of importance because activated Caspase 3 levels are increased in post-mortem PD brains compared to controls (Mogi et al., 2000; Tatton 2000). Similarly, work carried out in the current thesis showed that Caspase 3 levels are significantly higher in MSA Purkinje cells compared to control Purkinje cells (p<0.0001).

_Purkinje Cell Circularity_

This thesis also investigated the circularity of Purkinje cells, and found MSA cells to be less circular than control Purkinje cells (p=0.0033). This is of importance because studies have shown that cells change their cellular membrane morphology during the execution phase of apoptosis, producing cell bodies that lose their robust circularity.
(Coleman et al., 2001). Therefore, both Caspase 3 expression increases and changes in cell morphology indicate that MSA Purkinje cells are undergoing apoptosis. These results are compatible with the suggestion that CXCR4/SDF-1 are playing a central role in Purkinje cell death in MSA.

**Purkinje Cell Area**

The area of MSA Purkinje cells was measured and compared to control Purkinje cells, and it was found that MSA Purkinje cells have a significantly smaller area than control Purkinje cells (p=0.0116). As the previous results stated, MSA Purkinje cells had greater integrated density for the three proteins (SDF-1, CXCR4, Caspase 3) compared to control Purkinje cells. Since integrated density is the product of cellular area and mean gray value of stain, MSA Purkinje cells having less area than controls means that the amount of protein present was relatively higher compared to controls than integrated density measurements indicate.

**CXCR4/SDF-1 as Neurotoxic Molecules**

There have been studies that investigate whether CXCR4/SDF-1 are neurotoxic to neuronal populations by either a direct mechanism, or an indirect mechanism, such as induction of the release of neurotoxins from microglia (Hesselgesser et al., 1998; Meucci et al., 1998; Kaul & Lipton, 1999; Bezzi et al., 2001). Activated microglia secrete neurotoxins, such as pro-inflammatory cytokines that can cause neuronal injury (Shimoji et al., 2009). However, signaling by CXCR4 has the ability to cause neuronal death even when pro-inflammatory cytokines are not present, as illustrated by in vivo models of PD (Shimoji et al., 2009). This suggests that CXCR4/SDF1 signaling has the potential to
cause neuronal death by a direct signaling cascade mechanism, such as the pro-apoptotic Caspase 3 pathway. The fact that CXCR4/SDF-1 have the ability to cause apoptosis without other molecules present strengthens the idea that CXCR4/SDF-1 signaling are involved in apoptosis in MSA. Along with SDF-1 having the ability to induce Caspase 3, there may also be other pro-apoptotic signals involved such as MAPK (p38), as suggested by other studies (Vlahakis et al. 2002).

Involvement in Multiple Sclerosis

SDF-1 has been shown to play a role in inflammation. Calderon and colleagues have shown that SDF-1 is an important factor in the pathogenesis in multiple sclerosis (MS) (Calderon et al., 2006). MS is an autoimmune, inflammatory, demyelinating disease in which there is extensive loss of myelin axons in lesions found within the central nervous system (Calderon et al., 2006). This is of interest because MSA and MS share many similarities, such as oligodendrocyte damage, demyelination, and neuronal injury (Calderon et al., 2006). Specifically, the pathogenic nature of MS involves leukocyte chemotaxis into the central nervous system and the production of inflammatory molecules, thereby causing neuropathology (Calderon et al., 2006). Inflammation, demyelination, and neurodegeneration are intimately linked together (Geurts & Barkhof, 2008). SDF-1 is a chemoattractant for not only both resting and activated T cells, but also monocytes (Calderon et al., 2006). SDF-1 has been investigated in MS as being responsible for chemotaxis of T cells, as well as leukocytes during inflammation (Calderon et al., 2006). Studies have shown that SDF-1 is expressed at a higher level in active lesions, which are lesions that have the presence of inflammatory cell infiltration (Calderon et al., 2006). SDF-1 expression is also higher in active MS lesions when
compared to normal brain (Calderon et al., 2006). This suggests that SDF-1 expression may be an important component in the initial events of the development of MS lesions by recruiting inflammatory cells such as T cells and monocytes (Calderon et al., 2006). The recruitment of these cells further amplifies the inflammatory response which ultimately leads to damage (Calderon et al., 2006). Although MSA is mainly characterized as a neurodegenerative disease, it also has components of chronic inflammation, such as cellular infiltration, for example, CD8 leukocytes, which is similar to MS (Van Wagner et al., 2012). Both diseases present with oligodendrocyte and neuronal injury, as well as demyelination, which are consequences of inflammation. Therefore, it is very likely that the up-regulation of SDF-1 in MSA is playing a role in leukocyte infiltration which is causing damage in MSA, similar to what it has been shown to do in active MS lesions. SDF-1 plays a role in many important processes so it is not surprising that it is possibly amplifying inflammation in MSA as well as playing a role in Purkinje cell death.

Post-mortem analysis on multiple sclerosis brains revealed that there is significant loss of Purkinje cells in MS patients (Kutzelnigg et al., 2007). In a mouse experimental model of MS called experimental autoimmune encephalomyelitis (EAE), it was discovered that Purkinje cell loss clearly correlates with gray matter atrophy in the cerebellum (MacKenzie-Graham et al., 2009). This study found that Purkinje cell density was not compromised during early stages of EAE, but only during later stages of the disease (MacKenzie-Graham et al., 2009). This finding suggests for MSA a possibility that CXCR4/SDF-1 play a role in inflammation to begin with, and secondarily as being involved in neuronal death. These previously mentioned studies present another
similarity that MSA shares with MS, which suggests that studies performed on MS could also possibly be considered as relevant information for MSA.

**CXCR4 in Human Immunodeficiency Virus**

Another disease in which CXCR4 plays a role in is the human immunodeficiency virus (HIV-1) where it acts as a receptor for the glycoprotein gp120 (Lapham et al., 1996; Berson et al., 1996; Feng et al., 1996). Interestingly, it is believed that CXCR4/SDF-1 signaling plays a role in the neuronal injury that is associated with HIV-1 infection (Lavi et al., 1998; Miller et al., 1999; Gabuzda et al., 2000; Martin-Garcia et al., 2002; Rostasy et al., 2003). Specifically, studies have indicated that CXCR4 signaling plays an important role in HIV-induced dementia and neuronal apoptosis (Biard-Piechaczyk et al., 2000; Bezzi et al., 2001; Corasaniti et al., 2001). This further supports that CXCR4/SDF-1 may be causing neuronal death in MSA as it does in HIV.

**Future Analyses**

Immunohistochemistry performed in this study suggests there is a quantitative difference between protein expression of SDF-1, CXCR4, and Caspase 3 in the granule cells between MSA and controls, as well as the Purkinje cells. However, statistical analysis of the granule cells is beyond the scope of this thesis. Future analyses will be performed to investigate this topic in detail.

**Conclusion**

Although the cause of the pathogenesis of MSA is still a mystery, this study suggests a cause for the death of the Purkinje cells in the MSA cerebellum. This work
shows for the first time that the protein levels of CXCR4, SDF-1, and Caspase 3 are all up-regulated in the Purkinje cells of MSA patients compared to controls. In vivo studies on animals would be necessary in order to investigate if CXCR4/SDF-1 signaling is activating Caspase 3, thereby causing neuronal death, similar to what has been performed related to Parkinson’s disease. If CXCR4/SDF-1 are indeed causing Purkinje cell death in MSA as suggested by this study, new therapeutics might be developed to intervene in this pathway. This work not only benefits MSA, but also has the therapeutic potential for drugs that might be used to treat other diseases that involve CXCR4/SDF-1 such as Parkinson’s disease, multiple sclerosis, and HIV.
BIBLIOGRAPHY


From the desk of: Jennifer Wendling Holm

☐ Please take the time to look on every page for corrections and be aware that there may be more than one correction on any given page.

☐ Maintain consistent 1st – 2nd level headings. Those items in your TOC that are in ALL CAPS and flush with the left margin are 1st level headings and should all look the same in the manuscript and start on a new page. The initially capitalized and indented lines in your TOC are 2nd level headings and should all appear the same in your manuscript.

☐ 1.5" left margin throughout for the purposes of binding

☐ All headings should match the TOC exactly

☐ Figure and Table titles should match exactly between the TOC and the text and maintain consistent capitalization patterns

Please return this copy and corrected thesis to me by Wednesday, June 19.

Thanks,

Jennifer