Understanding the Mechanism Involved in Immune-mediated Cognitive Functioning

BY

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Nursing Sciences
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

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This dissertation is dedicated to: my loving husband, Maveni Palu, who provides endless support and sacrifice so that I may realize my dreams; our precious daughters, Olivia and Oreina, who are constant reminders of life’s true joys as well as the fulfillment and happiness attained through curiosity, discovery, and everyday enlightenment; and my parents, Roger and Toyoko Dinger, who exemplify dedication, perseverance, and excellence in all endeavors and emphasized the importance of education throughout my childhood.
ACKNOWLEDGEMENTS

Many graciously gave their time, shared their wisdom, and provided the support and encouragement I needed to complete this dissertation. The members of my dissertation committee: Drs. Mariann Piano, Mi Ja Kim, Colleen Corte, Michael Ragozzino, and Teresita Briones did so collectively, especially with the design and data interpretation. Dr. Piano was my committee chair and pivotal in the data analysis, writing, and presentation of this dissertation and became my advisor when I most needed her leadership and strength to succeed. From the onset of my doctoral preparation, Dr. Kim has been my mentor: I am honored by her support and guidance as a member of my committee as well as my overall academic development, and I am inspired by her amazing character, body of work, contributions to nursing, and world-wide legacy. Dr. Corte provided the basis of fundamental research design and dedicated time for recommendations and reviewing the project. Dr. Ragozzino contributed significantly to the research design and potential interpretation and limitations during its initial proposal and through my defense, providing key insight and expertise in the neurobiology of learning and memory. Most importantly, Dr. Briones has been my research advisor and mentor throughout the doctoral process, and her exceptional contributions have profoundly influenced my overall training, development, and progress and have been essential to the development and completion of this dissertation. Lastly, I also wish to acknowledge the contributions of Dr. Chang Park, who was instrumental during the data analysis and interpretation process, as well as Dr. Laurie Quinn, who facilitated my early biobehavioral research training; both extraordinary individuals provided the endless support and generous advice to complete this project.

I am grateful for the support of many individuals and wish to recognize their significant and memorable contributions toward this dissertation. The research project was conducted at
ACKNOWLEDGEMENTS (continued)

Wayne State University (WSU), in the laboratory of and through the resources and supplies generously provided by the Katharine Faville Endowed Chair of Dr. Briones, and through the generous contributions of the Rosen family through the Seth & Denise Rosen Graduate Student Research Award and through research funding awarded by the National Institute of Nursing Research, NINR (F31NR012096). Drs. Karyn Holm, Marquis Foreman, Mi Ja Kim, Yee-Kin Ho, Laurie Quinn, Michael Ragozzino, James Artwohl, and especially Tess Briones provided the necessary support I needed for the NINR award. In addition, the pilot study presented in the NINR application and in this dissertation was completed with the collaboration of Dr. Hala Darwish, Marilyn Cruz, and Melanie Ortiz. Both Susan Littau and Stephen Ponka provided the indispensable support needed to secure, maintain, and disperse research funds. Drs. Ke Ma and Peter Toth facilitated the training and analysis of all the immunofluorescence staining through UIC’s Research Resources Center, and Dr. Artwohl provided training in surgery and osmotic pump implantation as a veterinarian consultant to the project at UIC. At WSU’s Division of Laboratory Animal Resources, Karen Dean-Christie made significant contributions to additional animal training and research supplies and as a lead consultant to animal health and care, and both Carrie James and Amyre Sabbath provided direct animal care and advice. Lastly, I am grateful for Drs. Jen’nea Sumo and Elizabeth Florez, who I met at DePaul University when my career as a nurse began and whose lasting friendships have sustained me through the doctoral journey and commemorate a brighter and fulfilling future in research.
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<td>C-terminus</td>
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<td>DNA</td>
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<td>df</td>
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<td>ELR</td>
<td>Glutamic Acid-Leucine-Arginine</td>
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<td>Fab</td>
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<td>FITC</td>
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<td>GCL</td>
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<td>MANOVA</td>
<td>Multivariate Analysis of Variance</td>
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LIST OF ABBREVIATIONS (continued)

MCP-1 Monocyte Chemotactic Protein 1
min Minute
MIP-1 Macrophage Inflammatory Protein 1
mRNA Messenger Ribonucleic Acid
N Total Number of Positive Cells Relative to the Sample Volume
NeuN Neuronal Nuclear Antigen
N-terminus NH$_2$- or Amino-terminus
NMDA N-methyl-D-aspartate
NS Normal saline
NE Northeast
NW Northwest
PBS Phosphate Buffered Saline
PI-3 Phosphatidylinositol-3
PKA Protein Kinase A
PLC Phospholipase C
PSA-NCAM Polysiliated Form of the Neural Cell Adhesion Molecule
Q- Positive Stained Cells
RT Room Temperature
SDF-1 Stromal-Cell Derived Factor-1
SE Southeast
SEM Standard Error of the Mean
SGZ Subgranular Zone
SPSS Statistical Package for the Social Sciences
SW Southwest
SSC Saline-Sodium Citrate
SSF Section Sampling Fraction
T Lymphocyte Thymus Lymphocyte
TBS Tris Buffered Saline
TBS-TS Tris Buffered Saline with Triton X-100 and Serum
TSF Thickness of the Sampling Fraction
X Times
v. Version
SUMMARY

Cytokines and chemokines influence cognitive functioning but little is understood about the mechanisms involved. The purpose of this study was to examine the effects of CXCL12/CXCR4 signaling on the different phases of neurogenesis and its relationship to learning and memory using adult male Wistar rats. A unique biobehavioral design was used to evaluate the different stages of neurogenesis with immunostaining markers and to assess hippocampal-dependent learning and memory through behavioral performance in the water maze and Y-maze. Results from behavioral testing demonstrated that CXCR4 antagonism significantly decreased spatial learning and short-term temporal order memory; however, the results regarding neurogenesis as the mechanism associated with these memory impairments were inconclusive due to the study’s limitations. Future studies are needed that are powered and optimized to detect differences in the stages of neurogenesis and that optimally inhibit or enhance CXCL12 signaling function. Identifying the mechanisms involved in immune-mediated cognitive functioning will identify the targets for therapeutic modulation to optimize learning and memory, especially when impairments sequela disease and injury.
I. INTRODUCTION

A. Forward

The long-term objective of this study was to examine the role of chemokines, immune system molecules, in cognitive functioning. There is increasing evidence that communication exists between the brain and immune system in regulating behavioral responses such as cognitive functioning (Lorton et al., 2007; Yirmiya & Goshen, 2011). For example, evidence exists demonstrating the involvement of cytokines (immune system molecules) such as interleukin-1, in the cognitive impairments seen following central nervous system (CNS) injury (Schultzberg et al., 2007). Since chemokines are similar to cytokines in physiologic function, it is reasonable to suggest that chemokines may also have an affect on cognitive functioning. Chemokines are chemoattractant molecules, and they mediate immunity by directing cells to sites of inflammation (Banisadr et al., 2005; Furze & Rankin, 2008). Our preliminary data as well as reports from others suggest that the specific chemokine, CXCL12 (also known as stromal-cell derived factor, SDF-1), may be involved in cognitive functioning under normal physiological conditions (Kolodziej et al., 2008; Parachikova & Cotman, 2007). What is not clear to date is the underlying mechanism of how CXCL12 and its corresponding receptor, CXCR4, affect the complex CNS function of cognition.

B. Specific Aims and Hypotheses

Cognition is the active intellectual processes through which information is obtained, transformed, stored, and used to help define us as individuals. Cognitive functioning encompasses several domains such as attention, executive function, and learning and memory; however, memory is the axis upon which human cognitive ability revolves (Ryan et al., 1987). Our preliminary data and other findings (Kolodziej et al., 2008; Parachikova & Cotman, 2007)
suggest that CXCL12 is associated with learning and memory under normal physiologic functioning; therefore, the mechanism involving the role of CXCL12/CXCR4 on memory function was examined in this study. It was hypothesized that a possible mechanism underlying the role of CXCL12/CXCR4 on memory function involves the ability of CXCL12/CXCR4 to regulate the different phases of adult neurogenesis in the hippocampus (brain region involved in learning and memory). The proposed study is important because understanding the possible mechanisms that mediate learning and memory is clinically significant for the development of targeted research-based strategies to prevent or eliminate cognitive impairments.

Neurogenesis is the process of generating new neurons from progenitor cells in the mammalian brain and is traditionally viewed as a strictly developmental phenomenon; however, the expanding body of evidence to date demonstrates that new neurons are continually generated throughout adulthood (Altman & Das, 1964; Cameron & McKay, 2001; Eriksson et al., 1998). It is now well established that in several species, including humans, certain regions of the brain, such as the hippocampal formation, contain progenitor cells that continue to produce new neurons well into late adulthood (Altman & Das, 1964; Eriksson et al., 1998; Kempermann et al., 2000). Neurogenesis is a multi-step process that involves progenitor cell proliferation, migration and differentiation with a commitment to a neuronal phenotype, neuron maturation, and synaptic scaffolding into the existing neuronal circuitry. Recently, a relationship between neurogenesis and CXCL12 was observed by Liu et al. (2008). They reported that decreased proliferation of neuronal progenitor cells correlated with decreased CXCL12 levels in adult rats. However, the decreased cell proliferation as reported in this study may not be sufficient to explain the effects of CXCL12 on neurogenesis because evidence in rats shows that a large proportion of proliferating cells die before they reach maturation even under normal conditions and that not all
proliferating cells differentiate into neuronal phenotype (Cameron & McKay, 2001). What is lacking in the present literature are reports on the effects of CXCL12 on the cell maturation and synaptic scaffolding phases of neurogenesis, which are vital phases that address both the physiologic and morphologic maturation as well as the functionality of adult born neurons beyond proliferation. Moreover, current studies on the effects of CXCL12 on neurogenesis (Bhattacharyya et al., 2008; Liu et al., 2008) do not include a concurrent behavioral examination to evaluate the functionality of the newly generated neurons. This scientific gap underscores the importance of examining the effects of CXCL12/CXCR4 signaling on all phases of neurogenesis in parallel with behavioral evaluations specific to learning and memory. The brain region that was examined was the hippocampus because it is an area identified to have neuronal progenitor cells (Altman & Das, 1964; Cameron & McKay, 2001; Eriksson et al., 1998) and is primarily involved in memory processing (Burgess et al., 2002; Lisman, 1999; Squire & Zola, 1998).

The specific aim of the study was to examine the effects of CXCL12/CXCR4 signaling on the different phases of neurogenesis and its relationship to learning and memory. The hypotheses tested were: Rats given a CXCR4 antagonist (to block the receptor and CXCL12 function), when compared to control rats would demonstrate:

1. Decreased proliferation, migration and differentiation into neuronal phenotype, maturation and synaptic scaffolding of new cells produced in the hippocampus. Immunohistochemical markers were used to evaluate these different stages of neurogenesis.

2. Impaired behavioral performance on the learning and memory tasks associated with decreased cell maturation and synaptic scaffolding of new cells produced in the dentate gyrus of the hippocampus. Behavioral performance was evaluated using the water maze and Y-maze to assess hippocampal-dependent tasks associated with learning and memory.
C. Theoretical Framework

The framework guiding this study (Figure 1) is based on the modified theory on brain plasticity, a widely accepted theory on the physiologic basis of learning and memory.

Figure 1. CXCL12/CXCR4 function in learning and memory. CXCL12/CXCR4 function in the CNS involves the regulation of learning and memory. It is believed that the mechanism facilitating the effects of CXCL12/CXCR4 signaling on learning and memory is through the process of neurogenesis. It is possible that CXCL12/CXCR4 signaling can affect one or more processes involved in neurogenesis.

Brain plasticity is the ability to create and maintain memories based upon the brain’s unique properties to receive input from individual experiences and to adapt and remodel itself (Bruel-Jungerman et al., 2007). The area in the brain most often studied in the context of learning and memory is the hippocampus, where the unique electrophysiologic and anatomical properties of
brain plasticity are observed (Kitabatake et al., 2007). The morphological mechanisms that contribute to brain plasticity during learning are modifications in synaptic strength and neuronal network remodeling (Bruel-Jungerman et al., 2007). Adult neurogenesis is a form of brain plasticity and contributes to both synaptic strengthening and neuronal network remodeling because of the unique membrane characteristics of newly generated neurons (Schmidt-Hieber et al., 2004). Learning activities also influence neurogenesis through enhancement of the functionality of new neurons produced by the adult progenitor cells. That is, the excitability and thresholds of activity of the new neurons are enhanced by learning activities affecting their capability of responding to and interpreting new experiences (Bruel-Jungerman et al., 2007; Kitabatake et al., 2007; Ge et al., 2008).

Various molecular interactions direct the processes of neurogenesis (Kitabatake et al., 2007; Zhao et al., 2008), and it is possible that CXCL12/CXCR4 signaling may be one of them. The hypothesized role of CXCL12/CXCR4 signaling in regulating adult neurogenesis in this study was based on the evidence that this chemokine plays a role in mediating the proper formation of the hippocampus during development (Bagri et al., 2002; Lu et al., 2002). But despite evidence on the involvement of CXCL12/CXCR4 signaling during development, to date, there is a lack of information on the role of this chemokine in adult neurogenesis. Since CXCL12/CXCR4 expression in the hippocampus is constitutive and continues from development into adulthood (Lu et al., 2002; Strumm et al., 2002), it was reasonable to suggest that it may also be involved in regulating adult neurogenesis.

Correlative evidence shows the link between adult neurogenesis and learning and memory. For instance, studies demonstrate that spatial learning and memory is better in mice strains having more new neurons (Kempermann & Gage, 2002). Alternatively, reduced
production of new neurons in the hippocampus because of adverse experiences at an early age is associated with poor learning ability (Lemaire et al., 2000). Based on our preliminary data that CXCR4 receptor antagonism resulted in decreased CXCL12 expression and impaired learning and memory, in concert with the correlative findings between neurogenesis and learning and memory, it was hypothesized that activating CXCL12/CXCR4 signaling affects cognitive function by regulating the different phases of adult neurogenesis.

D. Study Rationale

Cognitive function consists of gains and losses: accumulation of knowledge as well as reduced or impaired knowledge acquisition respectively (Liu et al., 2009). Healthy cognitive functioning determines individuality and also quality of life. Factors such as injury and aging can affect normal cognitive functioning and with cognitive impairment, decreased quality of life, increased health care costs, and increased socioeconomic burden on families often ensues. Accordingly, the aim of the proposed study was to examine the mechanisms that mediate normal cognitive functioning so that results from the study may be used as a foundation for the development of scientifically sound strategies to minimize the negative consequences of cognitive impairments. The use of an animal model allowed for tight control of variables while all levels of biological and behavioral systems could be considered. Such an approach was necessary for a full understanding of the mechanisms that mediate cognitive functioning. It must be emphasized that because an intimate relationship exists between learning and memory and the different domains of human cognition, the major cognitive function that was examined in the proposed study was memory processing.
II. REVIEW OF LITERATURE

A. Introduction

Normal cognitive function shapes individuality, allowing adaptation to individual experiences and promoting well-being. Quality of life arguably depends on healthy cognitive functioning. A likely but not well-understood cellular mechanism regulating learning and memory is adult neurogenesis, a life-long process of generating new neurons, which must successfully integrate into an existing neuronal network and may represent new memories. The intricate processes of regulating adult neurogenesis involves many signaling molecules, and it is possible that the chemokine, CXCL12, and its corresponding receptor, CXCR4, may be involved. Thus, background literature on chemokines and the CNS, the role of CXCL12/CXCR4 in the CNS and the role of neurogenesis in learning and memory are reviewed in this section.

B. Chemokines and the Central Nervous System

1. Overview

In 1992, chemokines were first identified as a subclass of cytokines, a large family of polypeptides of the immune system (Rostene et al., 2007) from which they were named. This classification and subsequent designation to CHEMOnubic cytoKINES (Rostene et al., 2011) was based upon their involvement in neutrophil chemotaxis in response to inflammation (Yoshimura et al., 1987) but later discovered to also regulate the physiologic process of immune surveillance by controlling leukocyte migration between the circulatory and lymphatic systems (Bajetto et al., 2002; Banisadr et al., 2005). Until recently, research on chemokines focused on other areas besides the brain because immune system activity is comparatively limited in the CNS. Immune cell entry is restricted and the absence of antigen presenting cells prevents an initiation of immune response (Li & Ransonhoff, 2008). Therefore, studies involving
chemokines and the CNS are relatively few but rapidly growing.

As in other organ systems, the expression of chemokines in the CNS affect pathologic as well as physiologic processes. Most research in this area has concentrated on the pathologic mechanisms of chemokines that mediate inflammation and neurodegeneration in specific brain disorders or injury (Banisadr et al., 2005; Rostene et al., 2011) through leukocyte recruitment into the CNS and increased inflammation (Bajetto et al., 2002). Among these studies, a few have found a behavioral consequence of chemokines during these pathologic states, such as their involvement in cognitive functioning. Chemokine involvement with dementia in acquired immune deficiency syndrome (AIDS) was one of the first cases to demonstrate immune-mediated affects on cognitive impairment (Bajetto et al., 2002; Rostene et al., 2011); furthermore, recent in vitro findings with cerebroocortical cultures from rats suggest that CXCL12/CXCR4 binding and involvement with gp120 promotes neurotoxicity that may be counteracted by the neuroprotective affects of macrophage inflammatory protein 1 beta (MIP-1β) binding to its receptor, cysteine-cysteine chemokine receptor 5 (CCR5) (Kaul et al., 2007).

Another association between chemokines and cognitive functioning is with decreased CXCL12 levels in patients with dementia in Alzheimer’s disease (Parachikova & Cotman, 2007). There have also been correlations between elevated serum chemokines: eotaxin, macrophage inflammatory protein 1 alpha (MIP-1α), and monocyte chemotactic protein 1 (MCP-1) with delirium in post-operative patients after cardiac surgery (Rudolph et al., 2008) suggesting a systemic influences of these chemokines on cognitive functioning.

Chemokines are implicated in mediating cognitive impairments involving inflammatory and neurodegenerative conditions, but they are also constitutively present in the CNS during health and under normal physiologic conditions. Identifying and understanding how chemokines
mediate physiologic processes is an important step to the success of therapeutic interventions targeting pathologic conditions. These interventions aim to regulate chemokines associated with neuroinflammation and neurodegeneration; however, the potential to interrupt important physiologic processes also needs to be considered.

2. Chemokine Structure

Chemokines are 8 to 14 kDa in molecular weight and consist of 60 to 100 amino acids in which the N-terminus cysteine residue positions are conserved (Banisadr et al., 2005). These cysteine positions dictate the classification and nomenclature of the chemokines within four subclasses: CXC, CC, CX3C, and C (Murphy et al., 2000). The N-terminus of the chemokines is believed to bind corresponding chemokine receptors (Banisadr et al., 2005) and initiate signal transduction through receptor coupled G or guanosine triphosphate binding proteins (Neptune & Bourne, 1997). Binding specificity between chemokines and chemokine receptors is usually not specific; for example, CXCL12 binds two receptors: CXCR4 and CXCR7. However, the CXCR4 receptor only binds CXCL12. The CXCR7 receptor binds with high affinity to CXCL12 as well as CXCL11 (Burns et al., 2006). Therefore, there is potential overlap in receptor activation when these chemokines and receptors are localized and co-expressed on the same cell.

Chemokine receptors belong to the family of G protein coupled receptors (GPCRs) but share characteristics uncommon to the other GPCRs (Bajetto et al., 2001). Chemokine receptors are 340-370 amino acids long, are 25-80% homologous, have an acidic N-terminus, and have a C-terminal tail, which becomes phosphorylated after chemokine binding (Bajetto et al., 2001; Banisadr et al., 2005). Chemokines bind to their corresponding receptors with varying degrees of affinity, subsequently activating intracellular, heterotrimeric G proteins to begin signal transduction usually through the $\beta\gamma$ subunit rather than the $\alpha$ subunit (Bajetto, 2001).
uncoupling of the $\beta\gamma$ subunit is responsible for cell migration, the most common signal transduction response of chemokine receptors activation (Bajetto et al., 2002). This pathway, consisting of phospholipase C (PLC) activation and the elevation of both inositol triphosphate (IP$_3$) and intracellular calcium, is used to measure and verify chemokine receptor activity (Bajetto et al., 2002).

3. **Chemokine Expression and Function in the Central Nervous System**

Fifty three chemokines have been identified in humans (Li & Ransohoff, 2008), and at least 22 identified chemokine receptors are expressed on immune cells as well as other cell types (Bajetto et al., 2002; Guyon & Nahon, 2007). Chemokines and their receptors are present in mammals, birds, and fish, and their amino acid sequence among species is relatively conserved (Bajetto et al., 2002). This evolutionary preservation implies an important physiologic role.

Constitutively expressed chemokines and chemokine receptors in the CNS reside on glial, neuronal, and local immune cells (Rostene et al., 2011). Their expression pattern is either throughout or localized within specific regions of the brain and differs from that induced by pathologic conditions (Adler & Rogers, 2005). Neuronally expressed chemokines and chemokine receptors may be co-localized on the same neuron in select brain regions (Rostene et al., 2011). These chemokines are observed in vesicles of nerve endings (Bhattacharyya et al., 2008) and co-localized with traditional neurotransmitters (Banisadr et al., 2003) suggesting a direct role in neuromodulation and neurotransmission.

4. **Summary**

Mounting evidence regarding the constitutive expression of chemokines and chemokine receptors and their homology among species has increased research interest to understand the significance of chemokines and their role in neurophysiologic processes of the CNS. Current
findings indicate that the role of chemokines in physiologic processes may be generalized into
two main functions: 1) mediating cell proliferation and chemotaxis during cell migration and
differentiation, analogous to their role in the immune system, and 2) mediating functions unique
to the nervous system, such as neuromodulation and plasticity (Bajetto et al., 2002; Banisadr et
al., 2005). Examples of these roles are present in both the developing and adult brain. In this
regard, the most studied chemokine in the CNS is CXCL12 (Banisadr et al., 2005).

C. The Role of CXCL12/CXCR4 in the Central Nervous System

1. Overview

CXCL12 belongs to the CXC group, the largest within the chemokine family. The CXC
chemokines have four conserved cysteines, two of which are separated by one amino acid
residue, denoted by its C-X-C designation (Bajetto et al., 2002). Further subdivision of CXC
chemokines is based upon the amino acid sequence (ELR: glutamic acid, leucine, and arginine)
near the N-terminus, which conveys a chemotactic preference for particular immune cells
(Banisadr et al., 2005). CXCL12 is ELR-negative, so it preferentially targets lymphocytes and
monocytes unlike ELR-positive CXC chemokines, which target neutrophils (Bajetto et al.,
2002). Despite the designation and classification as proteins of the immune system, evolutionary
evidence suggests that the CXC family is primarily associated with the CNS since its presence is
specific to higher vertebrates (Guyon & Nahon, 2007).

CXCL12 was first identified as a secreted factor from a stromal cell line of bone marrow
(Tashiro et al., 1993) and thus referred to as stromal-cell derived factor-1 (SDF-1). Through
alternative messenger ribonucleic acid (mRNA) splicing, three isoforms of CXCL12 have been
characterized in the CNS: α, β, and, most recently, γ in rats (Gleichmann et al., 2000).

CXCL12α is the prototypic isoform, consists of 89 amino acids with a 19 amino acid signaling
peptide, and is the most studied (Lazarini et al., 2003). CXCL12β has four additional amino acid residues at the C-terminus, and CXCL12γ has an additional 30 amino acid C-terminal extension and differs from CXCL12β by a 2.5 kb extension after codon 89 (Lazarini et al., 2003; Stumm et al., 2002). Both CXCL12α and CXCL12β mRNA are predominantly expressed from embryonic to postnatal development, but the CXCL12γ mRNA is expressed mostly in the adult brain (Lazarini et al., 2003). In the brains of adult mice, CXCL12α mRNA are produced in neurons, and CXCL12β mRNA are produced in microvessel endothelial cells; however, neither were found on glia cells (Stumm et al., 2002). The primary CXCL12 structure is conserved across species with only one amino acid difference between humans and mice (Shirozu et al., 1995). The N-terminal region and core of CXCL12 convey the binding affinity and ability to internalize CXCR4, whereas the C-terminal end, an α-helix, is responsible for activating CXCR4 and inducing signal transduction (Tan et al., 2006).

CXCR4 was identified as co-receptor for HIV cell entry into CD 4+ T lymphocytes (Oberlin et al., 1996) and has since been targeted in various studies for its therapeutic potential. Unlike other chemokine receptors, CXCR4 binds exclusively to one ligand: CXCL12. As a GPCR, CXCR4 is a seven-span transmembrane receptor located on the plasma membrane of various cells. In humans and mice, an additional CXCR4 isoform exists (CXCR4-Lo and CXCR4-B, respectively) but whether these isoforms have a differential function from the prototypic receptor in the CNS is unknown (Lazarini et al., 2003). In the cerebral cortex and hippocampus of adult mice, CXCR4 mRNA was constitutively and exclusively expressed on neurons with axonal and somatodendritic targeting suggesting both pre- and post-synaptic receptor functioning (Stumm et al., 2002).
CXCL12 binding to and subsequent activation of CXCR4 leads to G protein coupled signal transduction, the activation/phosphorylation of various kinases and a transient intracellular calcium increase, specifically phosphatidyl-inositol-3 (PI-3) kinase in rodent neuronal progenitor cells and cortical neurons (Guyon & Nahon, 2007). On the membranes of neurons, CXCR4 activation modulates the opening of calcium channels resulting in increased intracellular calcium, activation of second messengers and signaling pathways that result in cellular proliferation, migration, and survival or apoptosis (Guyon & Nahon, 2007; Li & Ransohoff, 2008). Two studies using cultured neurons have shown as association between CXCR4 activation and opposite effects on the cyclic adenosine monophosphate (cAMP) pathway through the Gi protein: the cAMP pathway is inhibited in cultured hippocampal neurons (Liu et al., 2003) or an increase in cAMP elevation leads to protein kinase A (PKA) activation with subsequent CREB phosphorylation and translocation into the nucleus (Chalasani et al., 2003) likely to mediate epigenetic modifications. The diversity of CXCL12/CXCR4 responses and activation of second messengers may be attributed the ability of CXCR4 to homodimerize or heterdimerize as well as its two binding sites and the concentration of CXCL12 (Guyon & Nahon, 2007). CXCR4 has both a docking and signaling site, which may result in either a high or low CXCL12 concentration signaling response, or CXCR5 may desensitize and internalize with high CXCL12 concentrations (Guyon & Nahon, 2007).

Until recently, CXCR4 was the only known receptor binding CXCL12; however, CXCR7 was identified as another high affinity receptor (Burns et al., 2006). CXCR7 has been sequenced in humans, dogs, and rodents, and is highly conserved in these species (Thelen & Thelen, 2008). In humans, it is expressed by neurons in the cerebral cortex and hippocampus and co-expressed with CXCR4 on cultured neurons (Shimuzu et al., 2011). Unlike CXCR4, CXCR7 knockout
mice do not result in a specific brain phenotype of abnormal prenatal development, and receptor activation and signal transduction by CXCL12 binding is not mediated by G-protein coupling (Thelen & Thelen, 2008). However, CXCL12 binding does internalize CXCR7, and evidence suggests that CXCR7 and CXCR4 may heterodimerize and interact intracellularly to exert activity (Shimizu et al., 2011; Thelen & Thelen, 2008). Recently, CXCR7 activation has been reported to inhibit CXCR4 and scavenge CXCL12 (Uto-Konomi et al., 2013). Since little was known about CXCR7 expression and its functional significance in the CNS during the design of this study, only the CXCL12 and CXCR4 signaling system was considered.

2. CXCL12/CXCR4 in the Developing Brain

Evidence suggests that CXCL12/CXCR4 signaling is associated with the development of multiple organ systems, including the brain. CXCR4 is expressed on progenitor cells of various organ tissues (Kucia et al., 2004), and CXCL12/CXCR4 knockouts have defective hearts and large vessels (Kucia et al., 2004) as well as defective liver and hematopoietic systems (Ma et al., 1998). In the brain, CXCL12 and/or CXCR4 knockout mice result in identical, nonviable phenotypes with abnormal cerebellar development (Ma et al., 1998) as well as abnormal development of the neocortex as a result of defective migration and integration of interneurons (Stumm et al., 2003). CXCR4 knockout mice also have abnormal development of the hippocampal dentate gyrus due to defective migration of neural progenitor cells, decreased number of dividing cells, and premature differentiation of neurons (Bagri et al., 2002; Lu et al., 2002). These CXCL12/CXCR4 knockout studies suggest CXCL12/CXCR4 involvement in patterning the neuronal network of the CNS during development by mediating proliferation, migration, differentiation, and integration of neuronal cells.

*In vitro* studies support the significance of CXCL12/CXCR4 in patterning the CNS.
neuronal network. Pujol, Kitabgi, and Boudin (2005) used rat hippocampal cultures from 18 day old embryos and found that CXCL12 was involved in axonal patterning by regulating axon elongation and branching. Pujol et al. also observed a differential expression pattern of CXCR4 in these neurons as they matured, where CXCR4 redistributed from nerve tips to a more broad distribution along axons as well as dendrites. Using cultured cerebellar granule neurons from mice, Arakawa et al. (2003) determined that varying CXCL12 concentration mediates axon formation as well as elongation. A possible mechanism and signaling pathway of axonal elongation by CXCL12/CXCR4 was demonstrated by Chalasani, Sabelko, Sunshine, Littman, and Raper in retinal ganglion cells from chick embryos. Chalasani et al. (2003) demonstrated that CXCL12/CXCR4 mediates the elongation/guidance of growth cones, the growing tips of axons, through Gi protein induced elevation of cAMP, subsequent activation of protein kinase A (PKA), and phosphorylation and translocation of cAMP response element-binding (CREB) into the nucleus. These studies suggest CXCL12/CXCR4 involvement in forming a functional neuronal circuit by mediating neuronal morphogenesis and possibly synaptic integration during embryonic development.

3. **CXCL12/CXCR4 in the Adult Hippocampus**

CXCL12/CXCR4 expression in the CNS continues from embryonic development through adulthood. In the adult brain, CXCL12 and CXCR4 are constitutively expressed in various differentiated cells (Li & Ranshoff, 2008) and in specific brain regions, unlike the other chemokines and chemokine receptors which are more generally dispersed in the brain (Adler & Rogers, 2005). The cerebral cortex and hippocampal formation are two sites of constitutive CXCL12 and CXCR4 expression in adult rodents (Banisadr et al., 2002; Banisadr et al., 2003; Stumm et al., 2002). Moreover, the neurons and perhaps glia in these areas are the constitutive
source of CXCL12. For example, Cajal-Retzius cells, a transient neuron associated with supporting neuronal migration during CNS development (Frotscher et al., 2003) is located in the post-natal hippocampal dentate gyrus and expresses CXCL12 proximal to maturing granular neurons (Berger et al., 2007), suggesting that these cells may be the source CXCL12 that affects adult neurogenesis. Unlike constitutively expressed CXCL12, induced CXCL12 is primarily produced by astrocytes and endothelial cells, detected at non-specific locations and at higher concentrations, and associated with pathologic conditions (Li & Ransohoff, 2008). CXCR4 is constitutively expressed on all CNS cell types in the adult brain: neurons, microglia, astrocytes, oligodendrocytes, and endothelial cells (Li & Ransohoff, 2008). Neurons cultured from the adult human hippocampus and cortex also express CXCR4 (Shimuzu et al., 2011). The continued expression of CXCL12/CXCR4 in specific regions, such as the adult hippocampus, suggests a functional significance analogous to but beyond the role of CXCL12/CXCR4 in development.

CXCL12 and CXCR4 are constitutively expressed in the neurogenic zones of the pre-natal cortex and the pre- and post-natal hippocampus in humans and rodents. CXCR4 expression has been observed on neuronal progenitor cells isolated from the embryonic cortex of humans and rats (Ni et al., 2004; Peng et al., 2004). In mice, Tran, Ren, Veldhouse, and Miller (2004) found that neuronal progenitor cells from the hippocampus of both embryonic and adult mice express CXCR4 mRNA. Likewise, Tissir, Wang, and Goffinet (2004) traced CXCR4 mRNA expression from differentiating neurons in the pre-natal hippocampus to a more localized post-natal expression in the subgranular layer of the dentate gyrus. The dentate gyrus is the main afferent site for excitatory projections to the hippocampus and also one of the sites for ongoing neurogenesis into adulthood (Berger, Li, Han, Paredes, & Pleasure, 2007). Focusing exclusively on the post-natal dentate gyrus of mice, both Berger et al. (2007) and Tran et al. (2004) found
that CXCR4 is expressed by neuronal progenitor cells and immature dentate granule neurons and that CXCL12 is expressed by immature dentate granule neurons. The expression of CXCL12/CXCR4 on neuronal progenitor cells and newly generated granule neurons suggest a significant neurophysiologic role in the adult hippocampus associated to neurogenesis.

More recently, two post-developmental studies show that 1) CXCL12/CXCR4 signaling promoted cell proliferation in neuronal progenitor cells in the hippocampus and mediated GABAergic inputs in that brain region (Bhattacharyya et al., 2008) and that 2) activation of the CXCR4 receptor also supported the survival and differentiation of adult born, immature neurons in the hippocampus (Kolodziej et al., 2008). Together, these studies provide the earliest data on the possible role of CXCL12/CXCR4 in adult neurogenesis under normal physiologic conditions.

Recent evidence and our preliminary data suggest that CXCL12 and CXCR4 may also have a significant role in hippocampal-mediated behavior such as learning and memory. Kolodziej et al. (2008) demonstrated that CXCL12 activation of CXCR4 on newly generated hippocampal neurons in rats was associated with long-term memory. Parachikova & Cotman, (2007) blocked CXCL12/CXCR4 signaling using a pharmacological antagonist, resulting in learning and memory deficits in mice. Similarly, we blocked CXCL12/CXCR4 signaling in rats and also observed cognitive deficits in learning and memory tasks. These studies suggest the plausibility that CXCL12 and CXCR4 are involved in adult brain plasticity. The continued presence of CXCL12/CXCR4 in the adult dentate gyrus area of the hippocampal formation even in the absence of pathology (Lu et al., 2002; Strumm et al., 2002) strongly suggests its involvement in normal physiologic brain functioning.

4. **Summary**

Collectively, these reports provide a general consensus on the importance of
CXCL12/CXCR4 in the developing brain. Even though the findings in these studies are consistent, the major scientific knowledge gap that still exists is the role that CXCL12/CXCR4 plays in adult brain functioning, specifically memory processing. Furthermore, studies to date that show the influence of CXCL12/CXCR4 on adult neurogenesis are still lacking because they only examined the role of this chemokine on the proliferation and to some extent differentiation of neural progenitor cells in the hippocampus; therefore, the fundamental issue involving functionality of the newly generated cells were not addressed. That is, cell proliferation and differentiation are just two of the phases in the neurogenesis process and the other equally important phases (cell maturation and synaptic integration) have not been studied. It should be noted that even though cells proliferate, they do not automatically assume neuronal lineage (Cameron & Mckay, 2001), and more importantly, even under normal physiologic conditions, approximately 50% of these cells die before they mature and form appropriate synaptic connections and become functional neurons in rodents (Dayer et al., 2003).

D. Neurogenesis and Learning and Memory

1. Overview of Neurogenesis

It is now universally accepted that the phenomenon of adult neurogenesis occurs in the hippocampus and olfactory bulb (Balu & Lucki, 2009; Deng et al., 2010), where it is observed consistently in vivo (Zhao et al., 2008); however, neurogenesis has also been observed in other brain regions such as the neocortex (Dayer et al., 2005) and striatum (Bedard et al., 2006). The main afferent site for excitatory projections to the hippocampus is the dentate gyrus, which is part of the hippocampal formation and often studied for ongoing neurogenesis into adulthood as well as the functional relevance of the new neurons to learning and memory (Berger et al., 2007).

Adult neurogenesis is a multi-step process resulting in the production of new neurons in
the adult brain originating from neuronal progenitor cells (Balu & Lucki, 2009; Deng et al., 2010). Neurons are the most fundamental units of the nervous system which process and transmit information; whereas, glia (astrocytes, oligodendrocytes, and microglia) are the resident support cells and important for maintaining function (Zhao et al., 2008). In the adult CNS, neuronal progenitor cells are similar to stem cells except that their ability to proliferate (self-renew) and their multipotency (differentiation into various cell lineages) are limited (Balu & Lucki, 2009). Adult progenitor cells proliferate as they actively undergo mitosis and cell division, but their capacity to differentiate is restricted to one cell type (Balu & Lucki, 2009; Zhao et al., 2008).

The process of adult neurogenesis can be divided into four independent steps, beginning with 1) progenitor cell proliferation, followed by 2) migration and differentiation with a neuronal phenotype commitment, then 3) maturation of the new neuron, and ends with 4) synaptic scaffolding and integration of the new neuron (Balu & Lucki, 2009; Deng et al., 2010). These steps, distinguishable by cellular morphology and molecular markers, are reviewed by Deng et al. (2010) and Balu and Lucki (2009) as they pertained to adult neurogenesis in the hippocampal dentate gyrus.

a. **Proliferation**

Proliferation occurs in the subgranular zone (SGZ), a thin layer approximately three nuclei wide between the dentate granular cell layer (GCL) and dentate hilus. The proliferating adult progenitor cells can be subdivided into progenitors which express both nestin (precursor cell marker protein) and glial fibrillary acid protein (GFAP, an astrocytic marker) or only nestin. The nestin only progenitor cells actively undergo mitosis and incorporate BrdU. Intermediate neuronal progenitors are called D cells and stop expressing nestin and begin to express polysiliated form of the neural cell adhesion molecule (PSA-NCAM).
b. Differentiation and Migration

One week post-birth, new granule neurons differentiate and migrate to the inner GCL, extending some processes but not to full integration. These cells are activated by gamma-aminobutyric acid (GABA). The initiation of neuronal differentiation and migration is associated with doublecortin (DCX), a microtubule-associated protein associated with neuronal lineage in rodents as well as PSA-NCAM expression.

c. Maturation

Two weeks post-birth, the new granule neurons continue to differentiate post-mitotically with dendrites extending towards the molecular layer and axons extending through the hilus to the CA3. DCX is expressed transiently with calretinin (calcium-binding protein) and with neuronal markers neuronal nuclear antigen (NeuN) and calbindin. Because their dentritic spines have not reached the molecular layer, 2 week old granule neuron membranes lack glutamatergic input but receive GABAergic input, which is vital to their survival and maturation.

d. Synaptic Scaffolding

Three week old granule neurons form afferent and efferent connections with the local network. On adult born granule neurons, dendritic spines appear and synapses form from afferent axon fibers from the entorhinal cortex. Likewise, adult born granule neuron axons form synapses on CA3 pyramidal neurons. Between 2 to 3 weeks post-birth, adult born granule neurons transition from excitatory to inhibitory GABAergic inputs as well as to glutamatergic synaptic inputs with N-methyl-D-aspartate (NMDA) receptors associated with neuronal development and plasticity.

At 4 to 6 weeks old, adult born granule neurons continue to maturate and integrate, distinguishable from mature granule neurons by their increased NMDA receptor mediated
synaptic plasticity. After 8 weeks old, adult born granule neurons reach their full maturation and integration potential and are indistinguishable from existing granule neurons.

2. Adult Neurogenesis in the Hippocampus

In the adult hippocampus, adult neuronal progenitor cells reside in the dentate gyrus in an area designated the SGZ and eventually migrate to the GCL where they become granular cell neurons (Zhao et al., 2008) and integrate into the circuitry (Deng et al., 2010). Morphological and physiological maturation of these adult born granular neurons continues for weeks until they resemble the existing dentate granule cell neurons (Deng et al., 2010). Since the hippocampus is necessary for forming new memories and for some learned behaviors (Deng et al., 2010), it can be reasoned that adult neurogenesis may have a functional role in learning and memory.

Adult neurogenesis is a form of brain plasticity that renews the supply of adult progenitor cells and adult born neurons to an existing neuronal network. The unique membrane characteristics of adult born granule neurons contribute to both synaptic strengthening and neuronal network remodeling (Deng et al., 2010; Schmidt-Hieber et al., 2004). At 2 to 3 weeks old, adult born granule cells have high membrane resistance and resting potentials, which may contribute to increased excitability (Deng et al., 2010). At 4 to 6 weeks old, adult born granule cells have increased synaptic plasticity due to their lower thresholds for long-term potentiation (LTP) induction but higher LTP amplitudes (Deng et al., 2010). The unique excitability and increased plasticity of these adult born granule neurons affect their capability of responding to network activity and interpreting new experiences (Bruel-Jungerman et al., 2007; Kitabatake et al., 2007; Ge et al., 2008). Therefore, adult born granule neurons could potentially represent new memories.

3. The Role of Neurogenesis in Learning and Memory
Studies in birds were the first to provide evidence for a positive relationship between adult neurogenesis and learning (Goldman & Nottebohm, 1983; Nottenbohm, 1985). In mammals, several lines of evidence also suggest a correlation between adult neurogenesis and learning. For example, strain differences in the rate of adult neurogenesis in mice have been shown to parallel strain differences in learning. That is, the mice with the fewest number of new neurons performed most poorly during spatial navigation learning in the Morris water maze task (Kempermann & Gage, 2002). Also, numerous conditions that decrease adult neurogenesis in the dentate gyrus of rats are associated with learning impairments. These include, but are not limited to, stress (Mirescu & Gould, 2006), increased levels of circulating corticosteroids (Mirescu & Gould, 2006), and aging (Drapeau et al., 2003). Likewise, adverse prenatal or early life experiences produce persistent reductions in neurogenesis (Lemaire et al., 2000; Mirescu et al., 2004) and reduced learning abilities in adulthood (Huot et al., 2002; Lemaire et al., 2000). On the contrary, conditions that increase the number of immature neurons such as estrogen (Tanapat et al., 1999), environmental complexity (Kempermann et al., 1997), and physical exercise (van Pragg et al., 1999) tend to enhance performance on hippocampal-dependent learning tasks.

Despite the abundance of evidence suggesting a positive correlation between neurogenesis and learning, there are a number of reports in which this relationship has been dissociated or appears to be reversed. For example, strain-dependent differences in rats have been reported on the relationship between hippocampal neurogenesis and spatial navigation learning (Van der Borght et al., 2005). Moreover, conditions of elevated glucocorticoids such as stress and aging diminish cell proliferation in the dentate gyrus, but they do not necessarily result in learning deficits on hippocampal-dependent tasks (Akirav et al., 2004; Bizon & Gallagher, 2003). In fact, stressor exposure has been shown to enhance learning of certain hippocampal-
dependent memory tasks (Leuner et al., 2004; Wood et al., 2001), which may suggest an inverse relationship between the number of new neurons and learning. There are also studies demonstrating no stimulatory effect of learning on adult neurogenesis in that training on various learning tasks either does not alter the number of new neurons in the hippocampus (Snyder et al., 2005) or actually decreases it (Ambrogini et al., 2004; Dobrossy et al., 2003; Olariu et al., 2005).

4. Summary

Taken together, the available evidence on potential correlations between learning and neurogenesis although convincing is still not yet fully established. The reported discrepant findings on the relationship between learning and neurogenesis may be due to: 1) the use of different new cell labeling techniques where studies show that learning both increases and decreases in the number of new neurons, 2) differences in learning and memory measures used which ranged from the simple task of trace eyeblink conditioning to the more involved water maze and delayed-non-matching-to-sample tasks, and 3) different ages of animals used from young to aged adults. Also, incomplete understanding on the unequivocal role of neurogenesis in learning and memory may be partially attributed to the fact that appropriate in vivo methods to directly manipulate and monitor new neuron production in adulthood do not exist at this time. However, the possibility that adult neurogenesis serves some function cannot be ignored given the substantial number of new neurons that are produced in the adult hippocampus of mammals, from rodents to humans (Dayer et al., 2003; Eriksson et al., 1998), and those that mature become synaptically integrated into the brain circuitry and attain the morphological and biochemical characteristics of neurons (Toni et al., 2008).

E. Implications

Until recently, chemokine functions were exclusively associated with the immune system
mainly in mediating the inflammatory response. In the literature reviewed, evidence suggests that chemokines play a role not only in pathologic but physiologic functioning in the CNS. Specifically, the chemokine CXCL12 and its corresponding receptor, CXCR4, have been identified to play an essential role in normal brain development (Lu et al., 2002; Peng et al., 2004; Tran et al., 2004) and to some extent in the early stages of adult neurogenesis (cell proliferation and cell differentiation) (Bhattaharryya et al., 2008; Kolodziej et al., 2008). As well, CXCL12 and CXCR4 have been implicated in learning and memory in a limited number of studies (Kolodziej et al., 2008; Parachikova & Cotman, 2007). However, what is not addressed in the literature to date is the role of CXCL12/CXCR4 signaling on all stages of the neurogenesis process, which will help in our understanding on the role of chemokine signaling in memory function. Given the substantial correlative evidence linking adult neurogenesis to memory processing as presented above, in concert with the role of CXCL12/CXCR4 on memory functioning as seen in our preliminary study, it was hypothesized that CXCL12/CXCR4 signaling influences learning and memory by regulating the different phases of neurogenesis.
I. PILOT STUDY

A. Purpose

The pilot study was conducted to examine whether CXCL12/CXCR4 signaling is involved in learning and memory under normal physiologic conditions.

B. Method

Fourteen male, adult Wistar rats (Harlan Laboratories; Madison, WI) were included in the study and randomly assigned to receive either CXCR4 antagonist (n=7) or normal saline (n=7) injections. Lab personnel other than the investigator prepared the injections, so the investigator was blinded to the random assignments. The CXCR4 antagonist used was AMD3100 given through daily subcutaneous injections for a period of 7 days at 12 µl/day (30 µg/µl). AMD3100 is a specific CXCR4 antagonist (Fricker et al., 2006; Rosenkilde, 2004) and has been shown to have efficacy on CXCR4 receptor blocking in the CNS (Hartman et al., 2010; Rubin et al., 2003). Amount of antagonist treatment was based on the previous studies cited above. The vehicle solution used was normal saline, given as daily subcutaneous injections for a period of 7 days and in volume equal to AMD3100. Behavioral testing was conducted in the afternoon of the last subcutaneous injection. Water maze testing was performed for 4 days followed by 2 days of testing in the spontaneous object recognition task. Animals were sacrificed at the end of the behavioral testing, and brain tissues were analyzed using immunohistochemistry and the stereological method of area fractionation.

C. Results

Rats injected with the CXCR4 antagonists resulted in decreased CXCR4 and CXCL12 expression in the hippocampus (Figure 2).
Figure 2. CXCR4 and CXCL12 staining in the hippocampus. Rats injected with CXCR4 antagonist resulted in significant decreased CXCR4 staining (LEFT): $t=14.96$, df=12, $^*p<0.001$ (left), suggesting that the receptor was blocked. They also rats resulted in significant decreased CXCL12 expression (RIGHT): $t=5.69$, df=12, $^{**}p<0.001$, suggesting a negative feedback mechanism in which CXCR4 blockage results in decreased CXCL12 synthesis and impaired learning and memory. Values are mean ± SEM. $^*p \leq 0.05$. $^{**}p \leq 0.001$.

Decreased CXCR4 expression suggests that CXCL12 receptor binding was prevented. The decreased expression of CXCL12 is consistent with that reported by others and may be due to the negative feedback mechanism. That is, decreased availability of CXCR4 because of prolonged blockage resulted in decreased CXCL12 synthesis (Parachikova & Cotman, 2007). Furthermore, rats that received the CXCR4 antagonist showed increased mean swim latency in the water maze and decreased discrimination ratio in the novel object recognition task (Figure 3).
These data suggest that blocking the CXCR4 receptor leads to decreased CXCL12 availability in the brain resulting in learning and memory impairment.

D. Implications

These findings provide a direct link between CXCL12/CXCR4 signaling and memory processing. However, the mechanism whereby CXCL12 exerts its physiological actions on learning and memory is not clear, which is important to truly understand the role of this chemokine in cognitive function. This study, therefore, was conducted to examine the role of neurogenesis as the possible underlying mechanism on the effects of CXCL12/CXCR4 signaling on learning and memory.
IV. METHODS

A. Design

A cross-sectional design was used, and animals were randomized into groups. The main independent variable was the treatment with AMD3100 (a CXCR4 antagonist). The control group received an equivalent volume of normal saline (NS, 0.9). The dependent variables were the 3 phases of neurogenesis and the behavioral performance results (as determined with water maze and Y-maze) (Figure 4).

B. Sample and Setting

1. Sample

Adult male (3 months old, 350-375 g) Wistar rats (N=72) were used in all experiments. Power analysis was performed (G*Power, Power Analysis Software, v. 3.1, Universität Kiel, Germany) to determine the total sample size of 72 (n=9 per cell or subgroup). The analysis was based on a two-tailed t-test, difference between two independent means, estimating the power of the test (0.80), effect size (d=1.44), and alpha level set at 0.05. The estimated effect size of the main independent variable experimental condition was calculated using preliminary data (d=1.20) and then choosing 20% of this effect size (d=1.44) in order to minimize the number of animals but still maintain significance (Figure 5).
Figure 4. Study design. The three phases of neurogenesis were differentiated by the timing of the bromodeoxyuridine (BrdU) injections relative to euthanasia and are designated as Groups 1, 2, and 3: 1 = proliferation; 2 = migration and differentiation; 3 = maturation and synaptic scaffolding. *Only Group 3 underwent behavioral testing. **Two rats were excluded from the study after osmotic pump insertion.
Figure 5. Sample. **Independent variable: experimental group receives AMD3100 and control group receives NS. Dependent variables: the three phases of neurogenesis (Groups 1, 2, and 3) and behavioral testing (Group 3 only). **Half of the Group 3 rats were excluded from behavioral testing (Group 3’) to examine any confounding effects this testing may have on neurogenesis. **Two rats were excluded from the study after complications indirectly related to osmotic pump implantation.
2. **Setting**

Upon arrival from the breeder (Harlan Laboratories; Madison, WI), rats were housed in standard micro-isolator cages (16.5 x 22.5 x 13.5 cm) within the same animal housing room with controlled temperature (24 °C ± 1.5 °C), humidity, and lighting (12:12 hour light:dark cycle). Throughout the study, animals had free access to food and water. Animals’ baseline weights were taken pre-operatively and monitored throughout the study. All animal-related procedures were approved by the Institutional Animal Care and Use Committee (Protocol# A12-01-11) of Wayne State University and the Animal Care Committee (Protocol# 11-202) of University of Illinois at Chicago (Appendix A) and were in accordance with the U.S. National Institutes of Health “Guide for the Care and Use of Laboratory Animals.”

3. **Acclimation and Randomization**

After animal arrival, they were acclimated to their housing and environmental conditions for at least two weeks prior to beginning experiments. All animals were handled daily. During acclimation, the animals were randomized (Research Randomizer, v. 4.0; Urbaniak & Plous, 2011) to one of two groups (experimental, AMD3100 group or control, NS group) and also to one of three groups for the bromodeoxyuridine injection protocols, representing the three phases of neurogenesis. The randomized results were coded, and the investigator was blind to the random assignments.

4. **Exclusion Criteria**

Over the course of the study, weight loss of ≥ 20% and continuous lethargy were criteria for exclusion from the study; however, none of the animals exhibited these criteria. Two animals were excluded due to complications indirectly related to surgery: anesthesia and self-removal of the osmotic pump implant on post-operative day 1.
C. Materials and Procedures

1. Miniature Osmotic Pump Implantation

AMD3100 (Sigma-Aldrich; St. Louis, MO) is a specific CXCR4 antagonist (Fricker et al., 2006; Rosenkilde, 2004) and has been shown to antagonize CXCR4 in the CNS after subcutaneous administration in mice (Hartman et al., 2010; Redjal et al., 2006; Rubin et al., 2003). AMD3100 was diluted with sterile NS (8.5 mg/mL, 1.7 mg/200 uL) and loaded into osmotic pumps, (Model 2006, ALZET, Durect Co, Cupertino, CA); NS (200 µL) was loaded into pumps implanted in the control group (n=35). Amount of antagonist treatment was based on the pilot study and previous studies (Kolodziel et al., 2008; Rubin et al., 2003). Isoflurane was used for anesthetic induction and maintenance during surgical implantation of the pump subcutaneously in the back (above the scapula and behind the rib cage) of each animal. For short and long term pain management, local anesthetics was given after isoflurane induction via a subcutaneous injection at the incision site of lidocaine (0.5 mg/kg) and bupivicaine (1.5 mg/kg). In addition, 24 hr pre-operative and 24 hr post-operative acetominophen (200 mg/kg) was delivered in the drinking water.

2. Bromodeoxyuridine (BrdU) Injections

BrdU (Sigma-Aldrich; St. Louis, MO) is a synthetic nucleoside and thymidine analogue which incorporates into replicating cells during the S-phase of the cell cycle (Dayer et al., 2003). Because it substitutes thymidine in newly synthesized DNA, BrdU serves as a marker for newly generated cells and may be injected at various time points to monitor the different stages of neurogenesis. The dosage and route of BrdU injections is based on reports that show optimal labeling without triggering cell death (Taupin, 2007). BrdU was dissolved in warm, sterile 0.9 NS and injected intraperitoneally using a 22 µm filter at 75 mg/kg per injection in all animals.
Timing of the BrdU injections relative to euthanasia represents the stage of neurogenesis being examined (Figure 5). To examine cell proliferation, Group 1 rats (n=17) were given a single BrdU injection 4 hours prior to euthanasia. To examine cell migration and differentiation, Group 2 rats (n=18) were given three daily injections starting 10 days prior to euthanasia because newly generated cells differentiate into immature neurons and commit to neuronal lineage in approximately 10-14 days (Christi & Cameron, 2006). To examine cell maturation and synaptic scaffolding, Group 3 rats (n=35) were given three daily injections starting 28 days prior to euthanasia because newly generated cells become post-mitotic, mature neurons and form synapses in 24-28 days (Taupin, 2007; Kee et al., 2007).

3. Behavioral Testing

Only half of the Group 3 rats (cell maturation and synaptic scaffolding, n=17) were subject to behavioral testing beginning on Day 28, which was 3 weeks after the initial BrdU injection. The other half of Group 3, the Group 3’ rats (n=18), did not participate in behavioral testing to examine any confounding effects of behavioral testing on neurogenesis. Behavioral testing was conducted in the water maze and the Y-maze in the afternoon (rats’ active period). Water maze acclimation (1 day) and testing (4 days) was followed by Y-maze acclimation (1 day) and testing (1 day). One day of rest occurred after the final day of water maze testing and one day before Y-maze acclimation. All testing sessions were recorded and later analyzed using a computerized videotracking system (EthoVision® XT, v8.5; Noldus Information Technology, Leesburg, VA). Rats were transported by cart from their housing quarters to the testing area 30 minutes prior to the start of testing. Each rat was taken from their home cage (located outside the testing room) and delivered to the testing apparatus by the investigator. After delivering the
animal to the apparatus, the investigator walked to and remained at a fixed location for the duration of the trial. At the end of each test trial, the animals were removed from the apparatus and returned to their home cages. All handling throughout testing was completed by the same investigator. After daily behavioral testing was complete, the animals were transported by cart and returned to their housing quarters.

a. Water Maze

In the maze, each rat was allowed to swim freely in a large, black pool (180 cm diameter) to locate an escape platform (14 cm diameter) that was submerged 2 cm beneath the water’s surface and served as the task’s goal. The maze was divided into four quadrants: northwest (NW), northeast (NE), southeast (SE), and southwest (SW), and the goal remained in a fixed location (SW quadrant) throughout the test days. Large colored paper of various shapes and painted with black or white patterns were fixed on walls around the maze to serve as visual extra-maze cues. Other extra-maze cues such as room lighting and room noise were held constant. The maze was filled with tepid water (22 °C ± 2 °C) and emptied each day, and a black terra cotta pot was inverted and submerged to serve as the concealed escape platform. In addition, all animals were towel-dried and placed under a heating lamp (250 W, IR bulb) between trials and before returning to home cages, ensuring they were completely dry especially at the end of the testing day.

Total duration for the water maze study was five days: one pretrial day for acclimation and four days of actual testing. One day before the start of testing, a single swim pretrial for acclimation was conducted to decrease animal stress; wherein, each rat was allowed to swim for 10 seconds and then placed on the concealed escape platform, which was positioned in the NE quadrant and away from the designated location (SW quadrant) used throughout testing. The four
test days consisted of 4 trials per day for spatial learning and then one probe trial for memory retention after the final trial on Day 4.

During testing, all animals received 4 trials per day for 4 days with a different semi-random starting location (north, east, west, and south) of the tub for each trial. A minimum of two minutes between trials (retention interval) was used to provide a rest period for the animals and avoid any practice effect. Animals were allowed to swim for a maximum of 120 seconds per trial to prevent fatigue. When an animal did not locate the goal within 120 seconds, it was guided to the goal and allowed to remain there for 15 seconds to allow orientation to visual cues in the room. Likewise, animals that reached the goal within 120 seconds without guidance were allowed to remain there for 15 seconds.

For testing memory retention or long-term memory, one probe trial was included only on Day 4, the final day of water maze testing. The probe trial was conducted after the fourth trial of acquisition with at least a 15 min retention interval between the last acquisition trial and the probe trial. In the probe trial, the goal was removed, and each animal was placed in a pre-assigned randomly chosen starting location of the tub (north, south, east, and west) and allowed to swim freely for 60 seconds.

Performance measures that were analyzed for acquisition training were: escape latency (time for the rat to reach the platform from the start) and path distance (total distance traveled in the tub). For the retention test, quadrant durations, latency to the previous location of the escape platform, correct choice (entries made into the SW quadrant), and choice errors (entire body entries, excluding the tail, made into the NW, NE, and SE quadrants) were analyzed. Swim speed (velocity for the duration of each trial) was also analyzed. All measures were obtained from the videotracking software and independently verified by video playback.
b. **Y-maze**

The Y-shaped maze was used to test novel object recognition and temporal order memory, two forms of short-term memory. As it is named, the maze is a clear, Y-shaped apparatus consisting of a center and two arms, all of equal dimensions (56 cm x 10 cm). This design limits the influence of spatial and contextual information and allows direct assessment of object recognition. Since rats have a spontaneous tendency to explore with an inclination for novelty, they may be evaluated without a training and reward prerequisite. Each rat was placed in the center arm of the Y, within a confined “start” box (16.5 cm x 10 cm) prior to the beginning of the trial. Each trial began once the rat exited the start box through a trap door (raised and lowered by the investigator) and lasted for 1 minute. Animals were acclimated to an empty maze the day before actual testing wherein each rat was allowed to explore the apparatus (56 cm X 10 cm for each arm) for 10 seconds. During testing, five consecutive trials (Figure 6) were conducted (in one day) in which two objects were placed at the distal end of the arms and each rat was allowed free access to explore within the entire area of the apparatus (excluding the start box behind the lowered trap door).
Figure 6. Y-maze phases and trials. In one day, five trials are conducted with specific time delays between trials. During the sample phase, or trial 1 and 2, each rat was allowed to explore two objects (A) and (A’) as a set [AA’] with a 15 minute delay between trial 1 and 2. After a 15 minute delay, the choice phase began with trial 3 in which the animals’ recognition memory was tested by replacing one of the objects (A’) with a novel object (B), and each rat was allowed to explore the set of objects [AB]. On trial 4, after a 30-minute delay from completing trial 3, object (B) was replaced with object (C) for exploration of the set of objects [AC]. To evaluate temporal order memory on trial 5 (60 minutes after the end of trial 4), object A was replaced with object B (explored in trial 2) for exploration of the set of objects [BC].

Use of sample phase objects (A) and (A’) and choice phase objects (B) and (C) were not repeated over time to maintain novelty. To control for possible side preferences during testing, the location of the novel object was randomly alternated between the two arms of the maze for each trial but fixed for all animals to allow for comparison. Exploration time with the objects during the novel recognition memory (Trial 3 and 4) and temporal order memory (Trial 5) was measured and analyzed after sessions were complete using videotracking software (EthoVision® XT, v. 8.5; Noldus Information Technology, Leesburg, VA). Exploration of the object was
operationally defined as time spent sniffing, touching, climbing, and attending with the head directed toward and within 2 cm from the object. From the exploration time of each object, a discrimination ratio was calculated as the difference in exploration time of the novel versus the familiar object which was divided by the total exploration time of these two objects.

4. **Euthanasia and Intracardial Perfusion**

Animals from Group 3 and 3’ were sacrificed at the end of the behavioral testing (n=35) or Day 28 if in Group 1 or 2 (n=35) according to the recommendations of the Panel on Euthanasia of the Veterinary Medicine Association with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (7 mg/kg). After rats reached a surgical plane of anesthesia (absence of limb withdrawal in response to toe pinch), they received a median sternotomy incision to expose the heart for perfusion through the aorta. Prior to starting the infusion pump, a needle was inserted into the left ventricle and the descending aorta was clamped for CNS localization of the perfusate. An incision was made in the right atrium to drain venous blood and perfusate once the infusion pump was started for a continuous flow of: 100 mL of heparinized 0.1 M phosphate buffer (1000 U heparine/1000 mL of phosphate buffer, pH 7.4) and then 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2.

5. **Tissue Preparation**

The entire brain was harvested and preserved in fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) overnight at 4 °C and cyroprotected (30% sucrose in 0.1 M phosphate buffer, pH 7.4) at 4 °C until they were ready for sectioning. The entire hippocampus was mounted on a chuck using Tissue Tek OCT (EMS; Washington, PA) and frozen at -20 °C for sectioning. Coronal sections were made with the Leica CM1850 cryostat (Nussloch, Germany) at 40 μm thickness, collecting every other section rostral to caudal of the entire hippocampal
region. Each section was individually stored at -20°C in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer, pH 7.3).

6. Antibody Staining and Labeling

   a. Immunohistochemistry

   One of every eight sections (640 μm apart) was selected, so three sections from each rat were used for a particular staining and labeling protocol. For detection of BrdU-labeled nuclei, DNA was denatured to expose the antigen before incubation in the anti-BrdU primary antibody. The free-floating sections were initially washed X 3 for 10 min to remove sucrose in either newly prepared phosphate buffered saline (PBS, pH 7.4) for immunohistochemistry or Tris buffered saline (TBS, pH 7.5) for immunofluorescence and subsequently treated with: 1) 0.6% hydrogen peroxide in either PBS (immunohistochemistry) or TBS (immunofluorescence) at room temperature (RT) for 30 min to inactive endogenous peroxidase and washed for 5 min, 2) 50% formamide in 2 X saline-sodium citrate (SSC) at 85 °C for 2 hr to expose antigen binding sites and washed with 2 X SSC at RT for 5 min, 3) 2 N HCl at 37 °C for 30 min, 4) 0.1 M Boric acid, pH 8.5, at RT for 10 min, 5) Tris Buffered Saline (TBS, pH 7.5) at RT for 2 min X 4 to wash, and then 6) TBS-TS (0.2% Triton X-100 and 5% serum in TBS) at RT for 1 hr to incubate and block non-specific binding. Hippocampal sections were incubated overnight at 4 °C with an anti-BrdU purified monoclonal antibody (clone BU-33, Sigma-Aldrich; St. Louis, MO) diluted 1:400 in TBS-TS. To minimize intergroup and interbrain staining variability and to ensure reproducibility of results, tissues from all experimental groups were run simultaneously and under identical conditions. Controls were performed to rule out nonspecific immunostaining by eliminating the primary antibody from the protocols.

   After the overnight anti-BrdU antibody incubation, the sections were washed X 2 in PBS,
pH 7.3, rinsed for 10 min X 2 in TBS-TS, and incubated for 1 hr at RT with a biotinylated secondary antibody (horse anti-mouse IgG, Vector Laboratories; Burlingame, CA) diluted 1:200 in TBS-TS. The sections were washed for 5 min X 2 in PBS and for 10 min X 2 in TBS-TS and then incubated in avidin-biotin complex (ABC kit, Vector Laboratories; Burlingame, CA) at RT for 1 hr. Immunoreactions were visualized by treating sections with hydrogen peroxide and 3,3’-diaminobenzidine tetrahydrocholoride (DAB) in Tris buffer (pH 7.3). After thorough rinsing for 5 min X 5 in PBS, the sections were mounted on gelatin-coated slides (SouthernBiotech; Birmingham, AL), dried at RT for 3 hr, and coverslipped using DPX mountant (VWR; Leicestershire, England). To increase the staining visibility and include a counter-stain, the sections were outsourced and re-stained directly on the slides.

b. Immunofluorescence Labeling

After the overnight anti-BrdU purified antibody incubation, described previously in Antibody Staining and Labeling, the sections were washed (TBS for 10 min X 1 and for 5 min X 2) and blocked (TBS-TS for 10 min X 2) prior to incubating at RT for 2 hr with the appropriate fluorescence conjugated antibody, diluted 1:200 in TBS-TS, to detect BrdU labeling. Purified rabbit anti-mouse IgG Fab antibody (Jackson ImmunoResearch; West Grove, PA) was diluted 1:100 in TBS-TS and used when the primary antibodies were from the same species as the anti-BrdU antibody. These antibodies detect the specific phenotype acquired by the BrdU positive cells: doublecortin (DCX, cat. AB2253, Millipore; Temecula, CA) for differentiating neurons, synaptophysin (cat. MAB368, Millipore; Temecula, CA) for synaptic scaffolding, NeuN (cat. ABN78, Millipore; Temecula, CA) for mature neurons, and glia fibrillary acidic protein (GFAP, clone G-A-5, Sigma-Aldrich; St. Louis, MO) for astrocytes. For the final antibody labeling, sections were washed (TBS for 10 min X 1 and for 5 min X 2) and blocked
(TBS-TS for 10 min X 2) prior to incubating at RT for 2 hr with the appropriate fluorescence conjugated antibody, diluted 1:200 in TBS-TS. The fluorescent labels used were FITC (lots 102624 and 98581, Jackson ImmunoResearch; West Grove, PA) and either Rhodamine Red (lot 103577, Jackson ImmunoResearch; West Grove, PA) or Texas Red (cat. TI-7000, Vector; Burlingame, CA). The sections were washed in TBS for 10 min X 2 and TBS-TS for 5 min X 3, mounted and dried for 3 hr on uncoated slides, and coverslipped with ProLong Gold Antifade Mount (Invitrogen; Grand Island, NY).

7. **Quantification of Immunolabeling**

Three sections (640 μm apart) selected from the entire hippocampal region of each rat were analyzed and quantified. Within the hippocampus, the subgranular zone of the dentate gyrus was examined to detect BrdU positive cells in all the rats. The tissue sections examined for BrdU immunohistochemistry labeling were from Group 1 rats only, which represented cellular proliferation in neurogenesis. Using immunofluorescence labeling, the tissue sections from Group 2 rats, corresponding to cellular differentiation and migration in neurogenesis, and from Group 3 rats, representing cellular maturation and synaptic scaffolding in neurogenesis, were quantified.

For the immunohistochemistry staining, the Nikon Eclipse E400 confocal microscope at a magnification of 40X was used to visualize the BrdU labeled cells for each tissue section from a randomly positioned, unbiased counting frame on the dentate gyrus.

For the immunofluorescence labeling, the Zeiss Laser Scanning Microscope (LSM) 510 META was used to image the double-fluorescent signals at a magnification of either 25 X or 40 X from each tissue section and quantified. Specific laser settings and parameters were used for a given staining protocol to consistently capture fluorescent signals and to eliminate nonspecific
fluorescent emissions for each tissue section. The images were then used to quantify BrdU and phenotype labeling using the area fractionation formula \(N=\sum Q\times 1/SSF \times 1/ASF \times 1/TSF\), in which \(\sum Q\) is the sum of positive stained cells, SSF is the section sampling fraction (0.0625 µm), ASF is the area of the sampling fraction (0.00749 µm²), and TSF is the thickness of the sampling fraction, which was the cryostat section thickness (40 µm). N or the total number of positive cells relative to the sample volume was determined for the BrdU staining as well as the BrdU stained cells that acquired the phenotype of interest.

8. **Statistical Evaluation**

SPSS v. 21 (IBM SPSS; Armonk, NY) was used to conduct all statistical analysis for a significance level of \(p \leq 0.05\). Statistical procedures included: independent samples t-test, one-way and repeated measures analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and analysis of covariance (ANCOVA). To prevent experimenter bias during statistical evaluation, animal identification numbers were coded when appropriate. All values are reported as mean ± standard error of the mean (SEM) unless otherwise noted.
V. RESULTS

A. Baseline Measures

1. Body Weight

For Groups 1-3, baseline body weight and total weight gain of the NS and AMD3100 groups were compared within each group to determine if there were differences in body weight. Baseline (day 0) and ending (day 28) body weights were measured for each rat and also calculated was total weight gain (day 28 – day 0) (Table I).

Table I.

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Weight (g)</th>
<th>Weight Gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NS</td>
<td>360.1 ± 8.02</td>
</tr>
<tr>
<td></td>
<td>AMD3100</td>
<td>386.2 ± 5.69</td>
</tr>
<tr>
<td>2</td>
<td>NS</td>
<td>378.8 ± 4.71</td>
</tr>
<tr>
<td></td>
<td>AMD3100</td>
<td>375.3 ± 3.82</td>
</tr>
<tr>
<td>3</td>
<td>NS</td>
<td>377.2 ± 6.63</td>
</tr>
<tr>
<td></td>
<td>AMD3100</td>
<td>393.5 ± 8.90</td>
</tr>
</tbody>
</table>

The independent samples t-test was used for comparisons between the NS and AMD3100 groups. Group 1: baseline weight (t=-2.70, p=0.016) and total weight gain (t=-4.78, p=0.001). One-way ANOVA was used for group comparisons: AMD3100 total weight gain (F=3.399, p=0.030). Values are mean ± SEM. *p≤0.05. **p≤0.001.

With the exception of Group 1, no significant differences were found in baseline body weights or total weight gain within each group. Within Group 1, however, the baseline weight and total weight gain differed significantly between the AMD3100 and NS group. In addition, using a
one-way ANOVA, the total weight gain of Group 1 AMD3100 rats was significantly greater than Groups 2 and 3 AMD3100 rats. No differences were found in baseline weight or total weight gain among the NS groups (Table I). Overall, these results suggest that AMD3100 treatment did not affect the rats’ ability to gain weight and adversely affect health and well-being.

2. **Behavioral Testing**

Only Group 3 (n=17) rats underwent behavioral testing, first in the water maze and then in the Y-maze. Parameters evaluated between the groups were latency (time it takes to find the platform), distance, and velocity. Velocity was evaluated as a measure of both swimming and locomotor ability, which can be confounding variables on performance. To determine if there were baseline differences between the NS and AMD3100 groups, these former parameters (latency and distance) were compared using data from the first two trials (Trial 1 and 2) conducted on the first day of testing. Velocity was compared between groups for each day of behavioral testing (ie. days 1-4).
Figure 7. Baseline performance. Day 1 water maze testing comparing latency (LEFT) and distance (RIGHT) between the NS and AMD3100 groups using the independent samples t-test: Latency in Trial 1 ($t=-0.499$, $p=0.625$) and Trial 2 ($t=-0.687$, $p=0.503$); Distance in Trial ($t=0.460$, $p=0.652$) and Trial 2 ($t=0.496$, $p=0.627$). Values are mean ± SEM.
Figure 8. Velocity in the water maze. *Velocity on Day 1-4 was compared between the NS and AMD3100 groups using repeated measures ANOVA: (F(1,15)=1.195, p=0.292). Values are mean ± SEM.*

As shown in Figure 7, no significant differences were found in trial 1 or trial 2 latency or distance between the NS and AMD3100 groups. In addition, swimming velocity was compared on each of the four behavioral testing days, and no differences were found between groups (Figure 8), suggesting that swimming ability was also not a confounding variable in any of the performance results.

B. Water Maze

The water maze was used to test spatial learning and long-term memory. Water maze performance (latency and distance) across time (ie. days 1-4 and across the four daily trials) were compared between groups.
Figure 9. Latency across time. Latency across the four test days (LEFT) and across the four daily trials (RIGHT) was compared within the NS and AMD3100 groups using repeated measures ANOVA: within-subject effect across the days ($F_{(1,66)}=21.722$, $p \leq 0.001$) and across trials ($F_{(1,66)}=3.725$, $p \leq 0.05$); interaction of treatment with time: day*treatment ($F_{(1,66)}=0.401$, $p=0.529$) and trial*treatment ($F_{(1,66)}=0.688$, $p=0.410$). Values are mean ± SEM for each day (LEFT) and for each trial (RIGHT). *$p \leq 0.05$. **$p \leq 0.001$. 
Figure 10. Distance across time. Distance across the four test days (LEFT) and across the four daily trials (RIGHT) was compared within the NS and AMD3100 groups using repeated measures ANOVA: within-subject effect across the days ($F_{(1,66)}=22.677$, $p<0.001$) and across trials ($F_{(1,66)}=7.388$, $p=0.008$); interaction of treatment with time: day*treatment ($F_{(1,66)}=0.267$, $p=0.607$) and trial*treatment ($F_{(1,66)}=0.378$, $p=0.541$). Values are mean ± SEM for each day (LEFT) and for each trial (RIGHT). *$p<0.05$. **$p<0.001$. 
A significant within-subject effect was observed for latency across time (Figure 9) as well as for distance across time (Figure 10) but not for the interaction of treatment with time. For both the NS and AMD3100 groups, there are significant decreases from baseline through day 4, but no significant between group differences. Taken together, these results suggest that all the rats, regardless of treatment, learned to perform the task as demonstrated by the decreasing latency across time with similar decreases in performance magnitude.

Continuing the analysis by focusing on performance only across the four test days, the main effect (between-subject effect) of treatment on latency and distance was examined (Figure 11 and 12). Initially, the main effect of treatment was not significant for latency (p=0.087) or distance (p=0.779) except for a significant difference in latency on day 4 (Figure 11). That is, latency for the AMD3100 group was significantly increased on day 4. Subsequently, an outlier from the NS group was identified (performance was greater than 2*standard deviation above the group mean) and excluded while including velocity as a covariate in the analysis of the main effect of treatment on performance (Figure 12).
Figure 11. Main effect of treatment on performance. The main effect of treatment on latency (LEFT) and distance (RIGHT) was obtained using repeated measures ANOVA: latency ($F_{(1,66)}=3.013$, $p=0.087$) and distance ($F_{(1,66)}=0.080$, $p=0.779$). Post-hoc analysis with MANOVA revealed a significant difference for latency (RIGHT) on day 4 ($F_{(1,15)}=4.991$, $p=0.041$). Values are mean ± SEM. *$p \leq 0.05$. 
Figure 12. Main effect of treatment on performance excluding outliers with velocity as a covariate. The main effect of treatment on latency (LEFT) and distance (RIGHT) after outlier exclusion and including velocity using repeated measures ANCOVA: latency ($F_{(1,58)}=6.127$, $p=0.016$) and distance ($F_{(1,58)}=0.2724$, $p=0.104$). Post-hoc analysis with MANOVA revealed a significant difference in latency (LEFT): day 2 ($F_{(1,58)}=4.470$, $p=0.039$) and day 4 ($F_{(1,58)}=5.526$, $p=0.022$); and in distance (RIGHT): day 2 ($F_{(1,58)}=4.142$, $p=0.046$). Values are mean ± SEM. *$p \leq 0.05$. 
Outlier exclusion resulted in a significant main effect of treatment on latency (p=0.011) but not for distance (p=0.444) from repeated measures ANOVA (figures not included), revealing that the overall latency was significantly greater in the AMD3100 group. After velocity was included using repeated measures ANCOVA, these results remained consistent for latency (p=0.016) and distance (p=0.104) (Figure 13). That is, latency (but not distance) from baseline to day 4 was significantly greater in the AMD3100 group. Post-hoc analysis revealed that latency was significantly greater on day 2 and 4 and that distance was significantly greater on day 2 and approached significance for day 4 (p=0.065) for the AMD3100 group compared to NS group (Figure 13). These results suggest that spatial learning was impaired by AMD3100 treatment after outlier exclusion, demonstrated by the decrease in latency; however, overall distance with velocity as a covariate was not affected by AMD3100 treatment (p=0.104) although a significant decrease in distance was shown on day 2.

The retention test in the water maze was used to examine long-term memory or memory retention. This test consists of a probe trial (as previously described), in which the goal was removed, and latency to the goal location as well as individual quadrant durations were measured. The southwest (SW) quadrant previously contained the goal, so it is considered the target or correct quadrant; whereas, the northwest (NW), northeast (NE), and southeast (SE) quadrants were the incorrect quadrants. Latency and quadrant durations were compared between the NS and AMD3100 group (Figure 13).
Figure 13. Retention test. Latency to the former goal location (LEFT) and quadrant durations (MIDDLE and RIGHT) were measured and compared between the NS (n=9) and AMD3100 (n=8) groups using ANOVA: for latency, $F_{(1,15)}=0.990$, $p=0.335$; for duration in the SW quadrant, $F_{(1,15)}\leq0.001$, $p=0.996$). The paired samples t-test was used to compare the mean duration in the non-SW quadrant versus the SW quadrant within the NS and AMD3100 group (MIDDLE): NS $(t=4.32$, $p=0.003$) and AMD3100 $(t=2.996$, $p=0.020$).

$SW =$ southwest; $NE =$ northeast; $NW =$ northwest; $SE =$ southeast. Values are mean ± SEM. *$p\leq0.05$. 
Neither latency (p=0.335) nor target quadrant duration (p=0.996) were significantly different between the groups (Figure 14). Additional analysis using ANCOVA was completed to include velocity as a covariate; however, the difference between the groups in latency ($F_{(1,14)}=3.333$, p=0.089) and target quadrant duration ($F_{(1,14)}=0.017$, p=0.899) were also not statistically significant. Finally, the duration in the SW quadrant versus duration in the non-SW quadrants (time averaged among the NW, NE, and SE quadrants) were compared within the NS and AMD3100 groups (Figure 13, MIDDLE), and both groups had significantly greater duration in the SW quadrant. These results indicate that all the rats demonstrated long-term memory through spatial bias for the target quadrant; however, memory retention was not significantly impaired (p=0.089) by AMD3100 treatment.

C. Y-maze

Rats were tested in the Y-maze to assess novel object recognition (trials 3 and 4) and temporal order memory (trial 5), two forms of short-term memory. From the exploration time of each object, a discrimination ratio was calculated as the difference in exploration time of the novel versus the familiar object, which was divided by the total exploration time of these two objects. For trials 3-5, the discrimination ratios calculated for each group were compared (Figure 14).
Figure 14. Y-maze testing. Novel object recognition (Trial 3 and Trial 4) as well as temporal order memory (Trial 5) were examined by comparing the mean discrimination ratios between the NS and AMD3100 groups using the independent samples t-test: Trial 3 ($t=-0.895, p=0.385$), Trial 4 ($t=1.616, p=0.127$), and Trial 5 ($t=2.124, p=0.05$). Values are mean ± SEM. *$p<0.05$. 
In trials 3 and 4, no significant difference in the discrimination ratios was found between the groups (Figure 14, LEFT and MIDDLE); however, the difference between the NS and AMD3100 groups in trial 4 was 111% (calculated from mean discrimination ratio of NS, 0.590, and AMD3100, 0.1688, p=0.127) and likely not statistically significant due to the high variance within groups. In contrast, a significant difference was found between groups in trial 5 (Figure 14, RIGHT). These results suggest that AMD3100 treatment impaired short-term memory in the form of temporal order memory but not novel object recognition.

D. Neurogenesis

Three phases of neurogenesis were examined using BrdU staining (cell proliferation, cell migration and differentiation, and cell maturation and synaptic scaffolding), and these phases correspond to Group 1, 2, and 3, respectively. In addition, double staining to identify specific cellular phenotypes was completed using immunofluorescence staining in Group 2 and Group 3 rats. All the data obtained was analyzed to compare the differences in staining between the NS and AMD3100 groups within each respective overall group (Groups 1-3).

1. Cell Proliferation

BrdU immunohistochemical staining for Group 1 rats (n=17) was intended to examine cell proliferation. Because the original staining was undetectable using conventional methods described in the methods section, the tissue sections were outsourced for restaining. Despite these efforts, the BrdU immunohistochemical staining remained undetectable and could not be analyzed. Therefore, BrdU immunofluorescence labeling was used as a substitute to compare the relative difference in cell proliferation between groups (Figure 15).
Figure 15. Group 1: cell proliferation. *Independent samples t-test was used to compare the BrdU immunofluorescence staining between the NS and AMD3100 groups: t=-0.986, p=0.344. Values are mean ± SEM.*

No significant differences were found between the groups (Figure 15). The AMD3100 group showed 34% more BrdU positive cells (calculated from mean NS, 29035 cells, and AMD3100, 40990 cells, p=0.344). These results suggest that AMD3100 treatment may increase cellular proliferation.

2. **Cell Migration and Differentiation**

BrdU immunofluorescence labeling for Group 2 rats (n=18) was used to examine migrating and differentiating cells. The phenotypes of these cells were identified using antibodies specific for doublecortin (DCX), which identifies differentiating neurons (Figure 16),
and antibodies specific for glia fibrillary protein (GFAP), which identifies the astrocytic phenotype (Figure 17).
Figure 16. BrdU/DCX labeling in Group 2: cell migration and differentiation. Microscope images (40X) of DCX labeling (LEFT), BrdU labeling (MIDDLE), and double BrdU/DCX labeling (RIGHT) in a NS rat (TOP) and a AMD3100 rat (BOTTOM). Scale bars are 50 µm.
Figure 17. BrdU/GFAP labeling in Group 2: cell migration and differentiation. Microscope images (25X) of BrdU labeling (LEFT), GFAP labeling (MIDDLE), and double BrdU/GFAP labeling (RIGHT) in a NS rat (TOP) and a AMD3100 rat (BOTTOM). Scale bars are 50 µm.
Figure 18. Group 2: cell migration and differentiation. **DCX (LEFT) and GFAP (RIGHT) immunofluorescence staining of BrdU positive cells.** The independent samples t-test was used to compare differences between the NS and AMD3100 groups for: DCX staining of the BrdU positive cells ($t=1.725$, $p=0.112$) and the GFAP staining of the BrdU positive cells ($t=-1.605$, $p=0.145$). Values are mean ± SEM.
There were no significant differences between the groups in the immunofluorescence staining to identify newly generated migrating and differentiating neurons and astrocytes (Figure 18). The AMD3100 group showed 53% less DCX staining of BrdU positive cells (calculated from mean NS, 13349 cells, and AMD3100, 7743 cells, p=0.112), and 72% more GFAP staining of BrdU positive cells (calculated from mean NS, 8369 cells, and AMD3100, 17762 cells, p=0.145). Taken together, these staining results suggest that AMD3100 treatment may preferentially increase the migration and differentiation of new cells with the astrocytic rather than the neuronal phenotype.

3. Cell Maturation and Synaptic Scaffolding

BrdU immunofluorescence labeling for Group 3 rats (n=17) was used to examine mature neurons using antibodies specific for neuronal nuclear antigen (NeuN, a marker for mature neurons) (Figure 19) and synaptophysin (a marker for synaptic scaffolding).
Figure 19. BrdU/NeuN labeling in Group 3: cell maturation. Microscope images (40X) of NeuN labeling (LEFT), BrdU labeling (MIDDLE), and double BrdU/NeuN labeling (RIGHT) in a NS rat (TOP) and a AMD3100 rat (BOTTOM). Scale bars are 50 µm.
Figure 20. Group 3: cell maturation. *NeuN immunofluorescence staining of BrdU positive cells was compared between the NS and AMD3100 groups using the independent samples t-test: NeuN staining of the BrdU positive cells (t=1.164, p=0.263). Values are mean ± SEM.*
There was little to no staining of synaptophysin in the BrdU positive cells located in the dentate gyrus, so this phenotype was not included in the statistical analysis. No significant differences were found between the treatment groups in the NeuN staining of the BrdU positive cells (Figure 20). The AMD3100 group showed 39% less NeuN staining of BrdU positive cells (calculated from mean NS, 24266 cells, and AMD3100, 16419 cells, p=0.263), suggesting that AMD3100 treatment may decrease the maturation of neurons.

E. Confounding Effects of Behavioral Testing

The neurogenesis subgroups representing cell maturation and synaptic scaffolding are Group 3 and Group 3’, but only Group 3 underwent behavioral testing. Body weight and immunostaining data between the subgroups were compared to examine the potential confounding effect that behavioral testing may have on neurogenesis.

After the completion of behavioral testing and prior to euthanasia, an additional weight was measured only for the Group 3 and 3’ subgroups of neurogenesis on Day 35 to compare ability to gain weight and overall health. Using the baseline body weight from Day 0 (Table I), the overall weight gain before and after behavioral testing (from body weight measured on Day 28 and 35, respectively) was calculated and compared (Table II).
No significant differences in overall weight gain were found between Group 3 and 3’ before and after behavioral testing (Table II).

To determine whether behavioral testing influenced the cell maturation phase of neurogenesis, the immunostaining between Group 3 and 3’ was compared (Figure 21).
Figure 21. Behavioral testing and neurogenesis. *NeuN staining in BrdU positive cells were compared between Group 3 and 3’ rats using the independent samples t-test: NeuN staining in the NS (t=0.525, p=0.607) and AMD3100 (t=-1.215, p=0.243) groups. Values are mean ± SEM.*

There were no significant differences between Group 3 and 3’ in NeuN or GFAP staining in BrdU positive cells in either treatment or control group (Figure 21), suggesting that behavioral testing in the water maze did not have a confounding effect on the maturation of new neurons.
VI. DISCUSSION

A. Introduction

This study investigated the effects of CXCL12/CXCR4 signaling on learning and memory and its relationship to neurogenesis. The chemokine/receptor CXCL12/CXCR4 was primarily chosen because: 1) this chemokine is constitutively expressed in the adult rat brain (Banisadr et al., 2002; Su et al., 2011) and has been previously shown to modulate neuronal firing (Skryzdyelski et al., 2007) in the hippocampus (Bhattacharyya et al., 2008; Marchionni et al., 2010), 2) there is a characterized pharmacological agent available (AMD3100) which blocks CXCL12 function (Fricker et al., 2006; Rosenkilde et al., 2004), and 3) a pilot study demonstrated that systemic administration AMD3100 resulted in decreased CXCR4 and CXCL12 expression in the hippocampus as well as impaired learning and memory in hippocampal dependent tasks.

This study is unique in that it examined the effects of CXCR4 antagonism on all the phases of neurogenesis in parallel with behavioral testing in adult rats. To date, it is one of the most comprehensive examinations of CXCR4 effects on neurogenesis in the hippocampus and cognitive functioning. The hypotheses underlying this study were: 1) rats receiving a CXCR4 antagonist would demonstrate decreased neurogenesis, and 2) CXCR4-induced changes in the maturation and synaptic scaffolding phases of neurogenesis will correlate with decreased cognitive function. Using the water maze and Y-maze simultaneously with BrdU labeling and immunomarkers for neurogenesis was crucial for appropriately addressing the research aims and hypotheses of this study. These methods are the current research standards for examining hippocampal dependent learning and memory and adult neurogenesis in the dentate gyrus of the hippocampal formation.
B. Findings

1. Behavioral Performance

Results from the hippocampal-dependent behavioral testing demonstrated that CXCR4 antagonism significantly decreased spatial learning and short-term temporal order memory. These results correlate with other studies that observed impaired learning and memory during behavioral testing after AMD3100 administration (Kolodziej et al., 2008; Parachikova & Cotman, 2007). Long-term memory was also impaired but was not statistically significantly. Others have reported impaired long-term memory after AMD3100 treatment but were testing recognition of novel objects (Kolodziej et al., 2008) instead of long-term spatial memory using different testing parameters.

Baseline performance and weight comparisons between the NS and AMD3100 demonstrated that the groups were comparable (Figure 7 and 8; Table I) suggesting that AMD3100 treatment did not negatively impact health or locomotor ability. These are important considerations in the context of behavioral testing and performance because these factors could be confounding variables and also indicate that AMD3100 treatment may be exerting effects beyond CXCR4 antagonism and inhibiting CXCL12/CXCR4 signaling function.

In the water maze, rats in both groups over time (days 1-4) demonstrated spatial learning exemplified by a decrease in latency and distance to reach the concealed goal. However, this preliminary analysis did not demonstrate that learning was influenced by CXCR4 antagonism (Figure 9 and 10). Latency is the most robust measure of spatial learning in the water maze (Vohees & Williams, 2006) and was significantly increased with only outlier exclusion (p=0.011), demonstrating impaired spatial learning. After outlier exclusion and using velocity as a covariate, AMD3100 treatment significantly attenuated spatial learning with increased latency
on days 2 and 4 as well as distance on day 2 (Figure 12).

Similarly, all the rats, regardless of treatment, demonstrated long-term memory and learning capability in the probe trial by demonstrating spatial bias for the target quadrant (Figure 13); however, memory retention was not significantly affected \((p=0.330)\) by CXCR4 antagonism. Others report that memory retention is strongly dependent upon the retention interval (Vohees & Williams, 2006), suggesting that greater time delays between intervals corresponded to increased differentiation between the control and AMD3100 groups (Kolodziej et al., 2008). Kolodziej and others (2008) tested long-term memory of novel objects rather than spatial memory but found that increasing the retention interval from 15 min to 24 hr enhanced impairment detection in the AMD3100 group. In this study, the retention interval was at least 15 min but may have been inadequate to detect a significant group difference. By increasing the retention interval to 24 hr or adding additional probe trials at various retention intervals may increase the detection sensitivity for long-term spatial memory in the water maze.

During Y-maze testing, the difference between treatment groups was not significant for novel object recognition but was significant for temporal order memory (Figure 14), indicating that CXCR4 antagonism affects short-term memory in the form of relative recency or temporal order memory. Since novel object recognition was impaired after CXCR4 antagonism in the pilot study, this study finding was unexpected. During actual testing, increased variability in behavioral performance was observed. Some rats displayed decreased exploratory behavior despite pre-testing habituation, observed as delayed exit from the start box and increased time in the center arm. This variability may have contributed to the inability to detect a significant difference between the NS and AMD3100 groups with novel object recognition as detected in the pilot study. The impact of this variability was especially evident during trial 4 when the
difference between groups was 111\% (p=0.127) (Figure 14, MIDDLE) but not significant. Optimizing habituation to the Y-maze apparatus by increasing the duration of the acclimation period or adding habituation trials may decrease the behavioral variability during testing.

2. Neurogenesis

The results from the immunofluorescence staining and analyses suggest that CXCR4 antagonism does not significantly affect cellular proliferation (Figure 15). This and a 34\% increase (p=0.344) in proliferation were unexpected findings. Other studies examining neurogenesis observe a positive correlation between proliferation and CXCL12/CXCR4 signaling (Bhattacharyya et al., 2008). Also, decreased proliferation has been observed after AMD3100 treatment (Kolodziej et al., 2008) and has been correlated with decreased levels of CXCL12 (Liu et al., 2008). Given the contrary findings in other studies and the methodological issues encountered with this staining, the results are inconclusive, and proliferation after CXCR4 antagonism should be reevaluated. In addition, titrating the AMD3100 dosage may provide more conclusive evidence regarding a potential dose-dependent response of CXCR4 antagonism on cellular proliferation and neurogenesis.

The ability of newly generated neurons and astrocytes to migrate and differentiate, and mature (Figures 18) within the dentate gyrus of the hippocampal formation was not significantly affected by CXCR4 antagonism. Although not statistically significant, the observed trend was that CXCR4 antagonism decreased neuronal migration/differentiation by 53\% (p=0.112). This trend was consistent with the study hypothesis and other research findings that demonstrated a positive correlation between CXCL12/CXCR4 function and the migration of neuronal precursors (Tran et al., 2007) as well as the differentiation and maturation of new neurons in the dentate gyrus (Kolodziej et al., 2008). Increasing the sample size may increase the power, especially for
detecting differences in neuronal maturation. In contrast, astrocyte differentiation was enhanced
after CXCR4 antagonism by 72% (p=0.145). Astrocytes secrete CXCL12 and co-express
CXCR4 (Melik-Parsadaniantz & Rostene, 2008), and they mediate learning and memory via
interleukin-1 (IL-1) (Ben Menachem-Zidon et al., 2011). However, research regarding their role
in adult neurogenesis is very limited and needs further investigation.

Since the staining of synaptophysin was very weak in the dentate gyrus and few to no
BrdU positive cells co-expressed synaptophysin, the relationship between synaptic scaffolding
and CXCR4 could not be analyzed. However, the possibility that most of the newly generated
neurons did not form detectable levels of synaptic architecture by 4 weeks cannot be excluded. In
mice, morphological characteristics (including dendritic length/branching and spine density) of
new dentate gyrus neurons were measured, and all increased by 60% from 4 weeks to 4 months
old (van Praag et al., 2002). Therefore, synaptophysin staining should be conducted between 6
weeks to 4 months and optimized to improve detection. In addition, other detection methods such
as Golgi to detect dendritic branching or immunoelectron microscopy to detect synaptic
connectivity should be considered.

Chemokines may modulate neuronal signaling and affect neurogenesis as a result of
performance in cognitive tasks (Williamson & Bilbo, 2013). Increased neurogenesis in the form
of synaptic scaffolding was observed in the hippocampal formation of rats after water maze
testing (Su et al., 2011). Synaptic integration was not measured in this study, but others report
functional integration by 4 weeks (van Praag et al., 2002). Complete functional integration of
new neurons precedes morphological maturation because structural connectivity is experience
driven, occurs over many months as demonstrated in adult rats and mice (Ramirez-Amaya et al.,
2006; van Pragg et al., 2002), and supports hippocampal plasticity.
This study’s results suggest that behavioral testing did not have a confounding effect on the maturation of newly generated neurons of the dentate gyrus (Figure 21). Also, behavioral testing did not affect weight gain (Table II), suggesting that overall health and well-being were maintained regardless of testing.

In summary, we hypothesized that CXCR4 antagonism would demonstrate decreased neurogenesis in all phases and that decreased maturation and synaptic scaffolding would correlate with impaired learning and memory. This hypothesis could not be fully examined because significant differences in neurogenesis were not observed between the NS and AMD3100 groups; however, significant impairment in learning and memory was observed. This study succeeded in detecting impaired learning and memory with AMD3100 treatment since it was specifically powered to accomplish this using preliminary data to calculate the necessary sample size. Because CXCR4 antagonism decreased neuronal migration/differentiation (53%, \( p=0.112 \)) and maturation (39%, \( p=0.263 \)) at levels approaching significance, which correlates with others reporting increased CXCR4 function associated with increased neurogenesis (Kolodziej et al., 2008; Tran et al., 2007), increasing sample size may help reach a definitive conclusion as well as optimizing AMD3100 dosage.

C. Limitations

Subcutaneous dose responses of AMD3100 in the CNS of rodents have demonstrated efficacy without undergoing optimization. In this study, AMD3100 dosage was based on preliminary findings and other studies using mice that demonstrated CXCR4 antagonism in the CNS with systemic AMD3100 administration via subcutaneous injection or miniature osmotic pump delivery (Hartman et al., 2010; Redjal et al., 2006; Rubin et al., 2003). In rats receiving subcutaneous injections, maximum drug concentrations occurred within 1 to 2 hr with renal
excretion (72%) as the primary means of clearance, and a maximum dose limit of 120 μg/kg (42 μg for a 350 g rat) without signs and symptoms of adverse effects: sedation, spasms, and dyspnea (Hendrix et al., 2000). Oral administration in rats was poorly absorbed (Hendrix et al., 2000), but intravenous (IV) administration resulted in a 0.9 hr half-life after a single IV dose or 0.75 hr after repeat dosing (Hendrix et al., 2000). Likewise in humans, oral absorption was undetectable, but subcutaneous doses were similar to IV doses in concentration profiles with only 50% of the peak concentrations (Hendrix et al., 2000). Since the systemic AMD3100 dose response in the CNS is not well understood, optimizing the dose according the administration method as well as to the species, brain region, and phenomenon of interest should yield more robust results especially concerning group differences. This is particular important when antagonizing CXCL12/CXCR4 binding since the two binding sites of CXCR4 are implicated in a high and low CXCL12 response (Guyon & Nahon, 2007).

The ALZET pump was developed in 1970’s and is considered a reliable drug delivery system that delivers at a constant rate and independent of drug properties (Herrlich et al., 2012). Because of continuous administration, the osmotic pump theoretically reduces inconsistencies in dose delivery, confounding variables from animal handling and distress, and overall chronic psychological and physiologic pain and distress. Duration of AMD3100 administration (4 weeks) was necessary to examine all phases of the neurogenesis process. Furthermore, a continuous delivery of AMD3100 was used because it is a more consistent method of administration for the duration needed rather than the alternative of daily subcutaneous injections for 28 days with the potential for unpredictable plasma peaks and valleys. According to manufacturer recommendations (ALZET, Durect Co, Cupertino, CA), delivery may be verified by measuring plasma levels or residual volume. Plasma measurements are the most reliable method but induce
stress that may indirectly confound behavioral testing results. Measuring residual volume occurs after pump use and is less quantitative than measuring plasma levels. AMD3100 plasma levels and the pump residual volumes were not measured in this study. The technology, however, may be temperature sensitive and has been reported to have an increased release initially after implantation due to the temperature increase (Kerenyl & Hartgraves, 1987). Others have shown predictable drug delivery with constant plasma concentrations (Sykes et al., 2006) and do not report inconsistent results between daily injections or osmotic pump delivery of AMD3100 (Rubin et al., 2003).

Quantifying cellular proliferation using the immunohistochemistry staining was difficult, despite outsourcing and section restaining. The overall staining was weak, and BrdU positive cells could not be confidently identified. As an alternative, data from the immunofluorescence staining was analyzed to quantify cellular proliferation with some advantages. Imaging software enhanced fluorescence staining and counting through image brightness and contrast adjustments, and the relative proliferation between the NS and AMD3100 groups could be compared. However, immunohistochemical staining and quantification methods are the current standards to examine cellular proliferation; therefore, results obtained from any other method are lacking in detail and generalizability with published findings.

The immunofluorescence staining for BrdU proliferation was difficult to quantify relative to the other staining protocols. This may have resulted from a combination of factors, such as the low signal to noise ratio of the positive staining, inadequate antibody penetration of the tissue, and unclear cellular delineations without the presence of a positive stain. Also, a single injection of BrdU (75 mg/kg) was used for this group instead of three daily injections (75 mg/kg X3) received by the other neurogenesis groups.
The antibodies used in this study were previously optimized and used to demonstrate high specificity. Also, the free-floating section method was specifically used since it has been demonstrated to produce good antibody penetration on relatively thick sections with high specificity and low background (Briones et al., 2005; 2006). A counter-stain is usually reserved for a few specimens to verify positive, cellular staining; however, incorporating a counter-stain for all specimens would aid quantification during circumstances with a low signal to noise ratio. Employing other strategies to optimize positive staining without simultaneously increasing the background will increase the reliability and validity of quantification results. Possible strategies include: decreasing the section thickness or using an alternative section staining method, eliminating secondary antibodies or finding newer antibodies with higher specificity, and/or re-optimizing the staining protocol by altering antibody concentrations and incubation periods or increasing blocking strength and incubation periods.

Since its discovery as a second receptor to CXCL12 (Balabanian et al., 2005), CXCR7 expression has been discovered in the human hippocampus, in the hippocampus and dentate gyrus of the post-natal rat, and co-expressed with CXCR4 on cultured neurons (Schönemeier et al., 2008; Shimuzuet al., 2011). Little is known about its physiologic role in the adult dentate gyrus or its potential involvement in neurogenesis or learning and memory. A recent study suggests that CXCR7 activation results in CXCR4 inhibition and that CXCR7 is a CXCL12 scavenger (Uto-Konomi et al., 2013). It has also been reported that CXCL12 binding internalizes CXCR7 and proposed that CXCR7 may heterodimerize with CXCR4 and exert activity intracellular (Shimizu, Brown, Sengupta, Penfold, & Meucci, 2011; Thelen & Thelen, 2008). Because of the limited understanding of CXCR7 function proceeding CXCL12 binding and activation as well as its interaction with CXCR4, future studies need to consider CXCR7 when
examining CXCL12/CXCR4 signaling function because of the potential overlap when both receptors are co-expressed on the same neuron.

D. Conclusion

These findings are important in that CXCL12/CXCR4 signaling affects memory processing. However, the results are inconclusive regarding whether the affects of impaired memory processing are attributed to neurogenesis or not. Future studies are needed that are powered and optimized to detect differences in the stages of neurogenesis and that optimally inhibit or enhance CXCL12 signaling function. Other cytokines and chemokines are also involved in both the beneficial immune effects supporting memory, neural plasticity, and neurogenesis as well as the damaging effects characterizing disease, infection, and trauma; however, very little is understood about these influences, including the mechanisms involved (Yirmiya & Goshen, 2011). Using the comprehensive, biobehavioral design of this study will further reveal the role of the immune system in neurogenesis and learning and memory as well as other potential mechanisms that may impact immune-mediated cognitive functioning. Potential mechanisms besides neurogenesis that may affect CXCL12/CXCR4 mediated hippocampal-dependent learning and memory may involve neuromodulation, such as with GABA on granule cells (Bhattacharyya et al., 2008; Guyon & Nahon, 2007) or epigenetic modification through histone acetylation of chromatin, which integrates cell surface signaling, coordinates a transcriptional response, and has implication in learning and memory (Levenson & Sweatt, 2006). Identifying the mechanisms involved in immune-mediated cognitive functioning will isolate the targets for therapeutic modulation to optimize learning and memory, especially when impairments sequela disease and injury.


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Kaul, M., Ma, Q., Medders, K. E., Desai, M. K., & Lipton, S. A. (2007). HIV-1 coreceptors CCR5 and CXCR4 both mediate neuronal cell death but CCR5 paradoxically can also contribute to protection. *Cell Death and Differentiation, 14*, 296-305.


APPENDICES
Appendix A: Wayne State University, IACUC Protocol Approval

### INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

**PROTOCOL # A 12-01-11**

**ANIMAL WELFARE ASSURANCE # A 3310-01**

**TO:** Maria Palu
Adult Health Department
College of Nursing
344 Cohn Bldg.

**FROM:** Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

**SUBJECT:** Approval of Protocol # A 12-01-11
"Understanding the Mechanism Involved in Immune-mediated Cognitive Functioning"

**DATE:** February 14, 2012

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **February 14, 2012** through **December 31, 2014**. The listed source of funding for the protocol is **National Institute of Nursing Research**. The species and number of animals approved for the duration of this protocol are listed below.

<table>
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<tr>
<th>Species</th>
<th>Strain</th>
<th>Qty.</th>
<th>Cat.</th>
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<tbody>
<tr>
<td>RATS</td>
<td>Wistar</td>
<td>72</td>
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**Be advised** that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).
Appendix B: UIC, IACUC Protocol Approval

February 17, 2012

Maria Palu
Biological Sciences
M/C 802

Dear Dr. Palu:

The protocol indicated below was reviewed in accordance with the Animal Care Policies of the University of Illinois at Chicago on 2/15/2012. The protocol was not initiated until final clarifications were reviewed and approved on 2/17/2012. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: Mechanism in Immune-mediated Cognitive Functioning

ACC Number: 11-202

Initial Approval Period: 2/17/2012 to 2/15/2013

Current Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

Number of funding sources: 1

<table>
<thead>
<tr>
<th>Funding Agency</th>
<th>Funding Title</th>
<th>Portion of Proposal Matched</th>
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</thead>
<tbody>
<tr>
<td>NIH</td>
<td>Understanding the Mechanism involved in Immune-</td>
<td>Matched</td>
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<td></td>
<td>mediated Cognitive Functioning</td>
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<tr>
<th>Funding Number</th>
<th>Current Status</th>
<th>UIC PAF NO.</th>
<th>Performance Site</th>
<th>Funding PI</th>
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<tr>
<td>F31NR012096</td>
<td>Funded</td>
<td>2010-05576</td>
<td>Wayne State University</td>
<td>Maria Palu</td>
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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW’s "What Investigators Need to Know about the Use of Animals" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee
BM/5s

cc: BRL, ACC File, Mariann R. Piano, Teresita L. Briones, PAF # 2010-05576
VITA

Education
05/2013 PhD Nursing Science, University of Illinois, Chicago, IL
11/2006 MS Nursing, DePaul University, Chicago, IL
04/2000 BS Chemistry, Brigham Young University, Laie, HI

Employment History
Research Positions and Experience:
09/2012 Research Assistant, High fat diet and alcohol consumption on cognitive functioning, College of Nursing (Tess L. Briones, PhD, RN-Principal Investigator), Wayne State University, Detroit, MI
06/2008-08/2008 Pilot Study-PI, The role of chemokines in immune-mediated cognitive functioning, College of Nursing, University of Illinois at Chicago, Chicago IL
03/2006-11/2006 Master’s Thesis-PI, Isoflavone Regulation of Immune Response, Department of Nursing (Matthew Sorenson, PhD) and Department of Biological Sciences (Talitha Rajah, PhD), DePaul University, Chicago, IL
05/2000-12/2004 Research Associate II, R&D Immunology Department (Homero Sepulveda, PhD - Manager), BD Biosciences - Pharmingen, La Jolla, CA

Clinical Positions:
02/2007-03/2013 Clinical Nurse II/Registry (On-Call), Surgical and Level I Trauma ICU (Timothy Carrigan, PhD, RN, CCRN - Director), Advocate Illinois Masonic Medical Center, Chicago IL
10/2006-11/2006 Student Nurse in Clinical Leadership, Neuro ICU, Northwestern Memorial Hospital, Chicago, IL
06/2006-10/2006 Student Nurse Extern/Assistant, Neuro and Spinal Surgical Unit (Tom Piotroski, BSN, RN - Manager), Northwestern Memorial Hospital, Chicago, IL

Fellowships
01/2011-05/2013 Predoctoral Research Training Fellowship Individual National Research Service Award (F31NR012096), National Institute of Nursing Research, National Institutes of Health
08/2011-06/2012 Diversifying Higher Education Faculty in Illinois Fellowship, Illinois Board of Higher Education
12/2007-08/2009 Predoctoral Research Training Fellowship in Biobehavioral Nursing Research, Institutional National Research Service Award (T32NR007075), National Institute of Nursing Research, National Institutes of Health

Academic Honors and Scholarships
09/2006-05/2013 Nursing Education Scholarship Program, Illinois Department of Public Health
01/2007-05/2013 Bridges to the Doctorate for Minority Nursing Students Program, NIGMS & National Institutes of Health
09/2008-04/2012 Nursing Education Loan Repayment Program, US Department of Health and Human Services
05/2009  The Helen K Grace Diversity Award, Bridges to the Doctorate for Minority Nursing Students Program, College of Nursing, University of Illinois at Chicago

08/2007-12/2007 Board of Trustees Tuition Waiver, Graduate College, University of Illinois at Chicago

09/2005-11/2006 DePaul Nursing Organization Vice President, Department of Nursing, DePaul University

09/2005 Sigma Theta Tau Induction, National Nursing Honor Society

02/2005-08/2005 DePaul Nursing Organization Secretary, Department of Nursing, DePaul University

08/1998-04/2000 Japanese Language Skills Scholarship, Polynesian Culture Center

01/1999 The Katsumi Haruko Kazama Endowed Scholarship, Scholarship and Awards Board, Brigham Young University – Hawaii Campus

**Funded Research**


06/2010 Seth & Denise Rosen Graduate Student Research Award, College of Nursing, University of Illinois at Chicago ($1000). *Understanding the Mechanism Involved in Immune-mediated Cognitive Functioning*. Role: PI


**Presentations**


<table>
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<th>Date</th>
<th>Event Description</th>
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<tbody>
<tr>
<td>06/7-9/2012</td>
<td>PsychNeuroImmunogy Research Society’s 19th Annual Scientific Meeting, <em>Brain, Behavior, and Immunity in Health and Disease</em>, San Diego, CA</td>
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<tr>
<td>04/12/2012</td>
<td>DFI Conference and Diversity Research Forum, <em>Building a Community of Scholars</em>, Diversifying Higher Education Faculty in Illinois Program (DFI) and the Illinois African American and Latino Higher Education Alliance (IALHEA), Chicago, IL</td>
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<tr>
<td>09/13-15/2010</td>
<td>JBI 7th Biennial Joanna Briggs Colloquium, <em>Knowledge in Action: The Next Generation of Evidence-Based Practice</em>, Joanna Briggs Institute, Chicago, IL</td>
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**Certifications and Licensure**

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<th>Date</th>
<th>Certification</th>
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<tr>
<td>06/2011-Present</td>
<td>Pediatric Advance Life Support, American Heart Association</td>
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<tr>
<td>01/2007-Present</td>
<td>Registered Nurse, State of Illinois (041358261)</td>
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<tr>
<td>01/2005-Present</td>
<td>Advanced Cardiac Life Support, American Heart Association</td>
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**Professional Memberships**

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<th>Date</th>
<th>Membership</th>
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<tr>
<td>07/2009-Present</td>
<td>Midwestern Nursing Research Society</td>
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<tr>
<td>09/2005-Present</td>
<td>Sigma Theta Tau, National Nursing Honor Society</td>
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<tr>
<td>09/2004-Present</td>
<td>American Association of Critical Care Nurses</td>
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**Service Experience**

**Mentoring:**

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<th>Role</th>
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<td>05/2008-08/2008</td>
<td>City Colleges of Chicago, Bridges to Baccalaureate, research mentor, Chicago, IL</td>
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<tr>
<td>02/2005-11/2006</td>
<td>Master’s Entry into Nursing Practice, new student mentor, DePaul University, Chicago, IL</td>
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**Community Service:**

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<th>Date</th>
<th>Description</th>
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<tr>
<td>02/2005-03/2006</td>
<td>American Red Cross, hospital volunteer, San Diego, CA</td>
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<td>01/2001-10/2004</td>
<td>Church Youth Group, San Diego, CA</td>
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<td>05/2001-05/2004</td>
<td>Annual Kids’ Day, presenter and activities coordinator, BD Biosciences, La Jolla, CA</td>
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<td>07/2006</td>
<td>National Kidney Foundation of Illinois: Community Health Fair, screening and assessments, Back of the Yards, Chicago, IL</td>
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<tr>
<td>10/2006-01/2007</td>
<td>Community Outreach Program, STI workshop creation and screening services, Alivio Medical Center, Chicago, IL</td>
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<tr>
<td>07/2006</td>
<td>KIPP Ascend Charter School Health Fair, physical assessments, Chicago, IL</td>
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