THE MODULATION OF THE MESOLIMBIC AND NIGROSTRIATAL DOPAMINE PATHWAYS BY CXCL12 AND CXCR4

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Jordan Trecki
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Dissertation Committee:

Ellen M. Unterwald, Ph.D. (Advisory Committee Chair)
Nae J. Dun, Ph.D. (Examining Committee Chair)
Martin W. Adler, Ph.D.
Scott M. Rawls, Ph.D.
Ronald J. Tallarida, Ph.D.
Lynn G. Kirby, Ph.D. (External Reader)

ABSTRACT

THE ROLE OF CXCL12 AND CXCR4 IN THE MESOLIMBIC AND NIGROSTRIATAL DOPAMINE PATHWAYS

Jordan Trecki

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Doctoral Advisory Committee Chair: Ellen M. Unterwald, Ph.D.

The role of chemokines in immune function is clearly established. Recent evidence suggests that these molecules also play an important role in the CNS as modulators of neuronal activity. The chemokine CXCL12 has been identified in several regions of the adult rat brain including the substantia nigra, ventral tegmental area and caudate putamen. CXCR4, a receptor activated by CXCL12, is expressed by dopaminergic neurons in the substantia nigra. The research presented herein explored the behavioral modulation of CXCL12, expression of the CXCR4 receptor in the forebrain of the adult rat and the effect of CXCL12 administration on extracellular dopamine and glutamate release in the medial shell of the nucleus accumbens. This research furthered our understanding of how CXCL12 can affect behavior and suggested that the modulation of cocaine-induced behavior by CXCL12 is due to an interaction with CXCR4 receptors in the mesolimbic and nigrostriatal dopamine pathways.

The data presented tested the effects of intracranial injections of CXCL12 on cocaine-induced locomotion and stereotypic activity in adult male Sprague Dawley rats. Results demonstrate that intracerebroventricular administration of CXCL12 (25 ng/4 µl) 15 minutes prior to cocaine (20 mg/kg IP) produced a significant potentiation of both ambulatory and stereotypic activity as compared to cocaine alone. The effects of CXCL12 were blocked by administration of

the selective CXCR4 antagonist, AMD 3100. Administration of CXCL12 into specific brain regions was performed to further understand the site of action of CXCL12. Bilateral administration of CXCL12 (25 ng/0.5 μl) into the ventral tegmental area 15 minutes prior to cocaine (20 mg/kg IP) significantly potentiated cocaine-induced ambulatory activity, whereas microinjections of CXCL12 into the caudate putamen selectively increased stereotypy. Conversely, administration of CXCL12 into the lateral shell of the nucleus accumbens resulted in an inhibition of cocaine-stimulated ambulatory activity. No alterations in ambulatory or stereotypic activity were observed following CXCL12 administration into the core of the nucleus accumbens.

Immunohistochemistry results showed evidence of CXCR4 within the caudate putamen and lateral shell of the nucleus accumbens. Dual labeling immunofluorescence demonstrated that CXCR4 is co-expressed on cholinergic and GABAergic neurons, including co-localization with the D1 dopamine receptor in both the caudate putamen and lateral shell of the nucleus accumbens. Results demonstrated that CXCR4 is co-expressed with choline acetyl transferase, a marker for cholinergic neurons, with GAD C38, a marker for GABAergic neurons, and with the D1 dopamine receptor, also a marker for GABAergic medium spiny neurons.

High pressure liquid chromatography studies were conducted using brain dialysate collected from microdialysis probes surgically implanted in the medial shell of the nucleus accumbens. Results demonstrated no significant change in extracellular dopamine or glutamate following an acute administration of CXCL12.

The research presented herein sought to determine the behavioral modifications of CXCL12 as well as the localization of CXCR4 in the forebrain of the adult rat. This research also examined changes in extracellular dopamine and glutamate levels following CXCL12 administration. Results demonstrated that CXCL12 does alter the behavioral activity of cocaine. Results also showed that CXCR4 is localized on cholinergic and GABAergic neurons that could be contributing to the behavioral modification. These results have extended our understanding of the complex mechanisms of CXCL12 and CXCR4 in the mesolimbic and nigrostriatal dopamine pathways of the adult rat brain.

DEDICATION

~ To Rachel, for her constant love and support, and for keeping me sane through this process. She has been my rock unto which I was able to lean on and which lifted me higher. She is a magnificent person of whom I am for ever grateful.

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AIDS	acquired immunodeficiency syndrome
AMD 3100	octahydrochloride hydrate
ANOVA	analysis of variance
A/P	anterior / posterior
Asp	aspartic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
Ca	calcium
cAMP	cyclic adenosine 3',5'-monophosphate
ChAT	choline acetyltransferase
CNS	central nervous system
CPu	caudate putamen
DAPI	4′,6-diamidino-2-phenylindole
ddC	2',3'-dideoxycytidine
DAG	diacylglycerol
DAMGO	[D-Ala^2, N-Me-Phe^4, Gly-ol^5]enkephalin
DAT	dopamine transporter
D1R	dopamine receptor
DI	deionized
DOR	delta (δ) opioid receptor
ERK	extracellular signal-related kinase
ETA	ethanolamine
GABA	gamma (γ) amino butyric acid
GAD C38	glutamic acid decarboxylase C38
GFAP	glial fibrillary acidic protein
GIRK	G-protein-activated inward rectifier potassium
GPCR	G-protein coupled receptor
Glu	glutamic acid
gp120	glycoprotein 120
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HVA	high voltage-activated
ICV	intracerebroventricular
i.p.	intraperitoneal
IP_3	inositol 1,4,5 triphosphate
KOR	kappa (κ) opioid receptor
LAccSh	lateral accumbens shell
LE	leading electrolyte
MCH	melanin concentrating hormone

M/L	medial / lateral
MMP	metalloprotease
mRNA	messenger ribonucleic acid
MOR	mu (μ) opioid receptor
mPFC	medial prefrontal cortex
MS	multiple sclerosis
NAcc	nucleus accumbens
NMDA	N-methyl-D-aspartate
NRTI	nucleoside reverse transcriptase inhibitors
PBS	phosphate buffered saline
PE	polyethylene
PI-3	phosphatidylinositol-3
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
SDF-1	stromal cell derived factor-1
SEM	standard error of the mean
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulate
TE	trailing electrolyte
TH	tyrosine hydroxylase
V	ventral
VTA	ventral tegmental area

CHAPTER 1

GENERAL INTRODUCTION

Scientific Rationale

The investigation into the molecular mechanisms and neuromodulatory properties of chemokines includes cancer research, neuroinflammation, immune cell chemotaxis and their involvement in the response to various drugs of abuse. Currently, there are 53 different chemokine ligands binding to 23 known receptors throughout the mammalian system (Bhangoo et al. 2007b; Li and Ransohoff 2008). Since it is virtually impossible to study all of the chemokines simultaneously, studies must be conducted using individual chemokines to identify their mechanism of action as well as investigating their ability to modulate immune and neuronal function.

Chemokines are involved in the pathogenesis and maintenance of chronic pain in both disease-related conditions such as multiple sclerosis (MS) and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) as well as after trauma (White et al. 2005). Further understanding of the effects of chemokines could lead to the development of compounds that can interfere with chemokine function in the treatment of these chronic conditions. The chemokine CXCL12 and its receptor, CXCR4, have been implicated in various neurodegenerative and neuroinflammatory diseases such as HIV/AIDS, Parkinson’s, Alzheimer’s and MS, and thus are potential targets for drug development in the treatment of these diseases. Furthermore, it has been demonstrated that the receptors on leukocytes that mediate the infectivity of HIV-1 are chemokine receptors, specifically the CCR5 and CXCR4 receptors, making them viable targets in the treatment of AIDS.

CXCL12 is one of the few chemokines found in neurons and expressed constitutively, in contrast to many other chemokines found only under neuroinflammatory conditions (White et al. 2007). While previous research has identified CXCL12 and CXCR4 to be involved in many pathological conditions, the following work will explain some of the actions of this chemokine and its receptor in the central nervous system. From neuronal signaling to the immune system, and from pain hypersensitivity to the modulation of cocaine induced activity, CXCL12 and CXCR4 play vital roles in the brain.

The overall aim of the research described in this thesis was to evaluate some of the possible functions of CXCL12 within the central nervous system (CNS) of the adult rat. Specifically, this study explored the expression of CXCR4 in the adult rat brain and determined its cellular localization in the striatum. The studies also investigated the ability of CXCL12 to modulate behavioral activity both by itself and in combination with cocaine. Finally, studies were designed to evaluate extracellular dopamine and glutamate levels in the nucleus accumbens following administration of CXCL12 into the ventral tegmental area (VTA) to determine the effect of CXCL12 on the mesolimbic dopamine system. Key experiments presented herein determined:

1. the localization of CXCR4 within the nucleus accumbens and caudate putamen. CXCR4 localization was determined by immunohistochemical and immunofluorescent techniques.

2. the effects of CXCL12 on the behavioral response to acute cocaine administration. The modulation of cocaine-induced behavior was measured with the use of activity monitors following CXCL12 administration into specific brain regions.

3. the modulation of extracellular dopamine and glutamate within the medial shell of the nucleus accumbens following administration of CXCL12 into the VTA and/or cocaine systemically. Dialysate was collected via microdialysis probes. Dopamine was analyzed by high pressure liquid chromatography (HPLC) and glutamate was quantified using capillary electrophoresis.

Background

Chemotactic cytokines, also known as chemokines, are small secreted proteins that exert their effects through the activation of G protein-coupled receptors (GPCR's). Chemokines were originally discovered based upon their chemoattractant properties and their ability to control leukocyte migration. Most chemokines are not constitutively expressed at high levels, rather their production and secretion is associated with an inflammatory response (White et al. 2007). Chemokines have been shown to play a central role in the inflammatory response pathway and the pathogenesis of chronic inflammatory conditions such as atherosclerosis, arthritis, and irritable bowel syndrome (Charo 2006). Pathological conditions including autoimmune disorders such as MS, neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases, and virus based diseases such as HIV/AIDS and herpes simplex (Streit et al. 2001; Cartier et al. 2005; Ubogu et al. 2006).

Chemokines

Chemokines can be classified into C, CC, CXC or CX3C subfamilies based on the arrangement of the two cysteine residues near the N-terminus (Zlotnik and Yoshie 2000). Chemokine receptors are GPCR's with seven-transmembrane domains that have been highly conserved through their evolution (Fredriksson et al. 2003; Kawasawa et al. 2003; DeVries et al. 2006). To gain receptor designation, a GPCR must bind chemokines with high affinity and specificity and must engage in signaling, such as calcium mobilization (Zlotnik and Yoshie 2000; Li and Ransohoff 2008).

CXCL12

CXCL12, formerly known as SDF-1 (stromal cell derived factor-1), is a member of the CXC chemokine family of pro-inflammatory mediators and is a potent chemoattractant for T cells, monocytes and lympho-hematopoietic cells (Crump et al. 1997). The human gene for CXCL12 is located on chromosome 10, apart from the other CXC chemokines that are all located on chromosome 4 (Shirozu et al. 1995). A recent study has demonstrated that circulating levels of CXCL12 in the blood range from 1177-3572 pg/ml (Dimberg et al. 2007) Two forms of CXCL12 exist, formerly known as SDF-1α (68 residues) and SDF-1β (72 residues), with the four additional residues being found at the C-terminus. An additional form that has been processed at the C-terminus to generate a 67 residue protein was purified from stromal cells and is the commercially available form of CXCL12 (Bleul et al. 1996b). Chemokines average 69% homology between human and mouse, however CXCL12 is identical between these species except for a single amino acid change from valine to isoleucine at position 18 (Shirozu et al. 1995). This homology between species suggests a fundamental role of CXCL12 within mammalian systems.

CXCL12 is considered to be the most ancient member of the cytokine family, existing phylogenetically before any known immune system (Huising et al. 2003). Its early existence suggests the involvement of CXCL12 in roles other than its primary function of leukocyte chemotaxis (Knaut et al. 2003). The first CXCL12 cDNA clone was isolated from a murine bone-marrow stromal cell line via the signal sequence trap method and the gene was originally named SDF-1 (Tashiro et al. 1993).

CXCL12 can be distinguished from other chemokines due to its constitutive expression in neurons. Other chemokines are not expressed under normal condition, but are upregulated in response to a pathological event (White et al. 2007; Bhattacharyya et al. 2008). CXCL12 is expressed in astrocytes, microglia and neurons in various brain regions (Stumm et al. 2002; Banisadr et al. 2003) and has been shown to induce the migration of mouse microglial cells, but not astrocytes (Tanabe et al. 1997). Neuronal expression of CXCL12 has been found in the cerebral cortex, substantia innominata, medial septum, globus pallidus, hippocampus, paraventricular and supraoptic hypothalamic nuclei, lateral hypothalamus, substantia nigra, VTA, caudate putamen and oculomotor nuclei (Banisadr et al. 2003). CXCL12 is important in embryonic development and continues to be a vital component of the CNS through adulthood. In CXCL12 knockout models, animals develop abnormalities of the vasculature and heart (Nagasawa et al. 1996). Deletion of the CXCL12 gene also results in abnormal cerebellar and hippocampal development suggesting an important role of this chemokine in neurogenesis (Ma et al. 1998; Ohtani et al. 1998; Zou et al. 1998; Lu et al. 2002). Lastly, CXCL12 has been identified as a promoter of postmitotic neuron survival by regulating Rb protein. Rb is a transcriptional repressor which controls cell-cycle progression, differentiation and cell survival due to regulation of members of the E2F family of transcription factors. Rb is induced by CXCL12 due to enhanced expression of the neuronal Rb gene (Khan et al. 2008). The promotion of neuronal survival suggests a role for CXCL12 as a neuroprotectant, further accentuating the importance of CXCL12 within the central nervous system.

CXCR4

A receptor for CXCL12, an orphan GPCR receptor, was identified in 1996 and called LESTR/fusin (Oberlin et al. 1996; Bleul et al. 1996a). With the ability to bind to CXCL12, the designation CXCR4 was adopted to replace LESTR/fusin (Murphy et al. 2000). CXCR4 has been shown to be vital for the development of the embryo as CXCL12, acting through CXCR4, regulates the migration of progenitor stem cells that form various tissues (Tachibana et al. 1998; Lu et al. 2002). CXCR4 knockout mice develop abnormal blood vessels, heart and other organs. Mice lacking either CXCL12 or CXCR4 die shortly after birth and exhibit major defects during hematopoiesis, in their vasculature and in their CNS, particularly in the development of the cortex and cerebellum (Nagasawa et al. 1998; Tachibana et al. 1998; Zou et al. 1998; Stumm et al. 2003). CXCR4 is expressed on microglia, astrocytes and neurons of the mature CNS (Tanabe et al. 1997; Ohtani et al. 1998; Bajetto et al. 2001). Neuronal expression of CXCR4, as shown via immunohistochemistry, occurs in the cerebral cortex, globus pallidus, caudate putamen, substantia innominata, supraoptic and paraventricular hypothalamic nuclei, lateral hypothalamus, ventromedial thalamic nucleus, cerebeullum and substantia nigra pars reticulata (Banisadr et al. 2002). CXCR4 is co-localized with cholinergic interneurons in the substantia innominata (Banisadr et al. 2002), in arginine-vasopressin neurons of the supraoptic and paraventricular hypothalamic nuclei (Banisadr et al. 2003), within neurons expressing melanin-concentrating hormone in the lateral hypothalamus (Guyon et al. 2005), on dopaminergic neurons in the SN (Banisadr et al. 2002), on GABAergic neurons of the SN pars reticulata (Guyon et al. 2006) and in Purkinje neurons and granule cells of the cerebellum (Ragozzino 2002).

CXCR7

Until recently, CXCR4 has been thought of as the only receptor activated by CXCL12. However, a second receptor on T lymphocytes, CXCR7, has been identified (Balabanian et al. 2005). Recent studies have shown that CXCR7 is expressed by neurons in various brain regions (Schonemeier et al. 2008). The CXCR7 receptor binds both CXCL12 and CXCL11/ITAC with high affinity; however characteristic chemokine signaling has not been demonstrated (Burns et al. 2006). A connection between CXCR7 and other chemokine receptors was proposed based on the sequence similarity (43%) and identity (32%) with CXCR2 and its chromosomal location, where the gene is found in the vicinity of CXCR4, CXCR2 and CXCR1 on mouse chromosome 1 (Heesen et al. 1998; Moepps et al. 2006). Chemokine receptors have the ability to signal through pertussis toxin sensitive G_i-proteins (Thelen 2001), however coupling of CXCR7 to G-proteins has not been demonstrated (Thelen and Thelen 2008). Other criteria used to classify chemokine receptors includes chemotaxis, receptor-mediated calcium mobilization and the activation of intracellular cascades such as the MAP-kinase or PI-3 kinase pathways (Zlotnik and Yoshie 2000; Li and Ransohoff 2008), however these processes have not been consistently demonstrated for CXCR7 (Burns et al. 2006; Boldajipour et al. 2008; Mazzinghi et al. 2008). Few findings have been able to demonstrate a conclusive interaction between CXCL12 and CXCR7, although CXCR7 has been shown to be vital for CXCL12-mediated cell survival and shown to be involved in the recruitment of progenitor cells and in tissue repair (Mazzinghi et al. 2008). It is possible that CXCL12 could be exerting some of its effects through the CXCR7 receptor, especially under conditions of high concentrations of the peptide. For example, the decrease in dopamine release and depression of calcium currents in the substantia nigra observed following administration of high concentrations of CXCL12 are not blocked by the selective

CXCR4 antagonist AMD 3100 (Guyon et al. 2008), suggesting that CXCL12 under these conditions is working through mechanisms other than activation of CXCR4 receptors.

The CXCR4 receptor antagonist, AMD3100

Octahydrochloride hydrate, more commonly known as AMD3100, was first discovered during investigation into anti-HIV therapies. AMD3100 has anti-HIV activity due to its ability to block CXCR4, which is a co-receptor for X-tropic HIV strains into host cells (Schols et al. 1997; De Clercq 2003). Clinically known as Plerixafor®, AMD3100 is a specific antagonist of CXCR4 (Schols et al. 1997; Donzella et al. 1998). AMD3100 inhibits intracellular calcium signaling and chemotactic responses observed following CXCL12 binding to CXCR4 in vivo. Administered alone, AMD3100 is unable to elicit any intracellular calcium flux, chemotaxis, or to induce CXCR4 internalization, indicating that AMD3100 does not act as a CXCR4 agonist (Hatse et al. 2002). Although no longer being pursued for clinical use in HIV-therapy, AMD3100 is currently being tested in clinical trials (Phase III) to treat non-Hodgkins lymphoma due to its ability to mobilize $CD34^+$ hematopoietic stem and progenitor cells (DiPersio et al. 2009).

CXCR4 Binding Characteristics

CXCR4 contains two sites where CXCL12 binds. The initial interaction involves the binding of CXCL12 (amino acids 12-17) to the N-terminus of CXCR4 (amino acids 10-21), while residues 1-9 of CXCL12 bind to a second region of CXCR4. Amino acids 12-17 are important for optimal binding, however are not sufficient for receptor activation (Crump et al. 1997). It is not known which binding motif is responsible for CXCR4 activation or if binding to both sites could cause an inhibition of the receptor.

Studies have shown that different concentrations of CXCL12 can have opposite effects. At low concentrations, CXCL12 acts as a neuromodulator of dopaminergic neurons by potentiating potassium-induced dopamine release and high voltage-activated calcium currents, but at higher concentrations CXCL12 decreases dopamine release and inhibits calcium currents (Guyon and Nahon 2007). It has been suggested that CXCL12 binds to a single high affinity site at low concentrations (10-100 nM), while at high concentrations (> 1 μM), binding to a second lower affinity site occurs (Guyon and Nahon 2007). A similar dose dependence is observed in T cells, where CXCL12 (<100 ng/ml) acts as a chemoattractant for T-lymphocytes at low doses but is repulsive at higher levels (>1 μg / ml) (Zlatopolskiy and Laurence 2001).

Studies have demonstrated various cellular responses to CXCL12 based upon the concentration administered. There are many possibilities that could explain why CXCL12 can cause a response at a lower dose which is extinguished at higher levels. Theories include the affinity of binding sites, possible heterodimerization of the CXCR4 receptor, desensitization of the CXCR4 receptor, protease truncation of CXCL12 and receptor activation other than CXCR4.

Homodimerization has been suggested as a possible mechanism that could affect the binding properties of CXCR4. Following CXCL12 binding to CXCR4, CXCR4 has been shown to undergo dimerization which may be necessary for proper signaling (Mellado et al. 2001; Toth et al. 2004). This dimerization enables the activation of the JAK/STAT pathway, a regulator of cellular responses to cytokines and growth factors, allowing the subsequent triggering of G-protein dependent signaling events (Vila-Coro et al. 1999). CXCL12, a basic protein, can also dimerize in solution at non-acidic pH. Dependent upon its concentration, CXCL12 could be binding as a monomer or an oligomer to CXCR4 leading to different effects (Hirono et al. 2001; Sadir et al. 2004; Veldkamp et al. 2005). CXCL12 administration at low concentrations (10-100 nM) results in increased membrane current (Guyon et al. 2006) and an increase in locomotion (Skrzydelski et al. 2007; Trecki and Unterwald 2009). Administration of CXCL12 at high concentrations (100 nM – 1 μM) fails to affect membrane current (Guyon et al. 2006) and resulted in a decrease in dopamine release from neurons and depression of calcium currents in the substantia nigra (Guyon et al. 2008). Desensitization and internalization of CXCR4 at high concentrations of CXCL12 could help explain the bell-shaped dose response curve (Stumm and Hollt 2007).

Peptide metabolites are another factor that could alter the response observed following administration of CXCL12. Cleavage of CXCL12 by various enzymes leads to inactive peptides that still retain their binding properties for CXCR4. Dipeptidyl peptidase IV (DPP IV) cleaves the peptide into CXCL12 (3-68) (Proost et al. 1998), leukocyte elastase into CXCL12 (4-67) (Valenzuela-Fernandez et al. 2002), matrix metalloprotease (MMP)-2 into CXCL12 (5-67) (Delgado et al. 2001; Zhang et al. 2003) and cathepsin G into CXCL12 (6-67) (Delgado et al. 2001); all peptides that bind CXCR4 but do not activate the receptor. CXCL12 can be cleaved by leukocyte elastase into two truncated forms, CXCL12 (4-67) and CXCL12 (5-67). CXCL12 (4-67) can inactivate CXCR4 (Valenzuela-Fernandez et al. 2002) while CXCL12 (5-67) can produce neuronal cell death and inflammation following administration into the basal ganglia of mice (Zhang et al. 2003). It has been shown that the enzymes are secreted by cells that are attracted by CXCL12, such as in the case of MMP-2, which is produced by macrophages and activated by neuronal MT1-MPP (Zhang et al. 2003). CXCR4 activation by CXCL12 has been shown to further increase the secretion of MMP (Klier et al. 2001). Reports of cytokine stimulation have also been shown to lead to higher levels of CXCL12 as glial and endothelial cells can release chemokines following activation (Meucci et al. 1998; Ohtani et al. 1998; Lee et al. 2002).

CXCR4 Signaling Mechanisms

CXCL12 binding to the extracellular component of CXCR4 modifies the tertiary structure of the receptor, allowing the intracellular domain to activate heterotrimeric G-proteins (Rot and von Andrian 2004). CXCR4 undergoes desensitization and internalization following ligand stimulation (Haribabu et al. 1997). Signaling pathways activated by CXCL12/CXCR4 are mediated by pertussis toxin-sensitive $G_{\alpha i}$ and G_q proteins and give rise to two distinct signaling pathways. One pathway, involving phosphatidylinositol-3 (PI-3) kinase and extracellular signal regulated kinase (ERK 1/2), has been shown in rodent astrocytes, neuronal progenitors and cortical neurons (Bacon and Harrison 2000; Lazarini et al. 2000; Bajetto et al. 2001; Bonavia et al. 2003). Activated heterotrimer G proteins exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and dissociate into α- and βγ-subunits (al-Aoukaty et al. 1996; Kuang et al. 1996). The α subunit further activates phospholipase C (PLC), causing the generation of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP_3 binds its specific receptor in the endoplasmic reticulum and releases Ca^{2+} from intracellular stores. The calcium acts as a second messenger and its signaling is vital in ligand-induced chemokine receptor activation (Boutet et al. 2001; Gillard et al. 2002). A second pathway involves phospholipase Cβ whose activation leads to an increase in the intracellular calcium in astrocytes, neurons, microglia and cerebellar granule cells (Klein et al. 1999; Zheng et al. 1999; Bajetto et al. 1999a). CXCR4 activation has been shown to modulate high threshold calcium channels (Zheng et al. 1999; Guyon and Nahon 2007) which can stimulate intracellular calcium release and proline-rich tyrosine kinase (PYK2) (Lazarini et al. 2003). In addition, CXCR4 can inhibit cAMP pathways through G_i (Liu et al. 2003).

Modulation of neuronal activity

CXCL12 and CXCR4 are constitutively expressed both in the developing and mature CNS. During early development, CXCR4 is primarily detected in the ventricular zone, subventricular zone and the marginal zone, all areas which specialize in precursors for survival and proliferation (Li and Ransohoff 2008). Studies have shown that within the developing mouse CNS, expression of CXCR4 begins as early as embryonic day 8.5 (E8.5) and is sustained throughout adulthood (McGrath et al. 1999; Tissir et al. 2004; Lieberam et al. 2005). By approximately E15, both CXCL12 and CXCR4 are expressed within the cortex, olfactory bulb, hippocampus and cerebellum, as well as the meninges and endothelium (Jazin et al. 1997; Lavi et al. 1997; McGrath et al. 1999; Bajetto et al. 1999a; van der Meer et al. 2000; Tham et al. 2001; Banisadr et al. 2002; Banisadr et al. 2003; Krumbholz et al. 2006).

As described previously, CXCL12 and CXCR4 are found in various regions throughout the adult brain, including the hippocampus, cerebellum, substantia nigra, caudate putamen and VTA (Banisadr et al. 2002; Banisadr et al. 2003). CXCL12 increases the spontaneous release of glutamate and gamma amino butyric acid (GABA) in melanin concentrating hormone (MCH) neurons and activates a G-protein-activated inward rectifier potassium (GIRK) current (Guyon et al. 2005). CXCL12 modulates the action potential discharge of these neurons in a biphasic manner with effects varying as a function of concentration. At low concentrations (0.1 -1 nM), CXCL12 decreases the frequency of discharge and this effect is blocked by administration of the CXCR4 receptor antagonist, AMD3100. At higher concentrations, the frequency of discharge is increased, however it is not reversed by AMD3100, suggesting the effect is not mediated through CXCR4 (Guyon et al. 2005). CXCL12 (25 nM) has also been shown to enhance excitatory transmission within the adult rat hippocampus. The excitation enhancement was blocked by the

N-type calcium channel antagonist ω-conotoxin GVIA suggesting that CXCL12 acts through a CXCR4-mediated increase in intracellular calcium via N-type calcium currents (Zheng et al. 1999; Guyon and Nahon 2007). In embryonic cultures of rat hippocampus, high concentrations (50-100 nM) of CXCL12 reduce calcium signaling through CXCR4 by inhibition of cAMP (Liu et al. 2003). Studies have identified the expression of CXCR4 by dopaminergic neurons in the substantia nigra and VTA (Banisadr et al. 2002). Guyon and Nahon have demonstrated that CXCL12 can affect the pre- and postsynaptic mechanisms of dopaminergic neurons. Studies have shown that CXCL12 can mediate calcium currents, increase $GABA_A$ postsynaptic currents, and can modulate glutamatergic and GIRK currents all through the activation of CXCR4 (Guyon and Nahon 2007).

CXCL12 has been shown to increase glutamate and GABA synaptic activities in the lateral hypothalamus (Guyon et al. 2005), hippocampus (Zheng et al. 1999), cerebellum (Limatola et al. 2000) and substantia nigra (Guyon et al. 2006). Interestingly, the mechanism by which CXCL12 acts within each of these areas varies from one to another. In the cerebellum, the increase in frequency of $GABA_A$ postsynaptic events in response to CXCL12-mediated CXCR4 activation is thought to occur through an indirect presynaptic mechanism involving glutamate (Limatola et al. 2000). CXCR4 is localized on GABAergic interneurons and glial cells in the cerebellum (Banisadr et al. 2002). CXCR4, although classified as an inhibitory chemokine, can mediate calcium influx which then causes a release of glutamate from the glial cells of the cerebellum. Glutamate can then act upon GABAergic interneurons in the cerebellum, which increases the frequency of $GABA_A$ postsynaptic events. In the substantia nigra, CXCL12 activates CXCR4 and also increases the frequency of $GABA_A$ postsynaptic events, however by a separate and more direct mechanism (Guyon et al. 2006). CXCR4 activation is thought to increase these GABAergic postsynaptic events directly through an increase in calcium influx, which can mediate neurotransmitter release (Guyon et al. 2006). Similarly, glutamate release within the lateral hypothalamus is tetrodotoxin dependent (Guyon et al. 2005) while the glutamate release in the substantia nigra is tetrodotoxin-independent (Guyon et al. 2006). CXCR4 stimulation also modulates voltage dependent sodium and potassium channels in neurons, N-type high-voltage activated calcium channels in dopaminergic neurons of the substantia nigra and presynaptic glutamatergic terminals of the hippocampus (Zheng et al. 1999; Guyon et al. 2005).

Heterologous desensitization of CXCR4

Heterologous desensitization is defined as the desensitization of a GPCR following agonist binding to an unrelated receptor (Szabo et al. 2002). Intracellular signaling processes working via $G_{\alpha i}$ and protein kinases A or C (PKA or PKC) inhibit target receptor signaling and in some instances internalize the receptor (Lefkowitz 1998; Ali et al. 1999; Olbrich et al. 1999; Finley et al. 2008). Regardless of receptor internalization, the final result is the loss of receptor function.

Studies have demonstrated that chemokine signaling can down-regulate the function of another GPCR, the μ-opioid receptor (Szabo et al. 2002; Zhang et al. 2005). Down regulation occurs by heterologous desensitization resulting from the effects of chemokine receptor activation on μ-receptor function (Grimm et al. 1998; Chen et al. 2007). Studies have revealed the heterologous desensitization is bidirectional. Both μ (MOR) and δ-opioid receptors (DOR) can cross-desensitize CCR1, CCR2, CCR5, CXCR1 and CXCR2, but not CXCR4. Conversely, CCR1, CCR2, CCR5, CCR7, CX3CR1 and CXCR4, but not CXCR1 or CXCR2 cross-desensitize with MOR and DOR (Grimm et al. 1998; Honczarenko et al. 2002; Szabo et al. 2002;

Szabo et al. 2003; Zhang et al. 2003; Finley et al. 2008). Recently, it was shown that the κ-opioid receptor (KOR) is also involved in a bi-directional heterologous desensitization with CXCR4 (Finley et al. 2008). It has been shown in CXCR4+/MOR+ neuronal cultures that the selective MOR agonist DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) inhibits both the ERK1/2 and Akt intracellular signaling pathways, known to be activated by CXCL12 (Patel et al. 2006). Both ERK 1/2 and Akt are activated by chemokines in various cell types and are involved in the regulation of cell survival and proliferation (Meucci et al. 1998; Meucci et al. 2000). Furthermore, using cortical neuronal cultures, DAMGO abolished the neuroprotective effect of CXCL12 in N-methyl-D-aspartate (NMDA) neurotoxicity studies (Patel et al. 2006).

Role of CXCL12 and CXCR4 in Immune Function

Chemokines control cells of the immune system by modulating chemotaxis of lymphocytes and by interacting with antigen-presenting cells that reside within the tissues of the lymphatic system. Chemokines can either be inflammatory or homeostatic. During inflammation, they are released from a variety of cell types in response to a stimulus. In times of homeostasis, they are produced and released without any stimulation to the surrounding cells. Although constitutively expressed within the central nervous system (Stumm et al. 2002), CXCL12 has been found to be elevated in conditions such as HIV infection (Ikegawa et al. 2001), MS (Krumbholz et al. 2006) and in conditions that promote pain (Abbadie et al. 2003). CXCL12 and CXCR4 have the ability to modulate cells of the immune system by controlling cellular migration during an inflammatory response.

HIV

CXCR4 was the first identified co-receptor for human immunodeficiency virus (HIV-1) infection of CD4+ lymphocytes (Feng et al. 1996). More recently, it has been shown that microglia are the key cell type infected by HIV-1 in the CNS (Gonzalez-Scarano and Martin-Garcia 2005) and there are reports of an up-regulation of CXCR4 expression in microglia and neurons of AIDS patients (Sanders et al. 1998; Vallat et al. 1998; van der Meer et al. 2000; Petito et al. 2001). CXCL12 has been shown to be a highly efficacious chemoattractant for lymphocytes and monocytes, but not neutrophils (Bleul et al. 1996b). CXCL12 and CXCR4 also regulate the development of T and B lymphocytes, contribute to the survival of mature lymphocytes and aid in the generation of memory T cells (Klein and Rubin 2004). Administration of CXCL12 inhibits the infection of HeLa-CD4 cells by the T-trophic HIV-1 strain (Oberlin et al. 1996; Bleul et al. 1996a). HIV-1 gp120 is a glycoprotein localized on the surface of the HIV envelope. Gp120 enables HIV to infect any target cell with a CD4 receptor, particularly the helper T-cell, by binding to either CD4 or CXCR4 (Oberlin et al. 1996). The ability of gp120 to mediate HIV infectivity made it one of the first targets of HIV vaccine research. Gp120 however, is easily shed from the surface of the virus and subsequently be captured by T-cells, dampening efforts to develop vaccines against the protein.

Studies have described the actions of HIV-1 gp120 in neuronal apoptosis. P53 is a transcription factor that regulates the cell cycle and can activate DNA repair proteins when the DNA has sustained damage. Should the DNA be rendered unrepairable, p53 can initiate apoptosis, also known as programmed cell death. Gp120 stimulates p53 activity and induces expression of the p53 pro-apoptotic target Apaf-1 in cultured neurons. More specifically gp120 regulates the p53-induced phosphorylation and expression of other p53-responsive genes, such as

MDM2 (Hesselgesser et al. 1998). MDM2 is a trafficking protein which binds to p53 thus preventing its action during transport to the nucleus via the cytosol. Binding of gp120 to CXCR4 on sensory axons has also been shown to cause local axonal degeneration (Kury et al. 2003). Once gp120 binds to CXCR4, gp120 is internalized and is retrogradely transported to the neuronal soma by a CXCR4-mediated mechanism (Tarasova et al. 1998; Bachis et al. 2003; Bachis et al. 2006). Gp120 administered into the striatum is internalized by dopaminergic fibers and retrogradely transported to the substantia nigra where it causes apoptosis of dopaminergic neurons (Bachis et al. 2006). CXCL12 however, has been shown to promote neuronal survival by increasing p53 acetylation and p21 expression in neurons (Khan et al. 2005). This suggests that the stimulation of various p53 targets by CXCL12 could be important in determining the outcome of neruonal survival following the activation of CXCR4.

Brain-derived neurotrophic factor (BDNF) is a growth factor that promotes the survival, growth and differentiation of neurons (Huang and Reichardt 2001). BDNF can reduce the levels of CXCR4 and directly block the neurotoxic effect of CXCL12, hence protecting from gp120-mediated neuronal apoptosis and re-establishing the connection between CXCL12/CXCR4 and HIV/gp120 (Sanders et al. 2000; Bachis et al. 2003; Bachis and Mocchetti 2005). BDNF binds to the TrkB receptor, which is only found in neuronal cells. Pretreatment of adult rats striata with BDNF prior to gp120 prevents caspase-3 activation within the striatum and substantia nigra. CXCR4 expression is also decreased in striatal neurons of BDNF-treated rats (Nosheny et al. 2007). In addition, BDNF reversed the loss of tyrosine hydroxylase (TH) immunoreactivity normally induced by gp120 in both areas. This signifies that the protective role of BDNF against gp120 in combination with decreased CXCR4 expression is targeted specifically at neuronal cells (Fryer et al. 1996; Mocchetti and Bachis 2004).

Multiple Sclerosis (MS)

MS is an inflammatory disorder characterized by areas of demyelination with loss of oligodendrocytes and astroglial scarring in the central nervous system (Weiner 2004). CXCL12 is elevated in the cerebrospinal fluid of patients with MS as well as other neurological inflammatory conditions (Pashenkov et al. 2003; Corcione et al. 2004). CXCL12 has been shown to be elevated in both active and inactive MS lesions, with intense labeling in astrocytes. Astrocytes are commonly devoid of CXCL12 and CXCR4 under non-inflammatory conditions; however they display both the chemokine ligand and CXCR4 in neuroinflammatory disease states like MS (Krumbholz et al. 2006). Neuroinflammation results in elevated levels of CXCL12 and metalloproteases in the cerebrospinal fluid (Pashenkov et al. 2003). Cleavage by metalloprotease (MMP)-2 has been shown to convert CXCL12 into a neurotoxic form (Zhang et al. 2003). MMP-2 is also present in elevated levels in post-mortem tissue samples from MS patients. This suggests that elevated levels of CXCL12 in MS could be contributing to neuronal damage and axonal loss (Kornek and Lassmann 1999; Yong et al. 2001).

Pain

Chemokines and their receptors are expressed by sensory neurons and glia in the dorsal root ganglion and their expression is upregulated following peripheral nerve injury (Abbadie et al. 2003; Tanaka et al. 2004; White et al. 2005; Bhangoo et al. 2007a). Up-regulated expression of both CXCL12 and CXCR4 has been observed in populations of dorsal root ganglion neurons in chronic pain models (Bhangoo et al. 2007b). It has also been reported that administration of chemokines into the periaquaductal grey matter inhibits opioid-induced analgesia, specifically analgesia produced by mu- (Szabo et al. 2002) and kappa-opioid receptor agonists (Finley et al.

2008). Studies have shown that both chemokines and gp120 facilitate pain transmission via direct actions on chemokine receptors expressed by nociceptive neurons (Oh et al. 2001).

Nucleoside reverse transcriptase inhibitors (NRTI's), widely used treatments for HIV infection (Yeni et al. 2004), have been used to demonstrate that CXCL12 and CXCR4 are directly involved in painful neuropathies. Use of NRTI's produce pain and enhance states of pain hypersensitivity commonly produced by HIV-1 infection (Bhangoo et al. 2007b). A single administration of an NRTI, specifically the anti-retroviral drug 2',3'-dideoxycytidine (ddC), increases the levels of CXCR4 mRNA in glia and neurons and CXCL12 mRNA in glia. The increased CXCR4 mRNA expression was confirmed by measuring CXCL12-induced intracellular calcium increases in acutely isolated dorsal root ganglion neurons and glia. By using the CXCR4 antagonist AMD 3100, pain hypersensitivity produced by ddC was temporarily inhibited, demonstrating that an increase in pain hypersensitivity was mediated through CXCR4 signaling in the dorsal root ganglion (Bhangoo et al. 2007a).

Cocaine

Cocaine, a crystalline tropane alkaloid obtained from the leaves of the coca plant, is a stimulant of the CNS. The effects of cocaine include a heightened sense of energy and euphoria; however common toxicities can include myocardial infarctions, respiratory failure, strokes and seizures. In a study published in 2006, 6 million Americans age 12 and older had abused cocaine in any form and 1.5 million had abused crack at least once in the year prior to being surveyed (National Survey on Drug Use and Health, 2006). To date, there are no currently safe and effective pharmacological treatments for cocaine addiction.

In order to further investigate the mechanisms of CXCL12 and CXCR4 within the adult rat brain, CXCL12 was administered concomitantly with cocaine to determine if the chemokine could modulate the locomotor and stereotypic effects of cocaine. Cocaine is administered by a variety of routes, including insufflation, also known as snorting or sniffing, injection directly into the blood stream, smoked in the freebase form, and through oral consumption, commonly done with the raw coca leaf. Cocaine is metabolized in the liver and plasma and is broken down to benzoylecgonine and ecgonine among others (Fleming et al. 1990; Benowitz 1993). In humans, cocaine has a plasma half-life of approximately 0.5-1.5 hours (Fleming et al. 1990). Besides its rewarding effects, cocaine is used as a local anesthetic due to its ability to block sodium channels thus interfering with the conduction of action potentials (Crumb and Clarkson 1990). Cocaine also causes vasoconstriction and has been used to reduce bleeding during minor surgical procedures.

Lastly, cocaine can stimulate locomotor activity which is attributable to its involvement with the dopaminergic system of the brain. Following intraperitoneal (i.p.) administration of cocaine to the adult rat, cocaine rapidly accumulates in the brain which results in increased extracellular dopamine concentrations in the nucleus accumbens (Pettit et al. 1990), an area of the brain known to be involved in locomotion (Hedaya and Pan 1997). Dopamine is released under basal physiological conditions (Fig. 1.1) and is normally recycled via re-uptake by the dopamine transporter (DAT). The transporter pumps the dopamine from the synaptic cleft back into the presynaptic neuron, where it is re-packaged into storage vesicles or metabolized Fig. 1.2). Cocaine binds to the DAT inhibiting its function, allowing dopamine to accumulate in the synaptic cleft (Fig. 1.3) (van Rossum et al. 1962; McElvain and Schenk 1992). The result is an enhanced and prolonged pre- and postsynaptic effect of dopaminergic receptor signaling (Cragg and Greenfield 1997). The enhancement of dopamine receptor signaling produced from the excess of extracellular dopamine has been implicated in increased locomotion (Pettit et al. 1990; Hedaya and Pan 1997).

Dopamine

Cocaine inhibits the reuptake of dopamine, serotonin and norepinepherine into presynaptic terminals (Heikkila et al. 1975; Ritz et al. 1987; Nicolaysen and Justice 1988). Studies have identified the inhibition of dopamine reuptake as playing a vital role in the reinforcing properties of cocaine (Ritz et al. 1987). The cellular actions of dopamine are mediated by specific membrane receptors belonging to the class of seven transmembrane domain GPCR's. Five dopamine receptor subtypes, designated D1-D5 have been identified. Each dopamine receptor can be further categorized as D1-like (D1 and D5) or D2-like (D2, D3 and D4) based upon sequence homology (Kebabian et al. 1972; Sibley et al. 1993). Activation of D1-like receptors (acting through $G_{\alpha s/olf}$ G-proteins) stimulate whereas activation of D2-like receptors (acting through $G_{\alpha i}$ G-proteins) inhibit adenylyl cyclase and the production of cyclic adenosine 3',5'-monophosphate (cAMP). Following chronic cocaine administration, D1-receptor stimulated adenylyl cyclase activity is increased (Unterwald et al. 1996).

While cocaine inhibits the reuptake of norepinepherine, serotonin and dopamine resulting in increases of extracellular levels of these neurotransmitters, many studies have shown that the reinforcing properties of cocaine are mediated by increased dopamine transmission (Ritz et al. 1987; Anderson and Pierce 2005). Acute cocaine administration stimulates locomotor activity in animal models (Sahakian et al. 1975; Reith et al. 1985; Ushijima et al. 1995) and studies using

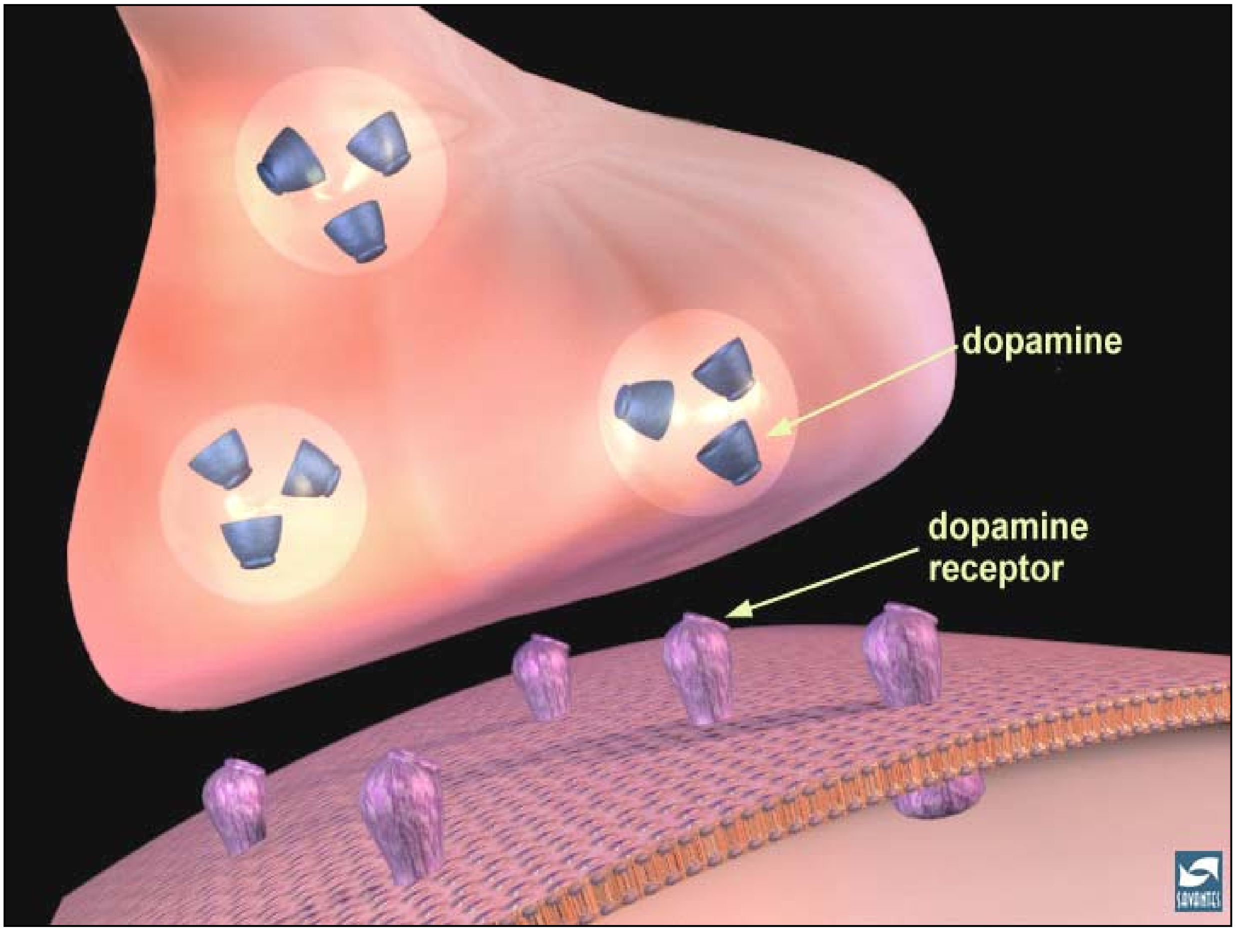

Figure 1.1 Dopaminergic Synapse

Cartoon representation of postsynaptic dopamine receptors (presynaptic dopamine receptors not shown) and dopamine packaged in presynaptic vesicles. (www.nida.nih.gov)

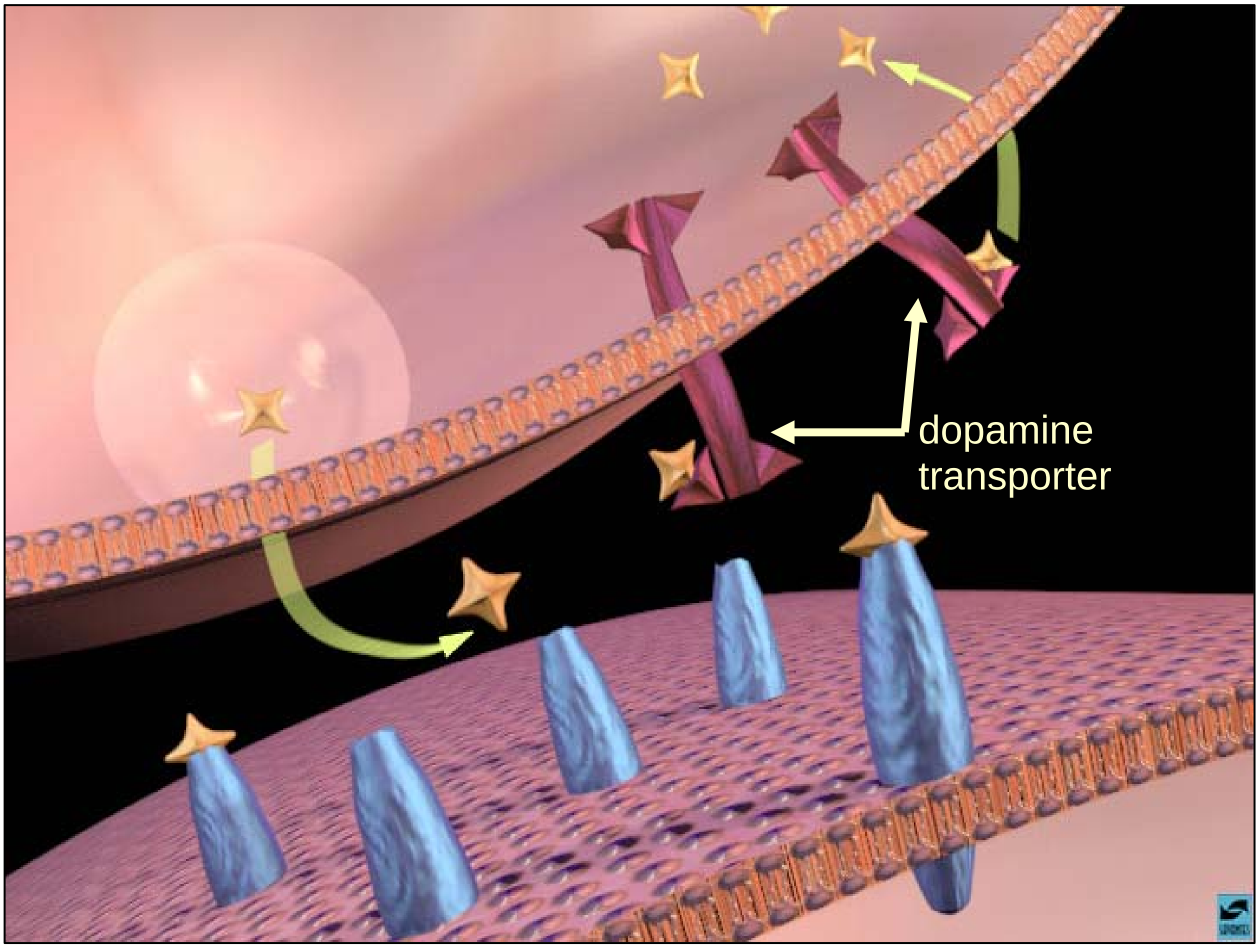

Figure 1.2 Dopamine Re-uptake

In the absence of cocaine, dopamine is removed from the synapse by reuptake into the presynaptic neuron by the dopamine transporter. (www.nida.nih.gov)

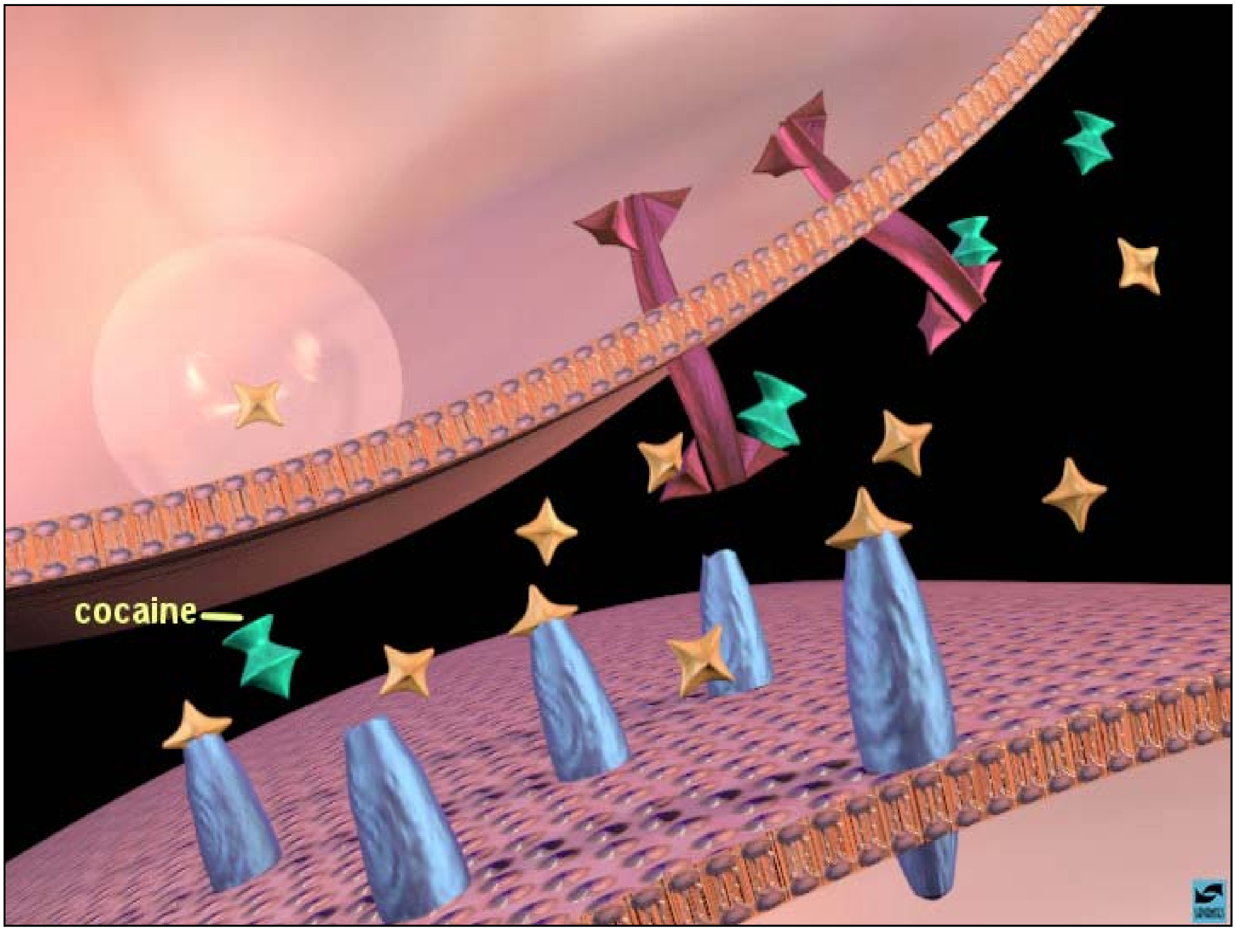

Figure 1.3 Blockade of the Dopamine Transporter by Cocaine

In the presence of cocaine, dopamine re-uptake is inhibited, leading to the increased concentration of extracellular dopamine. (www.nida.nih.gov) either conditioned place preference or self-administration models have demonstrated the rewarding effects of cocaine (Ettenberg et al. 1982; McKinzie et al. 1999). Repeated exposure to cocaine results in an enhancement in the locomotor activating and reinforcing effects of the drug, also known as behavioral sensitization (Kalivas and Duffy 1993; Miserendino and Nestler 1995). Genetic studies involving the modification of dopamine receptor subtypes have provided results suggesting a dopaminergic role in cocaine-induced behavior. Cocaine does not stimulate locomotor activity in D1 receptor knockout mice as compared to their wild-type littermates (Miner et al. 1995; Xu et al. 2000). D2 receptors are also involved in the locomotor stimulating effects of cocaine (Bhattacharyya et al. 1979; Arnt et al. 1988). Genetic deletions in D2 receptors results in a decreased locomotor response to cocaine (Chausmer et al. 2002). These data demonstrate the involvement of dopamine D1 and D2 receptors in the locomotor effects of cocaine.

The Mesolimbic System

The mesolimbic system is one of the dopaminergic pathways involved in cocaine-induced locomotor activity (Di Chiara and Imperato 1988). Dopaminergic neurons originate in the VTA and send projections to the nucleus accumbens, medial prefrontal cortex (mPFC), amygdala, hippocampus and ventral pallidum (Fig. 1.4) (Berendse et al. 1992; Klitenick et al. 1992; Brog et al. 1993; Heimer et al. 1997). High levels of D1 and D2 receptor mRNA are expressed in the nucleus accumbens, with moderate D1 mRNA expression in the frontal cortex and amygdala, and strong D2 mRNA expression found within the VTA (Camps et al. 1990; Bouthenet et al. 1991; Weiner et al. 1991; Hurd et al. 2001). The VTA can be divided into rostral and caudal portion: the rostral VTA contains a high percentage of GABAergic interneurons while the caudal VTA contains a higher amount of dopaminergic neurons (Johnson and North 1992). Inhibtory GABAergic interneurons in the VTA project to adjacent neurons in VTA and in the substantia nigra while dopaminergic neurons project to areas of the midbrain including the nucleus accumbens and caudate putamen.

The nucleus accumbens is located within the forebrain and thought to be responsible for reward, pleasure and addiction (Ikemoto and Panksepp 1999). The main cell type found within the nucleus accumbens is the medium spiny neuron which expresses GABA, one of the main inhibitory neurotransmitters of the central nervous system. These medium spiny neurons represent most of the efferent signals, projecting to the substantia nigra, VTA and ventral pallidum, of which the latter further projects to the thalamus, mPFC and striatum. The remaining neurons are of the nucleus accumbens are aspiny cholinergic interneurons or GABAergic interneurons. Axons of these adjacent interneurons project to nicotinic receptors at the terminal regions of dopaminergic neurons within the nucleus accumbens while inputs into the nucleus accumbens come from the mPFC, the basolateral amygdala and dopaminergic projections from the VTA (Kalivas et al. 1993).

The nucleus accumbens receives dopaminergic input from the VTA. The nucleus accumbens is the site of action of multiple drugs of abuse, including cocaine (Roberts and Koob 1982; Goeders and Smith 1983). The nucleus accumbens can be divided into two major subregions; the core and the shell, which have distinct cell morphology and function. The shell can be further sub-divided into the medial and lateral shell, and have different afferent and efferent neuronal projections (Schmidt et al. 2005). The cell bodies of dopamine neurons that project to the mPFC, medial accumbens shell, accumbens core or amygdala originate in the medial posterior portion of the VTA. Dopamine cells that project to the lateral shell of the

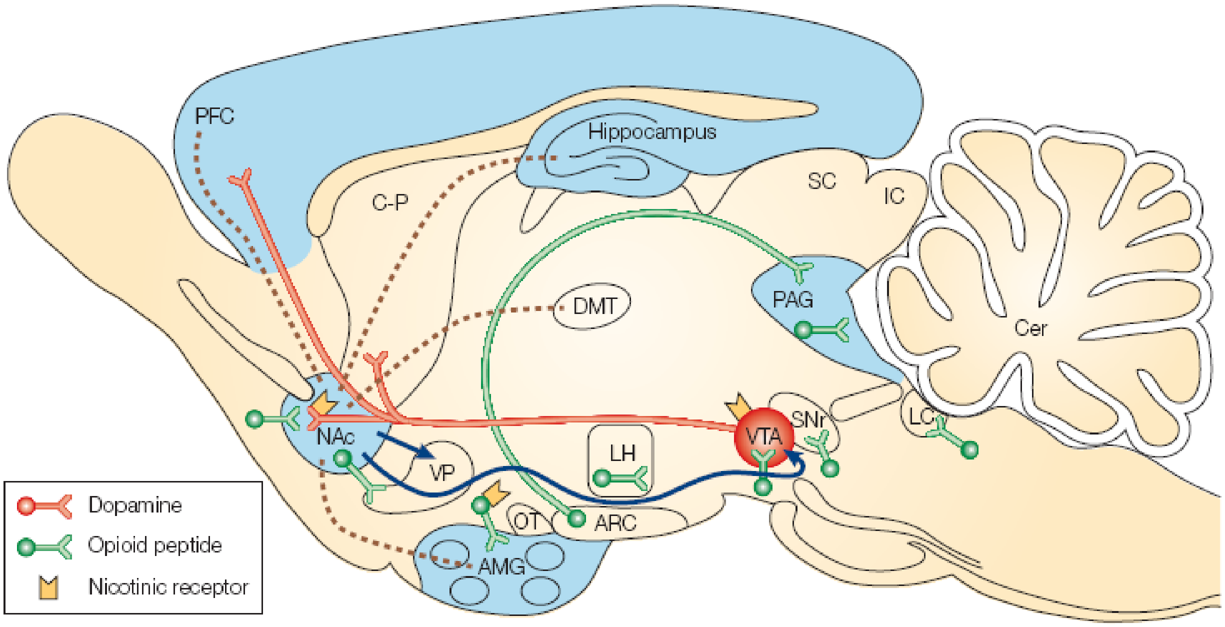

Figure 1.4 Saggital Section of the Adult Rat Brain

The mesolimbic and nigrostriatal dopamine pathways extend from the midbrain of the adult rat to regions in the forebrain. The mesolimbic dopamine pathway originates in the ventral tegmental area (VTA) and extends to the nucleus accumbens (NAc), amygdala (AMG), hippocampus and medial prefrontal cortex (PFC). The nigrostriatal dopamine pathway connects the substantia nigra (SN) with the caudate putamen (C-P; CPu) (Nestler EJ, 2001) nucleus accumbens are located in the lateral portions of the VTA, partially overlapping with substantia nigra cells that project to the dorsal striatum (Ford and Williams 2008). Dopaminergic cellular markers such as tyrosine hydroxylase, the rate limiting enzyme in the synthesis of dopamine, and DAT, are lower in the groups of neurons located in the medial aspect of the VTA as compared to neurons that projected to the lateral accumbens shell and dorsal striatum (Ford and Williams 2008). Some studies have suggested that administration of cocaine results in an increase of extracellular dopamine within the nucleus accumbens shell to a greater extent than the core (Pontieri et al. 1995; Aragona et al. 2008), while others demonstrate that the core of the nucleus accumbens is the site responsible for the increase in extracellular dopamine following cocaine administration (Cadoni et al. 2000). This increase in extracellular dopamine, however, is responsible for the modulation of cocaine-induced locomotion (Hooks et al. 1992).

The Nigrostriatal System

The nigrostriatal system consists of dopaminergic cells in the substantia nigra with afferents projecting to the caudate putamen (Fig. 1.4). The nigrostriatal dopaminergic pathway is thought to be directly involved in movement (Yamamoto and Freed 1984). The caudate putamen sends signals to the globus pallidus or back to the substantia nigra (Hattori et al. 1975). The substantia nigra can be divided into two subregions: the substantia nigra pars compacta (SNc) and the substantia nigra pars reticulata (SNr). The SNc contains interneurons and projection neurons which extend to other parts of the brain. The interneurons are primarily GABAergic while projection neurons are mostly dopaminergic. The GABAergic interneurons in the SNc affect neuronal activity in the SNr and are involved in the modulation of dopaminergic activity (Juraska et al. 1977). The neurons of the SNr are mainly GABAergic (Bolam et al. 2000;

Veliskova and Moshe 2006). In addition, the substantia nigra is a major source of GABAergic inhibition to many brain areas, contributing to its regulation of movement (Nicola et al. 2000). The substantia nigra and caudate putamen are also involved in the motor-stimulating properties of cocaine, specifically the stereotypic aspects of the behavior (Asher and Aghajanian 1974; Ushijima et al. 1995), as discussed in detail below.

Cocaine-Induced Hyperactivity

As discussed previously, hyperactivity as well as stereotypic behavior is induced by cocaine administration (Sahakian et al. 1975; Reith et al. 1985; Ushijima et al. 1995; White et al. 1998). Cocaine induced hyperactivity is related to the activation of brain dopamine systems as cocaine is an indirect agonist to dopamine, serotonin and norepinephrine receptors (Cabib et al. 1991). The inhibition of locomotor activity following administration of D1 and D2 antagonists suggests a role of both D1 and D2 dopamine receptors in the locomotor and reinforcing properties of cocaine (Kita et al. 1999). Administration of a dopamine antagonist such as haloperidol, have also been shown to abolish the psychomotor stimulant properties of cocaine (Reimer and Martin-Iverson 1994). Activation of the mesolimbic dopamine system, projecting from the VTA to the nucleus accumbens and the nigrostriatal dopamine systems, projecting from the substantia nigra to the caudate putamen, are directly involved in the locomotor-stimulating properties of cocaine (Kelly and Moore 1976). The importance of the mesolimbic dopamine system to cocaine-induced locomotion has been demonstrated following administration of 6-hydroxydopamine (6-OHDA). Destruction of mesolimbic dopamine neurons by an intra-accumbens injection of 6-OHDA can attenuate the locomotor response triggered by cocaine (Kelly 1975). Administration of a dopamine antagonist such as haloperidol, have also been shown to abolish the psychomotor stimulant properties of cocaine (Reimer and Martin-Iverson 1994).

Psychomotor stimulants such as cocaine also induce stereotypic behaviors (Schiorring 1981), especially at higher doses (Post et al. 1987). Stereotypic activities are defined as uncontrolled repetitive non-sense behaviors. In rats, stereotypy first increases sniffing and repetitive movements of the head, and is often accompanied by a decrease in both locomotor activity and rearing (Schiorring 1981; Rebec and Bashore 1984). Although both the mesolimbic and nigrostriatal dopamine systems are thought to be involved in cocaine-induced behaviors, studies have demonstrated that the nucleus accumbens is more important for the increased locomotion observed following cocaine, while the caudate putamen shows a stronger involvement in cocaine-induced stereotypic behaviors (Asher and Aghajanian 1974; Ushijima et al. 1995; White et al. 1998). Previous studies have shown the importance of dopamine in stereotypic behavior. Administration of dopamine to dopamine-deficient mice has been shown to induce stereotypic behaviors (Chartoff et al. 2001). Similarly, D1 (Gainetdinov et al. 1999) and D2 (Fetsko et al. 2003) receptor agonists induce stereotypy while D1 receptor antagonists have been shown to reduce stereotypic behaviors (Zhuang et al. 2001). Lastly, studies have shown mice with cocaine-resistant DAT do not demonstrate stereotypy, suggesting the involvement of DAT and dopamine in cocaine-induced stereotypic behaviors (Tilley and Gu 2008).

General Summary of Objectives

CXCL12 and its receptor CXCR4, are found in the mesolimbic and nigrostriatal pathways under basal conditions and neuroinflammatory conditions. By blocking the DAT on the presynaptic terminal of dopaminergic neurons, cocaine increases extracellular dopamine which can result in an increase in locomotion and stereotypic behaviors (Heikkila et al. 1975; Nicolaysen and Justice 1988). The CXCR4 receptor is localized on dopaminergic cell bodies in the substantia nigra and caudate putamen (Banisadr et al. 2002). Administration of CXCL12 in the substantia nigra has been shown to increase extracellular dopamine within the caudate putamen (Skrzydelski et al. 2007). The localization of CXCR4 on dopaminergic neurons of the mesolimbic pathway could likewise modulate dopamine release within the nucleus accumbens.

In this thesis, CXCL12 was co-administered with cocaine to evaluate the effects of CXCL12 on cocaine-induced activity. Adult male Sprague Dawley rats received intracerebral injections of CXCL12 into the right cerebral ventricle, the VTA, caudate putamen and the nucleus accumbens core or lateral shell. An i.p. injection of cocaine was then administered to determine if CXCL12 was able to modulate cocaine-induced activity and to determine the site of action of CXCL12. The hypothesis of this research is that CXCL12 can affect the mesolimbic and nigrostriatal pathways of the brain which contributes to the modulation of cocaine-induced behavior.

Various studies have shown the presence of CXCR4 receptors in select areas of the adult rat brain including the VTA, substantia nigra and caudate putamen, however results have not shown evidence of CXCR4 within the nucleus accumbens (Banisadr et al. 2002). A terminal site for neurons projecting from the VTA, the nucleus accumbens plays an important role in dopamine-related behaviors. For this thesis, it was hypothesized that CXCR4 was present in the nucleus accumbens. Free floating sections of adult rat brain were subjected to immunohistochemical and immunofluorescent tests to determine the localization of CXCR4 in the nucleus accumbens and caudate putamen. In addition, studies determined the neurochemical phenotype of cells expressing CXCR4, as well as examined the possible co-expression of CXCR4 with the D1 dopamine receptor.

A final goal of this research was to determine if CXCL12 modulates extracellular dopamine and glutamate levels in the medial shell of the nucleus. A previous study has shown that CXCL12 administered into the substantia nigra causes increases in extracellular dopamine in the caudate putamen as well as increases in contralateral turning (Skrzydelski et al. 2007). To test the hypothesis that CXCL12 can cause dopamine and/or glutamate release in the mesolimbic pathway, CXCL12 was administered into the VTA and extracellular dopamine and glutamate were collected from the medial shell of the nucleus accumbens using a microdialysis probe. Samples were analyzed using either high pressure liquid chromatography (HPLC) for quantification of dopamine or via capillary electrophoresis for quantification of glutamate.

This thesis will further the understanding of the mechanisms by which CXCL12 and CXCR4 can affect the dopaminergic pathways of the brain. The involvement of CXCR4 in neuroinflammatory diseases and chemotaxis provide an incentive to understand how chemokines can act as neuromodulators in the brain.

CHAPTER 2

Modulation of Cocaine-Induced Activity by Intracerebral Administration of CXCL12

Introduction

Chemokines are defined as small (8-14 kDa) chemoattractant cytokines involved in a variety of developmental and pathological conditions. Recent reports propose that endogenous chemokines within the brain act in conjunction with neurotransmitters to modulate brain functions (Adler et al. 2005). The chemokines themselves have been hypothesized to act as transmitters or neuromodulators in neuronal communication and have been shown to alter the actions of neuronally-active pharmacological agents such as opioids and cannabinoids (Steele et al. 2002; Adler et al. 2005; Burbassi et al. 2008).

CXCL12, formerly known as stromal cell derived factor one alpha (SDF-1α), is a CXC category chemokine secreted by bone marrow stromal cells as well as populations of neuronal and non-neuronal cells within the adult rat brain (Stumm et al. 2002). CXCL12 is involved in embryogenesis, cell migration and development of the brain, heart and large blood vessels (Moepps et al. 2000; Kucia et al. 2004). Until recently, CXCL12 was thought to signal through a single receptor, CXCR4. Vital during pre-natal development, CXCL12 or CXCR4 knock-out animals develop lethal cardiovascular, neuronal and blood vessel defects and die soon after birth (Ma et al. 1998; Zou et al. 1998). A second receptor that binds CXCL12, CXCR7 (orphan receptor RDC1), was identified via radio-labeled CXCL12 binding in vitro (Balabanian et al. 2005; Burns et al. 2006). Both CXCR4 and CXCR7 are G-protein coupled receptors that contain highly conserved transmembrane regions.

Studies have demonstrated the presence of CXCR4 mRNA in cultured rat neurons, astrocytes, glia and microglia (Ohtani et al. 1998; Bajetto et al. 1999). Both CXCL12 and CXCR4 have been found in regions of the adult rat brain including the cerebral cortex, caudate putamen and white matter of the corpus callosum (Banisadr et al. 2003). CXCR4 has been shown via immunohistochemistry to be localized within the substantia innominata in the basal forebrain, the caudate putamen and globus pallidus within the basal ganglia, and the substantia nigra and ventral tegmental area within the midbrain (Banisadr et al. 2002), all areas rich in dopaminergic neurons. Of note, CXCR4 has not been detected in the nucleus accumbens (Banisadr et al. 2002), an area highly innervated by dopaminergic terminals. Double immunostaining for CXCR4 and tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine, revealed that the majority of tyrosine hydroxylase-positive neurons in the substantia nigra were also immunoreactive for CXCR4, indicating that CXCR4 is expressed by dopaminergic neurons in this brain region (Banisadr et al. 2002). Two of the major dopaminergic tracks within the brain are the mesolimbic and nigrostriatal dopamine pathways. A major component of the mesolimbic dopamine system links the ventral tegmental area to the nucleus accumbens. The ventral tegmental area is comprised of dopamine-, GABA- and glutamate-containing neurons while the nucleus accumbens is comprised mainly (95%) of GABAergic medium spiny neurons. Most of the remaining neurons of the nucleus accumbens are cholinergic interneurons. The dopaminergic neurons projecting from the ventral tegmental area to the nucleus accumbens are involved in locomotor activity (Mogenson et al. 1979; Kalivas et al. 1981). The nucleus accumbens in conjunction with the ventral tegmental area are targets of psychostimulant drugs and have been widely implicated in mediating the rewarding and locomotor-stimulating actions of psychostimulants (Koob 1992). The nigrostriatal pathway consists of dopaminergic cell bodies in the substantia nigra which innervate the caudate putamen. Enhanced dopaminergic neurotransmission in the caudate putamen following psychostimulant administration is associated with hyperactivity, especially an increase in stereotypic behaviors (Asher and Aghajanian 1974; Kuczenski et al. 1991; Ushijima et al. 1995). Of particular relevance to the present study, administration of CXCL12 into the substantia nigra has been shown to increase both contralateral turning and extracellular dopamine within the caudate putamen (Skrzydelski et al. 2007).

Extensive research demonstrates the ability of cocaine to influence the activity of the mesolimbic and nigrostriatal dopamine systems. Cocaine blocks the dopamine transporter thereby inhibiting the reuptake of dopamine (Heikkila et al. 1975). By inhibiting the dopamine transporter, excess synaptic dopamine accumulates in the nucleus accumbens and caudate putamen thereby altering many functions including motivation, attention, reward and locomotor activity (Reith 1986; Ritz et al. 1987).

The present study investigated the potential of CXCL12, when injected into specific regions of the mesolimbic and nigrostriatal dopamine pathways, to modulate cocaine-induced behaviors. Results demonstrate site-specific actions of CXCL12 and its ability to modulate the locomotor- and stereotypic-activating effects of cocaine.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were utilized in all experiments. Animals were housed on a 12 h light-dark cycle (7 AM – 7 PM) with ad libitum access to food and water in groups of four per cage. Weight range upon arrival was 250-

300 g and upon testing was 300-350 g. Animals were allowed to acclimate to the animal facility for one week prior to surgery. Following surgery to implant indwelling intracranial cannula, animals were individually housed. Experimental testing began three days after surgery. Separate groups of rats were used for each study conforming to a between-subjects design. No animal was tested more than once. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine.

Drugs

Telazol (tiletamine/zolazepam) was purchased from Fort Dodge Animal Health (Overland Park, KS, USA). Recombinant mouse CXCL12/SDF-1α was obtained from R&D Systems (Minneapolis, MN, USA, 10 μg). Cocaine hydrochloride was generously supplied by the National Institute of Drug Abuse (Bethesda, MD, USA). Cocaine (20 mg/mL) and CXCL12 (12.5 – 200 ng/dose) were dissolved in sterile normal saline (0.9%) before use. AMD 3100 (2 μg/dose, octahydrochloride hydrate) was obtained from Sigma Aldrich (St. Louis, MO, 5 mg) and was dissolved in sterile normal saline (0.9%) before use.

Surgery

Rats were anesthetized with Telazol (40 mg/kg IP) and placed in a Kopf stereotaxic apparatus. Intracerebroventricular (ICV) cannulae were constructed of polyethylene tubing (PE 20, PE 10) and intracranial injection cannulae were constructed of stainless steel (22 GA guide, Plastics One, Roanoke, VA). A unilateral ICV cannula was inserted into the right lateral ventricle for the initial experiment. For subsequent experiments, cannulae were inserted bilaterally into the ventral tegmental area, caudate putamen, nucleus accumbens lateral shell or nucleus accumbens core. The following stereotaxic coordinates were utilized: ICV: A/P -0.9, M/L -1.4, V -4.0; caudate putamen: A/P 1.2, M/L ±2.4, V -4.7; lateral accumbens shell: A/P 1.3, M/L ±3.1, V -7.5; nucleus accumbens core: A/P 1.7, M/L ±1.5, V -6.8; ventral tegmental area: A/P -6.0, M/L ±0.7, V -8.0; and the incisor bar was adjusted to achieve a flat skull position (-3.9 ± 0.5) as determined according to the atlas of Paxinos and Watson (Paxinos and Watson 2007). The cannulae were secured with dental acrylic anchored to a stainless steel surgical screw inserted into the skull. Stainless steel stylets, 1.0 mm longer than the guide cannulae, were inserted to keep them free of debris. Cannula placements were verified at the conclusion of the experiment.

ICV and intracranial injections

Rats were placed into the activity monitors for 30 minutes prior to receiving an ICV or intracranial injection of either CXCL12 or normal saline. Injection volumes were 4 μl for ICV administration and 0.5 μl for intracranial injections. Injection cannulas were 28 GA and extended 1 mm below the 22 GA guide cannula (Plastics One, Roanoke, VA). Solutions were injected by hand at a rate of 1 μl/min with a Hamilton syringe connected to the cannula by polyethylene tubing. Rats were not restrained during injections. Fifteen minutes later, cocaine (20 mg/kg) or normal saline (1 mL/kg) was administered by the intraperitoneal (i.p.) route. Activity was recorded for 120 minutes post i.p. injection as described below. In one cohort of rats, AMD 3100 was administered via the ICV cannula (2 μg/4 μl) sixty minutes prior to an ICV injection of either CXCL12 or normal saline (Fricker et al. 2006). Fifteen minutes later, animals received an i.p. injection of either cocaine (20 mg/kg) or saline. Activity was measured for 120 minutes post i.p. injection as described below.

Behavioral activity

Activity was measured using a Digiscan D Micro System (Accuscan, Columbus, OH) and eight individual activity monitors. A single activity monitor consists of an aluminum frame equipped with 16 infrared light beams and detectors whereby a standard clear plastic animal cage (20 x 20 x 42 cm) is placed. As the animal moves within the plastic cage, light beams are broken and recorded by a computer connected to the Digiscan system. Activity was recorded as total activity, ambulatory activity and stereotypy. Total activity represents all beam breaks compiled by a single animal, and is the sum of the ambulatory and stereotypy counts. Ambulatory activity represents successive beam breaks while stereotypy counts are defined as bouts of repetitive beam breaks. Stereotypic counts do not identify specific stereotypic behavior, rather repeated breaks of the same beam indicative of a stationary animal engaged in a repetitive behavior as opposed to ambulation. Activity was measured over 120 minutes with 5 minute collection intervals.

Cannula placement

At the conclusion of each study, ICV cannula placements were verified by injecting 4 µL of methylene blue (Taylor Pharmaceuticals, Decatur, IL) into the cannula. Thirty minutes later, rats were decapitated and inspected for cannula placement. A positive result for correct ICV cannula placement was evident by even distribution of dye throughout the ventricles of the brain. For intracranial cannula placements, brains were excised, frozen and cut in 40 µM sections for determination of cannula placements. Final depths of individual cannula for all animals are marked by an enclosed circle in Figure 2.1. The rostral-caudal extent of cannula locations from bregma for each brain region investigated were as follows: caudate putamen 1.08 mm to 1.32

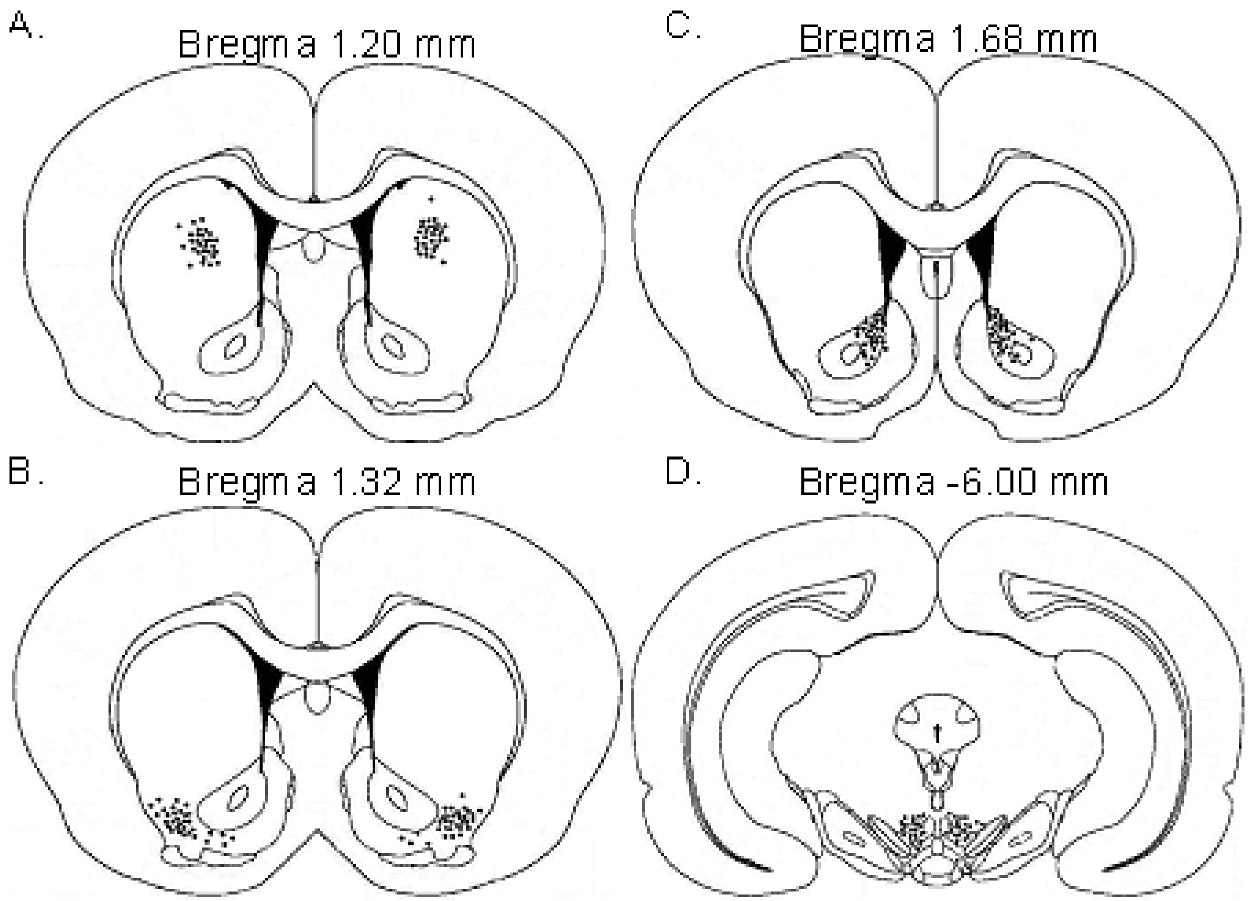

Figure 2.1 Cannula Placements

Injection sites for (A) caudate putamen, (B) lateral shell of the accumbens, (C) nucleus accumbens core, and (D) ventral tegmental area. Locations of the tip of the cannulae are indicated by black circles. Figures reproduced from Paxinos and Watson (2007) with permission.

mm, lateral accumbens shell 1.22 mm to 1.44 mm, nucleus accumbens core 1.56 mm to 1.80 mm and ventral tegmental area -5.88 mm to -6.12 mm.

Data analysis

Data are expressed as the mean ± S.E.M. Data were examined for significance by repeated measures ANOVA followed by Bonferroni post-tests (GraphPad Prism V.4). Statistical significance was achieved when $p<0.05$.

Results

ICV administration of CXCL12 and cocaine-induced activity

CXCL12 was administered ICV 15 minutes prior to an injection of cocaine (20 mg/kg). Using individual cohorts of rats, a range of doses for CXCL12 were evaluated including 12.5, 25, 50, 100 and 200 ng in 4 µL of sterile saline (0.9%). Differences in the mean total activity counts from 0 to 60 minutes between the rats receiving CXCL12 ICV plus cocaine and those receiving saline ICV plus cocaine for 5 doses of CXCL12 are shown in Figure 2.2. The differences between groups were calculated to determine which dose of CXCL12 provided the largest increase in cocaine-induced activity. The greatest potentiation of cocaine-induced activity was seen with the 25 ng dose of CXCL12.

CXCL12 potentiated cocaine-induced activity which was blocked by AMD 3100

To establish that CXCL12 was producing its effects through CXCR4, the selective CXCR4 antagonist AMD 3100 (0.2 or 2 µg/4 µl ICV) was administered 60 minutes prior to CXCL12 (25 ng/ 4 µl ICV) followed by either cocaine (20 mg/kg IP) or saline. Repeated measures ANOVA showed a significant difference between groups ($F(6,174) = 28.66$, $p<0.0001$) (Fig. 2.3). Bonferroni post hoc analysis confirmed the ability of CXCL12 to potentiate the

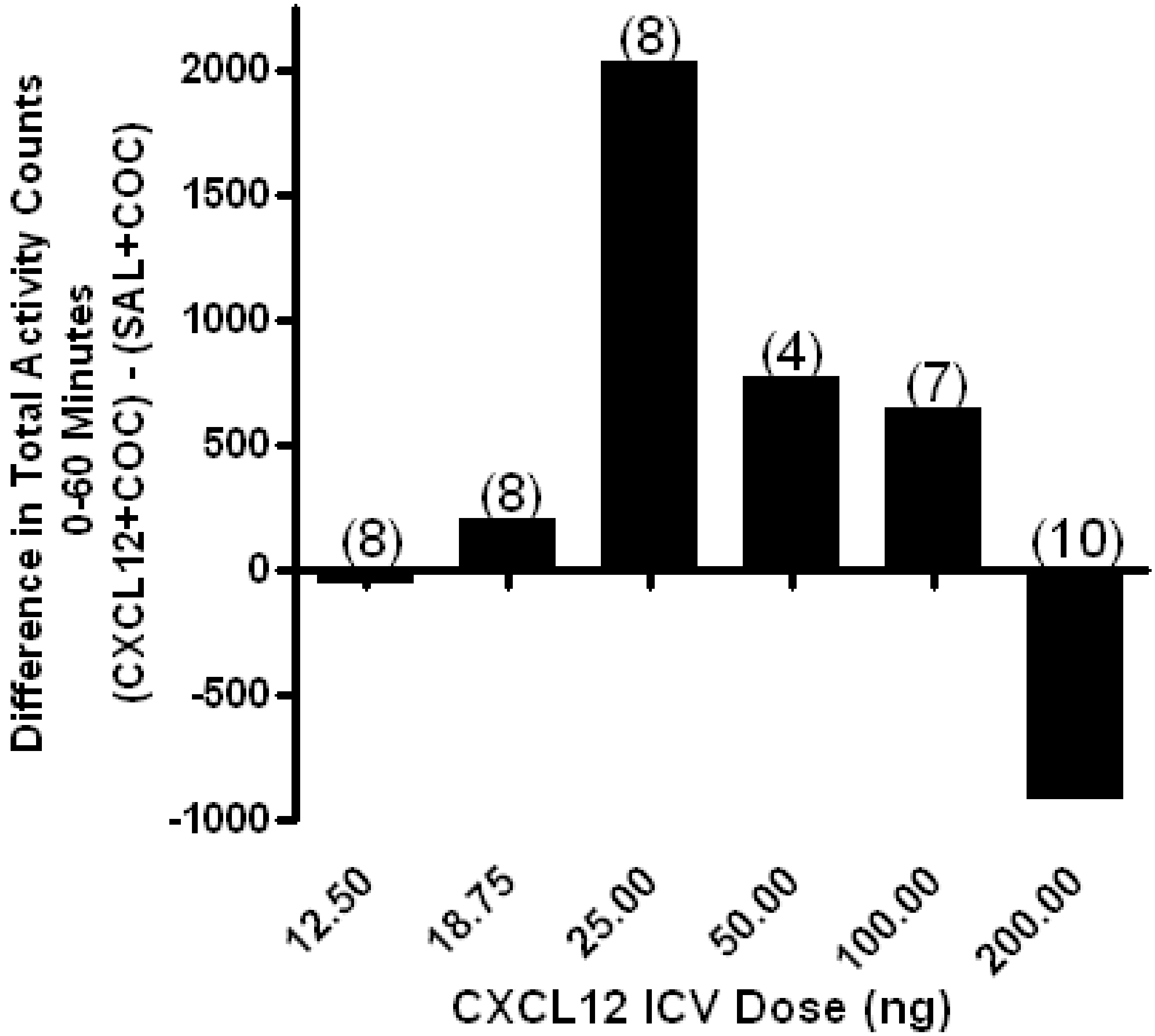

Figure 2.2 Effect of i.c.v. CXCL12 on Cocaine-Induced Activity

CXCL12 (12.5-200 ng/4 µl) was administered 15 minutes prior to cocaine (20 mg/kg i.p.) and activity was measured for 60 min. Data are expressed as the difference in total activity counts ([CXCL12 i.c.v. + cocaine i.p.] minus [saline i.c.v. + cocaine i.p.]). Results show that 25 ng of CXCL12 produced the greatest increase in cocaine-induced activity. Number of animals used in each treatment group is shown in parenthesis above each dose.

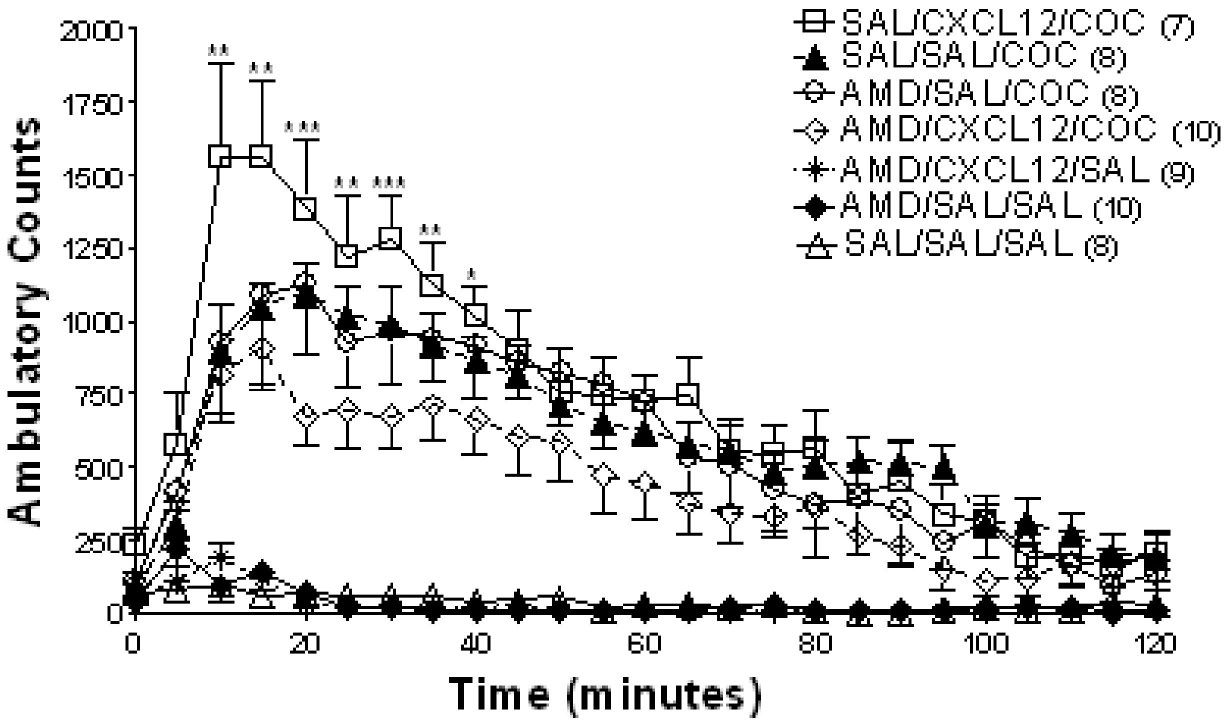

Figure 2.3 Effect of ICV AMD 3100 and CXCL12 on Cocaine-Induced Ambulatory Activity.

The selective CXCR4 antagonist AMD 3100 (2 µg/4 µl) was administered 60 minutes prior to administration of CXCL12 (25 ng/ 4 µl ICV), and then followed by saline or cocaine (20 mg/kg IP). Repeated measures ANOVA followed by Bonferroni post-hoc analysis showed a significant difference between the AMD/CXCL12/COC and the SAL/CXCL12/COC groups indicating that AMD 3100 blocked the CXCL12-induced increase in cocaine-stimulated ambulatory activity. Data are expressed as mean ± SEM beam breaks/five minute period. N= 7 – 10 per group as indicated in parentheses on the figure for each group. (AMD/CXCL12/COC vs. SAL/CXCL12/COC; *$p<0.05$, **$p<0.01$, ***$p<0.001$)

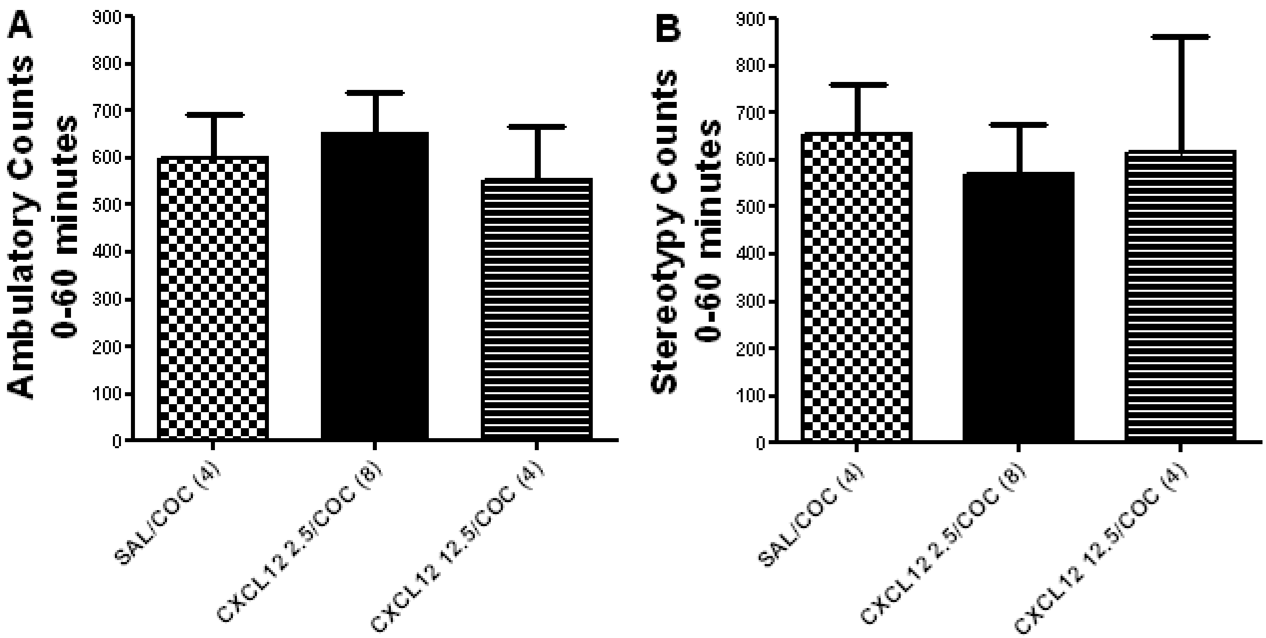

Figure 2.4 Dose-Response of CXCL12 Administered into the Ventral Tegmental Area.

Administration of CXCL12 (2.5 or 12.5 ng/0.5 µl) bilaterally into the ventral tegmental area concomitantly with cocaine failed to produce a significant increase in A) ambulatory activity or B) stereotypy when compared rats receiving cocaine alone. N = 4 – 8 per group as indicated for each in parentheses. (SAL/COC vs. CXCL12 2.5/COC or CXCL12 12.5/COC; $p>0.05$.)

ambulatory activity of cocaine (SAL/CXCL12/COC vs. SAL/SAL/COC; $p<0.05$). ICV AMD 3100 (2 μg/4 μl ICV) prior to CXCL12 and cocaine administration inhibited the potentiation of cocaine-induced ambulatory activity (AMD3100/CXCL12/COC vs. SAL/CXCL12/COC; *$p<0.05$, **$p<0.01$, ***$p<0.001$, Fig. 2.3). Administration of the lower dose of AMD 3100 (0.2 μg/4 μl ICV) had no significant effect on activity alone (AMD/SAL/SAL vs. SAL/SAL/SAL $p>0.05$) or on cocaine-induced activity (AMD/SAL/COC vs. SAL/SAL/COC $p>0.05$). These results demonstrate that CXCL12, by activating CXCR4 receptors, can enhance cocaine-induced activity.

CXCL12 administration into the ventral tegmental area

Microinjections of CXCL12 into the ventral tegmental area in conjunction with IP injections of cocaine were used to evaluate their interactions within the cell body region of the mesolimbic dopamine system. Doses of 2.5, 12.5 and 25 ng CXCL12 injected bilaterally into the ventral tegmental area were tested with and without cocaine (20 mg/kg i.p.). Results demonstrated that 2.5 and 12.5 ng CXCL12 failed to illicit a significant difference between cohorts receiving CXCL12 plus cocaine as compared to cocaine alone (Fig. 2.4; ANOVA: ambulatory activity $F(2,15) = 0.27$, $p>0.05$; or stereotypy $F(2,15) = 0.09$, $p>0.05$). However, 25 ng/0.5 μl CXCL12 injected bilaterally into the ventral tegmental area significantly enhanced cocaine-induced ambulatory activity. Repeated measures ANOVA showed a significant difference in ambulatory activity between the treatment groups ($F(3,99) = 70.12$, $p<0.0001$) (Fig. 2.5). Bonferroni post hoc analysis revealed that bilateral administration of CXCL12 (25 ng/0.5 μl) into the ventral tegmental area concomitantly with cocaine (20 mg/kg i.p.) resulted in significantly higher ambulatory activity as compared to cocaine alone (CXCL12/COC vs. SAL/COC; *$p<0.05$,

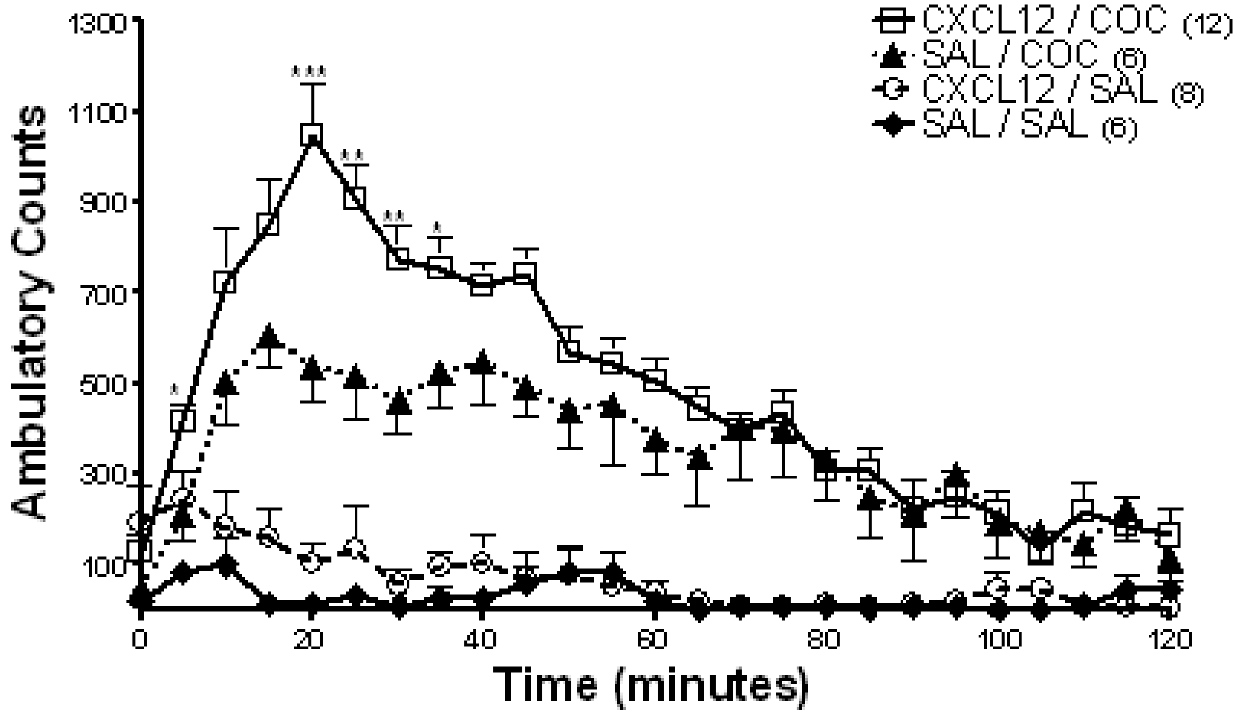

Figure 2.5 Effect of CXCL12 Administration into the Ventral Tegmental Area on Ambulatory Activity.

Administration of CXCL12 (25 ng/0.5 μl) into the ventral tegmental area 15 minutes prior to cocaine (20 mg/kg IP) resulted in a significant increase in ambulatory activity as compared to cocaine alone. Data are expressed as mean ± SEM beam breaks/five minute period. N= 6 – 11 per group as indicated for each. (CXCL12/COC vs. SAL/COC; *p<0.05, **p<0.01, ***p<0.001)

$**p<0.01$, $***p<0.001$). CXCL12 alone had no effect on ambulatory activity (CXCL12/SAL vs. SAL/SAL; $p>0.05$).

In contrast to its effect on ambulatory activity, CXCL12 injected directly into the ventral tegmental area did not alter cocaine-induced stereotypic activity. Stereotypy was significantly different between treatment groups ($F(3,99) = 15.83$, $p<0.0001$; data not shown). Administration of cocaine alone increased stereotypy counts as compared to saline controls (SAL/COC vs. SAL/SAL $p<0.05$). Post hoc analysis showed no significant difference in the stereotypic activity of rats administered CXCL12 (25 ng/0.5 µl) into the ventral tegmental area and cocaine (20 mg/kg i.p.) as compared to cocaine alone (CXCL12/COC vs. SAL/COC $p>0.05$). CXCL12 administered into the ventral tegmental area alone did not significantly alter stereotypy (CXCL12/SAL vs. SAL/SAL $p>0.05$).

CXCL12 administration into the caudate putamen

Bilateral administration of CXCL12 (25 ng/0.5 µl) into the caudate putamen 15 minutes prior to cocaine (20 mg/kg IP) resulted in a potentiation in stereotypy as compared to cocaine alone (Fig. 2.6). Repeated measures ANOVA showed a significant difference between treatment groups ($F(3,99) = 36.31$, $p<0.0001$). Bonferroni post hoc analysis showed that CXCL12 administration into caudate putamen prior to a systemic injection of cocaine produced greater stereotypy than that produced by cocaine alone (CXCL12/COC vs. SAL/COC; $*p<0.05$, $**p<0.01$, $***p<0.001$).

Ambulatory activity was significantly different between treatment groups ($F(3,99) = 33.76$, $p<0.0001$; data not shown). Administration of cocaine (20 mg/kg IP) produced a significant increase in ambulatory activity as compared to saline controls (SAL/COC vs. SAL/SAL $p<0.05$). Administration of CXCL12 (25 ng/0.5 µl) into the caudate putamen

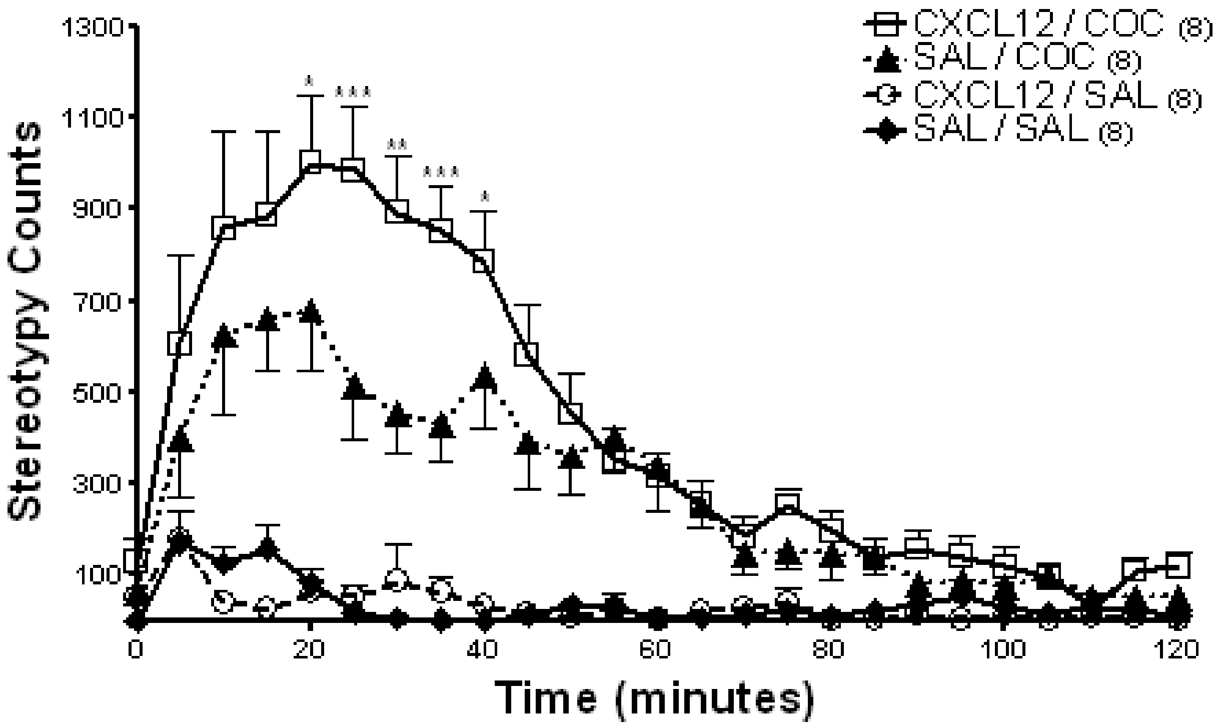

Figure 2.6 Effect of CXCL12 Administration into the Caudate Putamen on Stereotypy Activity.

Administration of CXCL12 (25 ng/0.5 µl) into the caudate putamen 15 minutes prior to cocaine (20 mg/kg IP) resulted in significantly higher stereotypic activity as compared to cocaine alone. Data are expressed as mean ± SEM beam breaks/five minute period. N= 8 per group. (CXCL12/COC vs. SAL/COC; *p<0.05, **p<0.01, ***p<0.001)

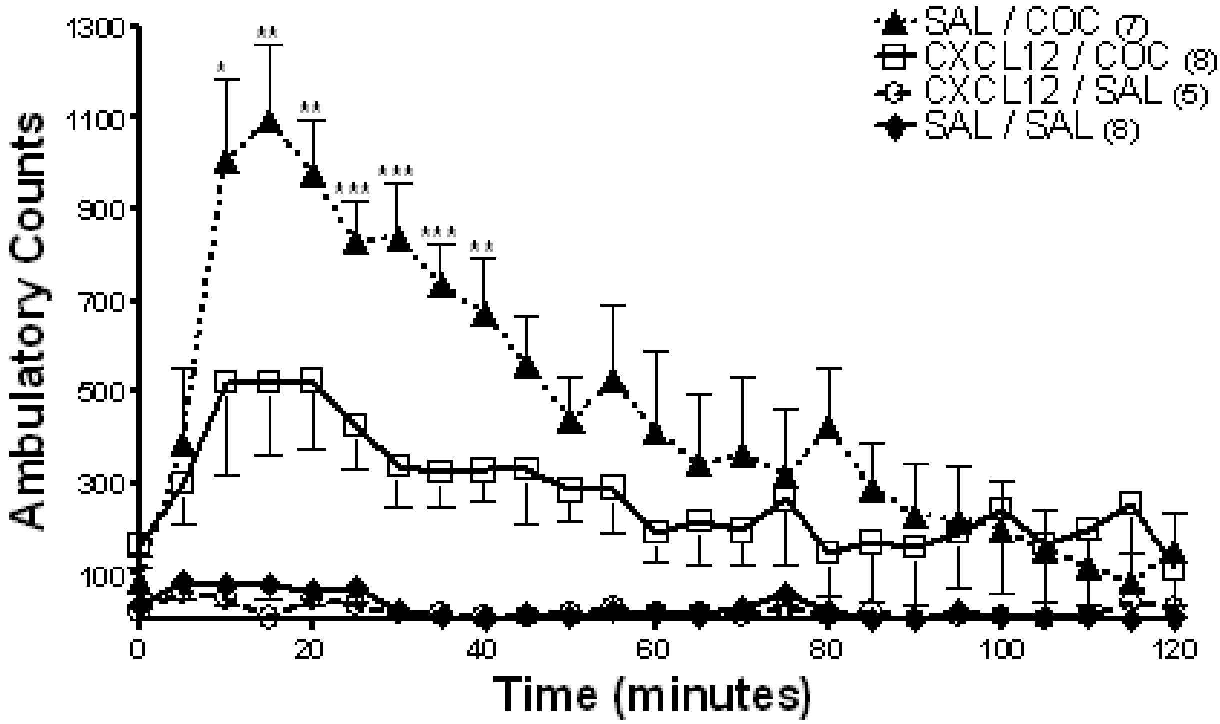

Figure 2.7 Effect of CXCL12 Administration into the Lateral Accumbens Shell on Ambulatory Activity.

Administration of CXCL12 (25 ng/0.5 μl) into the lateral accumbens shell 15 minutes prior to cocaine (20 mg/kg IP) produced a significant inhibition in ambulatory activity as compared to cocaine alone. Data are expressed as mean ± SEM beam breaks/five minute period. N= 5 – 8 per group as indicated for each. (CXCL12/COC vs. SAL/COC; *$p<0.05$, **$p<0.01$, ***$p<0.001$)

concomitantly with cocaine (20 mg/kg i.p.) produced a similar increase in ambulatory activity as that produced by cocaine (20 mg/kg i.p.) alone (CXCL12/COC vs. SAL/COC $p>0.05$). CXCL12 (25 ng/0.5 µl) administered into the caudate putamen alone did not significantly alter ambulatory or stereotypy activity as compared to saline controls (CXCL12/SAL vs. SAL/SAL $p>0.05$).

CXCL12 administration into the lateral shell of the nucleus accumbens

Bilateral administration of CXCL12 (25 ng/0.5 µl) into the lateral accumbens shell 15 minutes prior to cocaine (20 mg/kg IP) resulted in an inhibition in ambulatory activity as compared to cocaine alone (Fig. 2.7). Repeated measures ANOVA showed a significant difference in ambulatory activity between the groups ($F(3,99) = 38.45$, $p<0.0001$). Bonferroni post hoc analysis revealed that CXCL12 administration into the lateral accumbens shell prior to a systemic injection of cocaine produced significantly less ambulatory activity as compared to cocaine alone (CXCL12/COC vs. SAL/COC; *$p<0.05$, **$p<0.01$, ***$p<0.001$).

CXCL12 injected into the lateral accumbens shell did not alter cocaine-induced stereotypy. Stereotypy was significantly different between treatment groups ($F(3,99) = 36.04$, $p<0.0001$; data not shown). Administration of cocaine (20 mg/kg IP) significantly increased stereotypy (SAL/COC vs. SAL/SAL $p<0.05$). Administration of CXCL12 (25 ng/0.5 µl) into the lateral accumbens shell concomitantly with cocaine (20 mg/kg IP) did not alter cocaine-induced stereotypy (CXCL12/COC vs. SAL/COC $p>0.05$). CXCL12 (25 ng/0.5 µl) administered into the lateral accumbens shell alone did not significantly alter ambulatory activity or stereotypy as compared to saline controls (CXCL12/SAL vs. SAL/SAL $p>0.05$).

CXCL12 administration into the core of the nucleus accumbens

Bilateral administration of CXCL12 (25 ng/0.5 µl) into the core of the nucleus accumbens 15 minutes prior to cocaine (20 mg/kg IP) did not alter cocaine-induced ambulatory

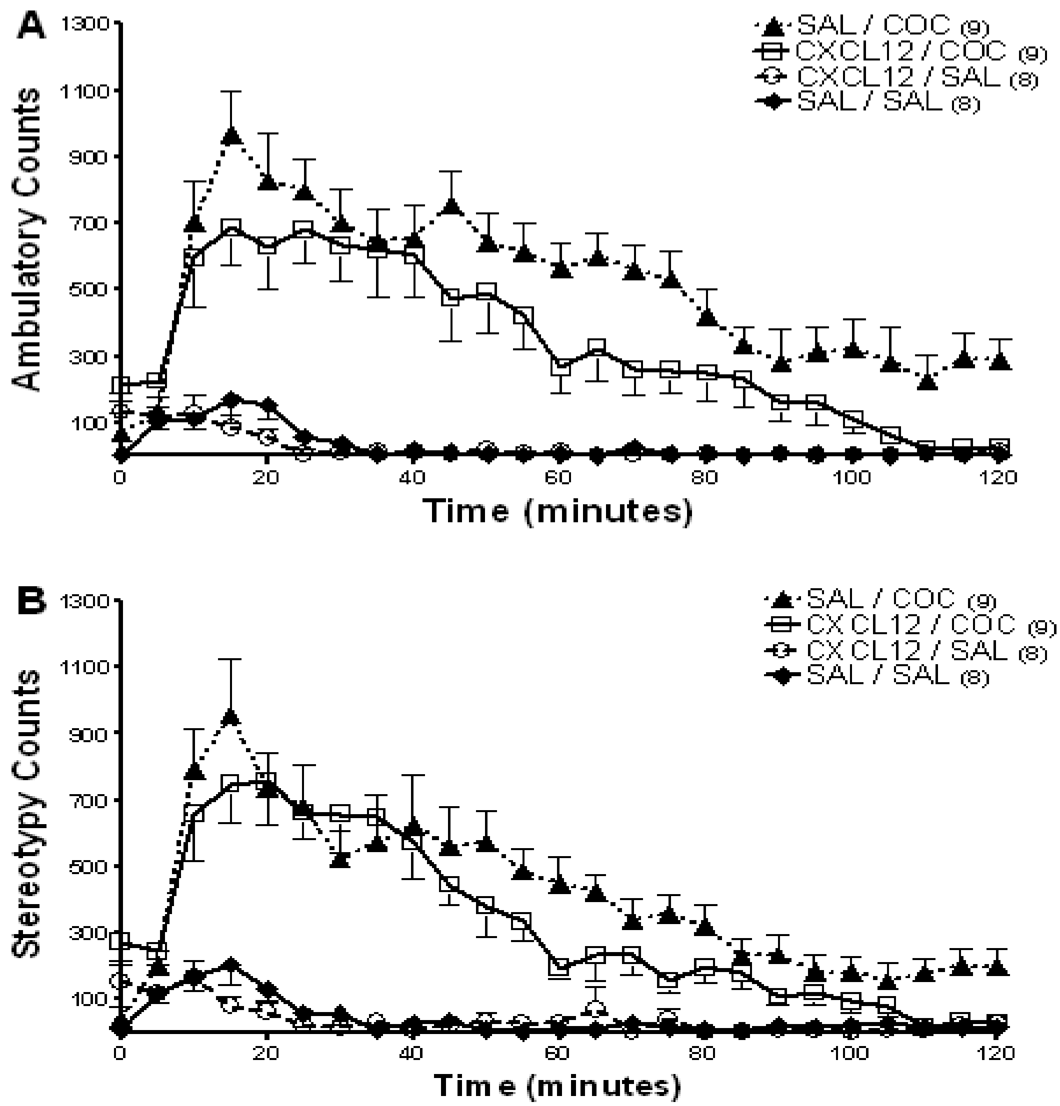

Figure 2.8 Effect of CXCL12 Administration into the Core of the Nucleus Accumbens on Ambulatory Activity and Stereotypy.

Administration of CXCL12 (25 ng/0.5 µl) into the core of the nucleus accumbens 15 minutes prior to cocaine (20 mg/kg IP) did not significantly alter A) ambulatory activity or B) stereotypy as compared to the cocaine alone. Data are expressed as mean ± SEM beam breaks/five minute period. N= 8 – 9 per group as indicated for each. (CXCL12/COC vs. SAL/COC; $p>0.05$)

activity or stereotypy (Fig. 2.8A, 2.8B). Repeated measures ANOVA showed there was a significant difference in ambulatory activity between groups ($F(3,99) = 73.48$, $p<0.0001$). Bonferroni post hoc analyses of ambulatory activity showed CXCL12 administration into the core of the nucleus accumbens prior to a systemic injection of cocaine was not significantly different than cocaine alone (CXCL12/COC vs. SAL/COC; $p>0.05$, Fig. 2.8A). CXCL12 (25 ng/0.5 μl) administered into the core of the nucleus accumbens alone did not significantly alter ambulatory activity as compared to saline controls (CXCL12/SAL vs. SAL/SAL $p>0.05$).

Repeated measures ANOVA showed a significant difference in stereotypy between the groups ($F(3,99) = 30.32$, $p<0.0001$). Bonferroni post hoc analyses showed cocaine (20 mg/kg IP) significantly increased stereotypy (SAL/COC vs. SAL/SAL $p<0.05$) however CXCL12 administration into the core of the nucleus accumbens did not significantly alter cocaine induced stereotypy (CXCL12/COC vs. SAL/COC; $p>0.05$, Fig. 2.8B). CXCL12 (25 ng/0.5 μl) administered into the core of the nucleus accumbens alone did not significantly alter stereotypy as compared to saline controls (CXCL12/SAL vs. SAL/SAL $p>0.05$).

Discussion

The present study evaluated the ability of CXCL12 to modulate the behavioral effects of cocaine. In the initial studies, CXCL12 was administered ICV. Ambulatory and stereotypic activities of rats administered concomitant ICV CXCL12 and IP cocaine were significantly higher than those given cocaine alone. The selective CXCR4 receptor antagonist AMD 3100 prevented CXCL12 from increasing cocaine-induced activity indicating that CXCL12 potentiated the effects of cocaine by activating CXCR4 receptors. AMD 3100 had no effect on baseline activity at the doses tested suggesting that CXCR4 does not tonically regulate activity

Brain Region	Ambulatory	Stereotypy
Lateral Ventricle	+	+
Ventral Tegmental Area	+	0
Caudate Putamen	0	+
Nucleus Accumbens		
Core	0	0
Lateral Shell	-	0

+, increase in cocaine-induced activity (p<0.05); -, decrease in cocaine-induced activity (p<0.05); 0, no change in activity (p>0.05)

Table 2.1 Modulation of Cocaine-Induced Ambulatory and Stereotypic Activity by CXCL12 (25 ng)

Animals receiving intracerebral administration of CXCL12 (25 ng) in the lateral ventricle or ventral tegmental area followed by cocaine (i.p.) demonstrated a significant increase in ambulatory activity as compared to animals receiving cocaine alone (Sal/Coc vs. CXCL12/Coc, $p<0.05$). Animals receiving intracerebral administration of CXCL12 (25 ng) in the lateral ventricle or caudate putamen followed by cocaine (i.p.) demonstrated a significant increase in stereotypic activity as compared to animals receiving cocaine alone (Sal/Coc vs. CXCL12/Coc, $p<0.05$). In contrast, animals receiving intracerebral administration of CXCL12 (25 ng) in the lateral shell of the nucleus accumbens followed by cocaine (i.p.) demonstrated a significant decrease in ambulatory activity as compared to animals receiving cocaine alone (Sal/Coc vs. CXCL12/Coc, $p<0.05$). Animals receiving intracerebral administration of CXCL12 (25 ng) in the core of the nucleus accumbens followed by cocaine (i.p.) showed no change in either ambulatory or stereotypic activity (Sal/Coc vs. CXCL12/Coc, $p>0.05$).

under normal physiological conditions. AMD 3100 is a CXCR4 receptor antagonist and does not inhibit calcium influx, stimulate guanosine triphosphate binding or have any effects on chemotaxis (Fricker et al. 2006). Although AMD 3100 alone failed to alter baseline activity in the present study, it should be noted that baseline activity was low as it usually is when measured during the animals' light cycle (inactive period), therefore any suppressant effect of AMD 3100 on activity could have been masked.

Hyperactivity produced by cocaine is due to its ability to enhance dopaminergic neurotransmission (Kuczenski et al. 1991). Therefore, one potential mechanism by which CXCL12 could potentiate cocaine-stimulated activity is through an interaction with dopaminergic neurons. Previous studies have demonstrated that CXCR4 is expressed by dopaminergic neurons within the substantia nigra (Banisadr et al. 2002; Banisadr et al. 2003). Here, cocaine-induced activity was used to broaden the understanding of CXCL12/CXCR4 pathways in the brain and investigate their interaction with dopaminergic neurons. Based upon the initial findings of a potentiation of cocaine-induced activity following ICV administration of CXCL12, subsequent studies investigated specific brain regions to determine the site of action of CXCL12. Using individual cohorts of animals, CXCL12 was injected bilaterally into the ventral tegmental area, caudate putamen, lateral accumbens shell and core of the nucleus accumbens both alone and in combination with cocaine, and the effects on activity determined. These findings are summarized in Table 2.1. It was somewhat surprising that the effective dose (i.e., 25 ng) of CXCL12 to enhance cocaine-stimulated activity was similar when administered ICV and into specific brain regions such as the ventral tegmental area. However, dose-response curves for both were performed which help to validate the findings. Further, the effective dose of CXCL12 when administered into the ventral tegmental area was similar to that published by Skrzydelski and colleagues. In their study, 50 ng CXCL12 was injected into the substantia nigra and this resulted in an increase in both contra-lateral turning and extracellular dopamine in the caudate putamen (Skrzydelski et al. 2007).

The ventral tegmental area has previously been shown to be an important site for mediating many of the behavioral effects of cocaine, including its reinforcing and locomotor-activating effects (Roberts and Koob 1982; Bozarth and Wise 1986). Dopaminergic cell bodies of the mesolimbic dopamine pathway originate in the ventral tegmental area and project to limbic forebrain regions including the nucleus accumbens. Positive CXCR4-immunoreactivity and CXCR4 mRNA have been identified in the ventral tegmental area (Banisadr et al. 2002). In the current study, a significant potentiation of cocaine-induced ambulatory activity was observed when CXCL12 was injected into the ventral tegmental area prior to cocaine. Cocaine-induced stereotypy was not affected by intra-ventral tegmental area injection of CXCL12. Although the mechanism by which CXCL12 could enhance the behavioral effects of cocaine has not been elucidated, it is possible that CXCL12 is activating CXCR4 receptors on dopaminergic neurons within the ventral tegmental area, stimulating a calcium current within these neurons and thus increasing dopamine release in the terminal regions. Another possibility involves the co-expression of CXCR4 on GABAergic interneurons of the ventral tegmental area (Banisadr et al. 2002). Activation of CXCR4 receptors could inhibit these GABAergic neurons, thus causing a disinhibition of the dopaminergic pathway.

The effects of CXCL12 administered directly into the terminal areas of the mesolimbic dopamine pathway were also evaluated. In contrast to its action in the ventral tegmental area, a significant decrease in cocaine-induced ambulatory activity was seen when CXCL12 was injected into the lateral shell of the nucleus accumbens. Stereotypy was unaltered by CXCL12 into the accumbens shell, similar to the ventral tegmental area. Preliminary results from our laboratory have provided evidence of CXCR4 immunoreactivity in the lateral shell of the nucleus accumbens (unpublished data). The nucleus accumbens contains a high percentage of GABAergic medium spiny neurons, some of which project back to the ventral tegmental area. If CXCR4 were present on these GABAergic neurons within the nucleus accumbens, activation of this receptor could cause feedback inhibition of dopaminergic neurons in the ventral tegmental area thus reducing subsequent release of dopamine (Guyon and Nahon 2007). In contrast to its effects in the lateral shell of the accumbens, administration of CXCL12 into the core of the nucleus accumbens did not affect cocaine-induced ambulatory or stereotypy activity. A previous report suggests that the core of the nucleus accumbens is devoid of CXCR4 receptors (Banisadr et al. 2002), and our preliminary immunohistochemistry data agree with this assessment (unpublished data). Thus, the present finding of no effect of CXCL12 when administered into the core of the nucleus accumbens agree with the lack of CXCR4 receptors in this brain region.

Previous research has shown that administration of CXCL12 into the substantia nigra induces contralateral turning and increases extracellular dopamine concentrations in the striatum (Skrzydelski et al. 2007), providing evidence that activation of CXCR4 can augment dopaminergic transmission in the nigrostriatal pathway. Studies have also shown that stereotypic activity is primarily controlled by the caudate putamen (Asher and Aghajanian 1974; Ushijima et al. 1995). In the present study, administration of CXCL12 into the terminal region of the nigrostriatal pathway, the caudate putamen, significantly potentiated cocaine-induced stereotypy.

CXCR4 is localized in the substantia nigra on both dopaminergic neurons and GABA axonal processes (Banisadr et al. 2002). CXCL12, by activating CXCR4, can influence the function of dopaminergic neurons through its actions on GABA- and glutamate transmission

(Guyon et al. 2006) and can increase intracellular calcium via N-type calcium currents (Zheng et al. 1999; Guyon et al. 2005). Multiple pre- and post-synaptic effects of CXCL12 on dopamine neurons in the substantia nigra have been shown including (1) an increase in the frequency of $GABA_A$ postsynaptic events in response to CXCL12 produced by activation of CXCR4 receptors on GABAergic neurons of the substantia nigra, (2) a glutamatergic inward current likely due to glutamate release from non-neuronal cells, (3) CXCL12 activation of a G-protein activated inwardly rectifying K+ channel (GIRK) current via CXCR4 and (4) in increase in the amplitude of total high voltage-activated calcium currents through CXCR4 activation at low concentrations of CXCL12 (.1-10 nM) (Guyon et al. 2006; Guyon and Nahon 2007). Together, these effects of CXCL12 in the substantia nigra result in increased dopamine release in the striatum (Skrzydelski et al. 2007).

In the present investigation, a dose-response study was performed to determine the optimum dose of CXCL12 ICV to alter cocaine-induced activity. Results revealed an inverted U-shaped dose-response function. This is in agreement with previous studies that have documented dose-dependent actions of CXCL12. For example, CXCL12 at concentrations of 0.1 – 1.0 nM depolarizes dopaminergic neurons by inducing the stimulation of glutamate receptors, whereas high concentrations (≥10 nM) hyperpolarize dopaminergic neurons by stimulating GABA receptors (Guyon et al. 2006). CXCL12 has also been shown to exert a biphasic effect on high voltage-activated calcium currents of dopaminergic neurons: a potentiation at low concentrations (0.1-10 nM) and a depression at higher concentrations (50-100 nM) (Banisadr et al. 2005; Guyon et al. 2006). Results from the present study with CXCL12 (25 ng) are consistent with the effects seen at low concentrations in terms of potentiating calcium currents of dopaminergic neurons (Guyon et al. 2006). Our finding that higher doses of CXCL12 were not effective in potentiating cocaine-induced activity may be due to its reported depression of calcium currents at high concentrations or activation of CXCR7 in addition to CXCR4 at higher doses. It has been shown in cultures of dopaminergic neurons, that the effect of high concentrations of CXCL12 (up to 1 μM) are not blocked by AMD 3100 (Guyon and Nahon 2007). Other possibilities include differential binding to various sites on the CXCR4 receptor, truncation of CXCL12 by proteases leading to active peptides that could differentially bind CXCR4 or activation of the CXCR7 receptor. Recent data have identified CXCR7 mRNA expression within the shell of the accumbens and the caudate putamen (Schonemeier et al. 2008). Alternatively, evidence of desensitization and internalization of CXCR4 at high concentrations of CXCL12 (Haribabu et al. 1997; Stumm and Hollt 2007) could also help explain the inverted U-shaped dose-response curve.

The results of our experiments demonstrate that CXCL12 can modulate the behavioral effects of cocaine differently depending upon its site of action within the brain. CXCL12, acting through its receptor CXCR4, can influence the function and behavioral output of both the mesolimbic and nigrostriatal dopamine pathways. Furthermore, the ability of CXCL12 to increase stereotypy when injected into the caudate putamen but inhibit locomotion following administration into the lateral accumbens shell demonstrates that activation of CXCR4 on different neuronal populations dictates its effect on behavior. The influence of CXCL12 on these dopaminergic systems provides further evidence of neurotransmitter/chemokine interactions in the brain. Future research into the central mechanisms of CXCL12 and both the CXCR4 and CXCR7 receptors is needed to elucidate the variety of cellular interactions and impact this chemokine has within the adult brain.

CHAPTER 3

Localization of CXCR4 within the Forebrain of the Adult Rat

Introduction

Chemotactic cytokines, also known as chemokines, are small secreted proteins that exert their effects through the activation of G protein-coupled receptors (GPCRs). Chemokines have been studied for their chemoattractant properties and their ability to control leukocyte migration, as well as their involvement in the neuropathology of multiple sclerosis, HIV/AIDS, Alzheimer's and Parkinson's disease (Streit et al. 2001; Cartier et al. 2005; Ubogu et al. 2006). Most chemokines are not constitutively expressed, rather their production and secretion is induced by an inflammatory response (White et al. 2007). CXCL12, formerly known as stromal cell derived factor-1 (SDF-1), is one of the few chemokines that is expressed constitutively (Tham et al. 2001; Banisadr et al. 2003; Tissir et al. 2004). CXCL12 is a potent chemoattractant for T cells, monocytes and lympho-hematopoietic cells (Crump et al. 1997). CXCL12 binds to and activates two receptors, CXCR4 and CXCR7. CXCR4 is vital for embryonic development as CXCL12, acting through CXCR4, regulates the migration of progenitor stem cells (Tachibana et al. 1998; Lu et al. 2002). Mice with a genetic deletion of CXCR4 develop abnormal blood vessels, heart and other organs. Mice lacking either CXCL12 or CXCR4 exhibit major defects within their vasculature and central nervous system (CNS) and die shortly after birth (Nagasawa et al. 1998; Tachibana et al. 1998; Zou et al. 1998; Stumm et al. 2003).

CXCR4 is expressed by microglia, astrocytes and neurons of the mature CNS (Tanabe et al. 1997; Ohtani et al. 1998; Bajetto et al. 2001). Neuronal expression of CXCR4, as shown via immunohistochemistry, occurs in the cerebral cortex, globus pallidus, caudate putamen, substantia innominata, supraoptic and paraventricular hypothalamic nuclei, lateral hypothalamus, ventromedial thalamic nucleus, cerebellum and substantia nigra pars reticulata (Banisadr et al. 2002). CXCR4 is expressed by cholinergic interneurons in the substantia innominata and caudate putamen (Banisadr et al. 2002), arginine-vasopressin neurons of the supraoptic and paraventricular hypothalamic nuclei (Banisadr et al. 2003), neurons expressing melanin-concentrating hormone in the lateral hypothalamus (Guyon et al. 2005), dopaminergic neurons in the substantia nigra (Banisadr et al. 2002), on GABAergic neurons of the pars reticulata and substantia nigra (Guyon et al. 2006) and Purkinje neurons and granule cells of the cerebellum (Ragozzino 2002). The expression of CXCR4 with various cell types indicates its involvement in both neuromodulation and neurotransmitter release in the adult brain.

Recent studies have suggested a role for CXCL12 and CXCR4 in the dopaminergic pathways of the brain. The mesolimbic dopamine pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens while the nigrostriatal dopamine pathway projects from the substantia nigra to the caudate putamen. Research has suggested the involvement of both the mesolimbic and nigrostriatal dopamine pathways in the modulation of behavioral activity following CXCL12 administration. Injection of CXCL12 into the substantia nigra increases the release of dopamine in the caudate putamen and results in an increase in locomotor activity (Skrzydelski et al. 2007). Previous results from our laboratory have demonstrated the ability of CXCL12 to modulate the behavioral effects of cocaine. CXCL12 administered into the VTA or caudate putamen prior to intraperitoneal (i.p.) cocaine potentiated cocaine-induced ambulatory and stereotypic activity, respectively. In contrast, injection of CXCL12 into the lateral shell of the nucleus accumbens reduced cocaine-induced ambulatory activity (Trecki and Unterwald 2009).

The mechanisms by which CXCR4 activation can modulate the behavioral effects of cocaine are not clearly understood. As described above, CXCR4 has been identified on dopaminergic and GABAergic neurons in the substantia nigra and on cholinergic neurons within the caudate putamen (Banisadr et al. 2002; Guyon et al. 2006). The present study utilized single staining immunohistochemistry to further evaluate expression of CXCR4 in the forebrain of the adult rat. Dual labeling immunofluorescent studies were used to determine the localization of CXCR4 on cholinergic and GABAergic neurons. The aim of this research was to determine the neurochemical phenotype of cells expressing CXCR4 in the caudate putamen and nucleus accumbens.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 325-350g were used in this study. Animals were housed in groups of four per cage on a 12 h light-dark cycle (7 AM – 7 PM) with ad libitum access to food and water. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine.

Immunohistochemistry

Animals were anesthetized with Telazol (tiletamine/zolazepam, 40mg/kg, i.p., Fort Dodge Animal Health, Overland Park, KS) and intracardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were removed, post-fixed in 4% paraformaldehyde for two hours, and stored in 30% sucrose/PBS solution overnight.

Single antibody labeling immunohistochemistry studies and dual labeling immunofluorescent studies were conducted using eight adult male Sprague Dawley rats.

Tissues were processed for CXCR4-immunoreactivity (CXCR4-ir) by the avidin-biotin complex procedure (Dun et al. 1993). The brain was embedded in agar and coronal sections of 50 µm thickness were cut on a vibratome. Sections were taken from 1.56 to 1.08 mm (Fig.3.1) (Paxinos and Watson 2007). Tissues were treated with 3% H_20_2 to quench endogenous peroxidase, washed several times, blocked with normal horse serum (1:30 dilution), and incubated in anti-CXCR4 antibody (1:100 dilution), which was an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of CXCR4 of human origin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After rinsing, sections were incubated in biotinylated antigoat IgG (1:150 dilution; Vector Laboratories, Burlingame, CA) for two hours, rinsed with PBS and incubated in avidin-biotin complex solution for 1.5 hours (1:100 dilution; Vector Laboratories, Burlingame, CA). After several washes in Tris-buffered saline, sections were developed in 0.05% diaminobenzidine/0.001% H_20_2 solution and washed with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air dried, dehydrated with absolute alcohol followed by zylene and cover-slipped with Permount.

A similar avidin-biotin complex procedure was used to detect the dopamine D1 receptor (D1R), choline acetyltransferase (ChAT), glial fibrillary acidic protein (GFAP) and glutamic acid decarboxylase C38 (GAD C38). For D1R, ChAT and GAD C38, sections were blocked with normal goat serum (1:100) and incubated in either anti-D1R antibody (1:500; rabbit polyclonal, Millipore/Chemicon International, Billerica, MA), anti-ChAT antibody (1:300; rabbit polyclonal, Millipore/Chemicon International, Billerica, MA) or anti-GAD C38 (1:500; a rabbit polyclonal directed against soluble and membrane bound GAD generously provided by Dr. JY Wu (Wu et al. 1986; Nathan et al. 1994; Hsu et al. 1999) to Dr. NJ Dun). For GFAP, sections were blocked with normal horse serum (1:30) and incubated with anti-GFAP antibody (1:300; mouse monoclonal antibody, Millipore/Chemicon International, Billerica, MA). After rinsing, sections were incubated in biotinylated antigoat IgG (1:50 dilution; Vector Laboratories, Burlingame, CA) for D1R, ChAT and GAD C38, while GFAP was incubated in biotinylated antimouse IgG (1:150 dilution; Vector Laboratories, Burlingame, CA).

For double labeling immunofluorescent studies, sections were blocked with normal horse serum (1:30 dilution in PBS, 0.5% BSA, 0.4% Triton X-100) and then incubated with anti-CXCR4 antibody (1:50) for 48 hours. Following several washes with PBS, sections were incubated with biotinylated antigoat IgG (1:50, Vector Laboratories), washed with PBS, and incubated with fluoroscein avidin D. After rinsing with PBS, sections were blocked with normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and incubated with anti-D1R antibody (1:250 dilution), anti-ChAT antibody (1:150 dilution), anti-GAD C38 antibody (1:250 dilution) or anti-GFAP antibody (1:150 dilution) for 48 hours. Following rinsing, tissues were incubated in appropriate IgG Texas Red antibodies. After rinsing with PBS, select sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:5000 dilution) for 10 minutes, washed with PBS, and mounted in Citifluor and cover-slipped (Brailoiu et al. 2007). Sections were examined under a confocal scanning laser microscope (TCS SL; Leica Microsystems Inc., Exton, PA) with excitation emission wavelengths set to 488 / 520 nm for fluorescein isothiocyanate, 543 / 620 nm for Texas Red and 405 nm for DAPI in the sequential mode.

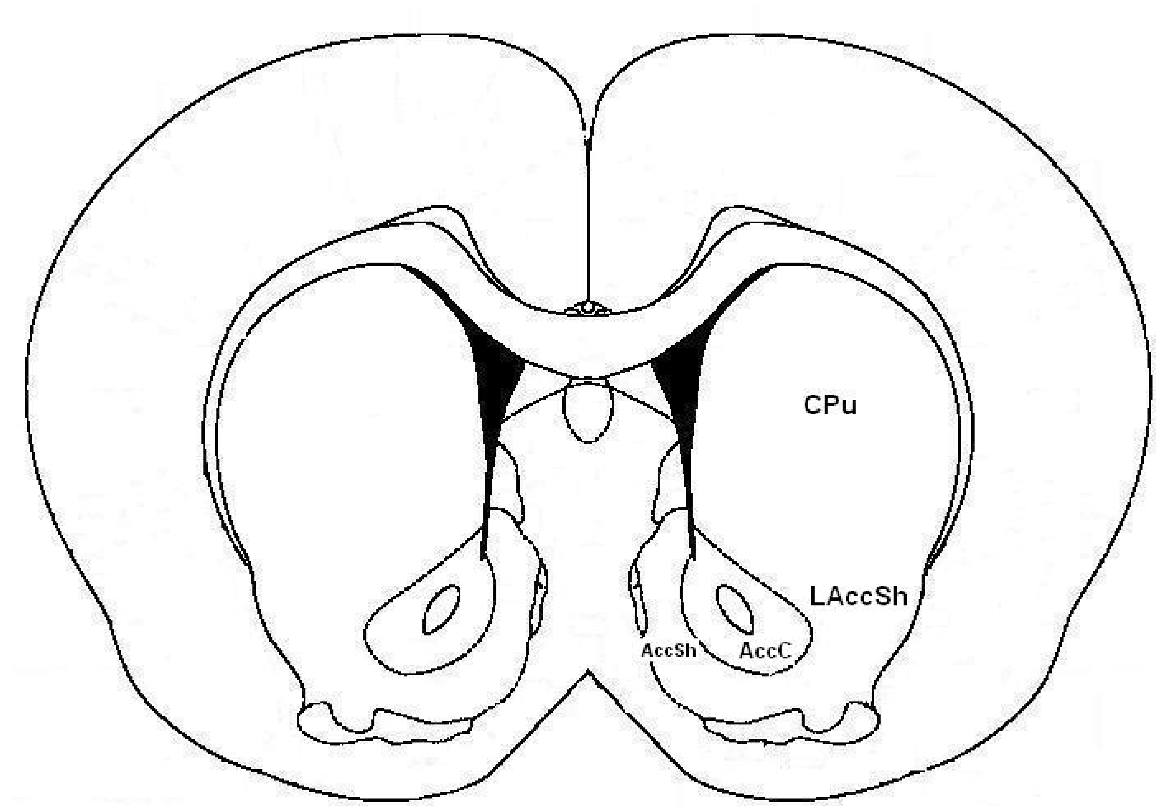

Figure 3.1 Immunohistochemical Analysis Was Conducted Using Coronal Sections From Bregma 1.56 to 1.08 mm.

Areas of interest are labeled. Figures reproduced from Paxinos and Watson (2007) with permission. (CPu – caudate putamen, LAccSh – lateral accumbens shell, AccSh – medial accumbens shell, AccC – accumbens core)

Results

Immunohistochemistry

Brain sections ranging from Bregma 1.56 to 1.08 mm from four adult Sprague Dawley rats were examined for the presence of CXCR4-ir in the caudate putamen and lateral shell of the accumbens. A representative section is shown in Fig. 3.1 (bregma 1.32 mm) (Paxinos and Watson 2007). Coronal brain sections were first incubated with anti-CXCR4 antibody to determine its expression in the caudate putamen and nucleus accumbens. Results demonstrate that CXCR4 is expressed on cells and processes in the caudate putamen (Fig. 3.2A, 3.2B) and lateral accumbens shell (Fig. 3.2C, 3.2D). No CXCR4 staining was observed in the medial shell or core of the nucleus accumbens. Separate sections were incubated with the following antibodies to show the localization in the forebrain and to optimize concentrations for dual-labeling immunofluorescence: D1R, the dopamine D1 receptor; ChAT, choline acetyl transferase, a marker for cholinergic neurons; GFAP, glial fibrillary acidic protein, a marker for astrocytes; and GAD C38, a marker for GABAergic neurons, shown to cross react with both soluble and membrane-associated glutamic acid decarboxylase. A more extensive description of the distribution of CXCR4-ir neurons and associated markers are presented below.

Caudate Putamen

Cells positively stained with the antibody to CXCR4 were found in the caudate putamen (Fig. 3.2A, 3.2B). Visual analysis revealed a homogenous pattern of CXCR4-positive cells throughout the rostral and caudal portion of the caudate putamen. No differences were observed between medial and lateral aspects, or between dorsal and ventral areas. Control studies, conducted in the absence of either primary (CXCR4) or secondary (biotinylated) antibody, resulted in no immunoreactivity in any of the sections examined (data not shown). Sections

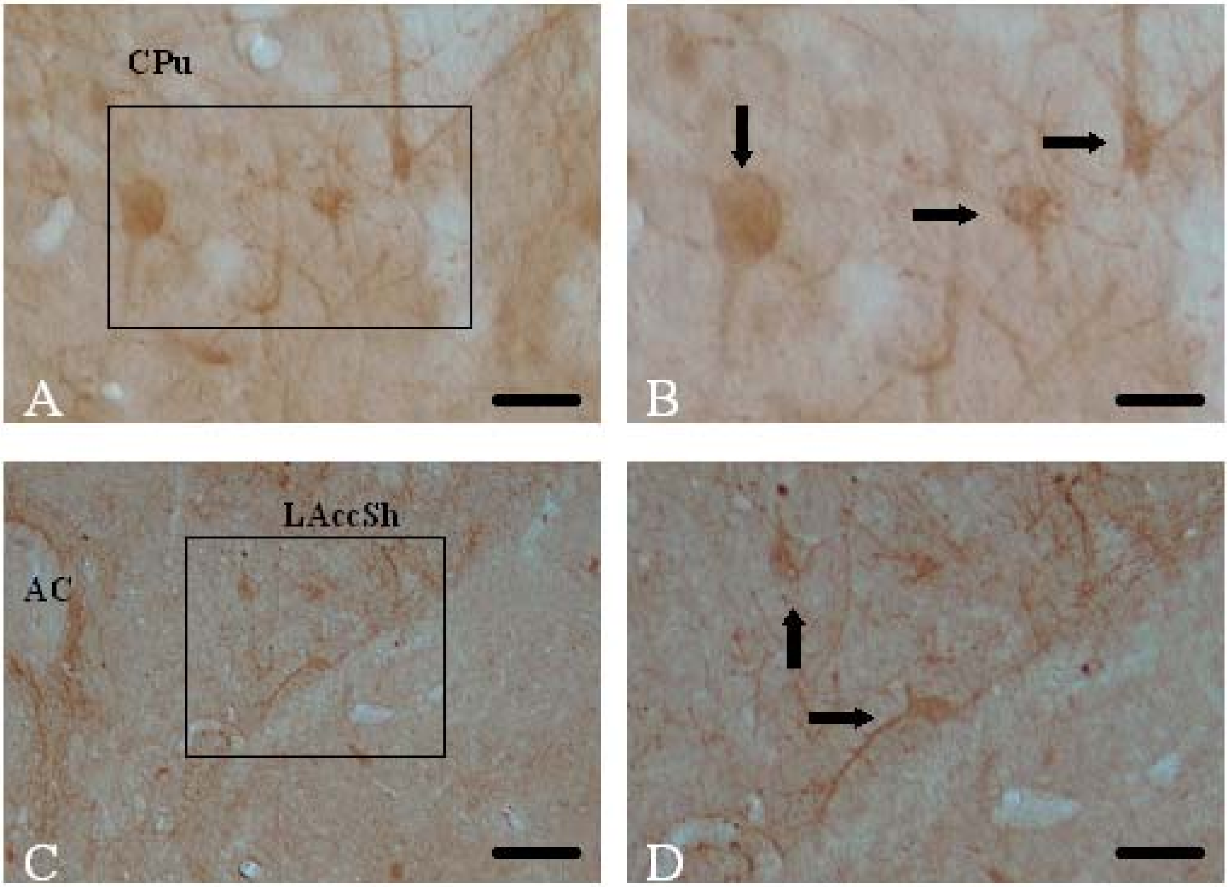

Figure 3.2 Forebrain Sections Labeled with CXCR4 Antibody.

A. CXCR4-ir positive neurons can be seen the caudate putamen. B. A higher magnification of the area outlined by the black box in A shows labeled neurons (black arrows) and processes. C. CXCR4-ir positive neurons within the lateral accumbens shell. D. A higher magnification of the area outlined by the black box in C shows neurons (black arrows) and processes labeled with CXCR4. *Scale bar*, A, C, 100 μm; B, D, 50 μm.

incubated with the anti-D1R antibody revealed staining of cell bodies throughout the caudate putamen. Sections incubated with antibody to either GAD C38 or GFAP demonstrated staining of both cell bodies and adjacent processes within the caudate putamen. Sections incubated with anti-ChAT antibody contained heavily stained processes and cell bodies.

Nucleus Accumbens

CXCR4-positive cells were identified within the lateral accumbens shell (Fig. 3.2C, 3.2D). Both cell bodies and processes positive for CXCR4 were observed throughout the rostral and caudal portions of the lateral accumbens shell. No differences in CXCR4 staining were observed from medial to lateral, or from dorsal to ventral. No CXCR4-positve cells were observed in the medial shell or the core of the nucleus accumbens. Control studies, conducted in the absence of either primary (CXCR4) or secondary (biotinylated) antibody, resulted in no immunoreactivity in any of the sections examined (data not shown). Sections incubated with antibody against the D1R revealed staining on cell bodies throughout the nucleus accumbens (Fig. 3.3A). Sections labeled with either anti-GAD C38 (Fig. 3.3B) or anti-GFAP (Fig. 3.3C) antibodies stained both cell bodies and adjacent processes within the lateral shell of the accumbens. Cells labeled with the ChAT antibody (Fig. 3.3D) contained heavily stained cell body regions and processes.

Dual Labeling Immunofluorescence

Results from dual immunofluorescence experiments demonstrate that cholinergic and GABAergic cells of the caudate putamen and lateral shell of the accumbens express CXCR4. Double labeling of striatal sections with CXCR4 and D1R antiserum revealed that a population of D1 receptor positive neurons of the caudate putamen and lateral shell of the accumbens express CXCR4 (Fig. 3.4C, 5C). Sections incubated with CXCR4 and GAD

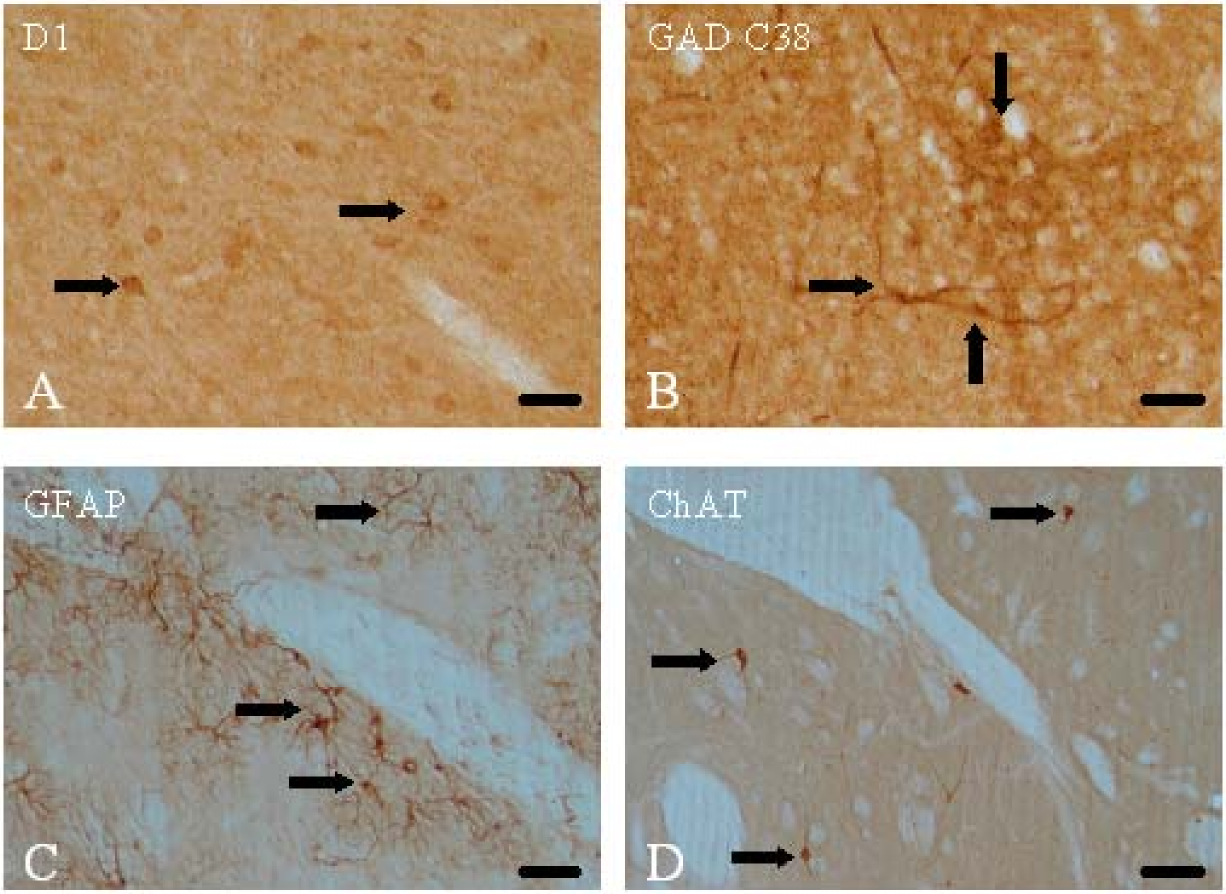

Figure 3.3 Single Labeling in the Nucleus Accumbens.

A. Labeling with D1 dopamine receptor antibody showing positive medium spiny neurons (20x, black arrows). B. Labeling with GAD C38 antibody demonstrating positive GABAergic cell bodies (20x, black arrows) and processes. C. Astrocytes labeled with GFAP antibody (20x, black arrows) D. ChAT-positive cholinergic neurons (20x, black arrows) *Scale bar*, A-D 100 µm.

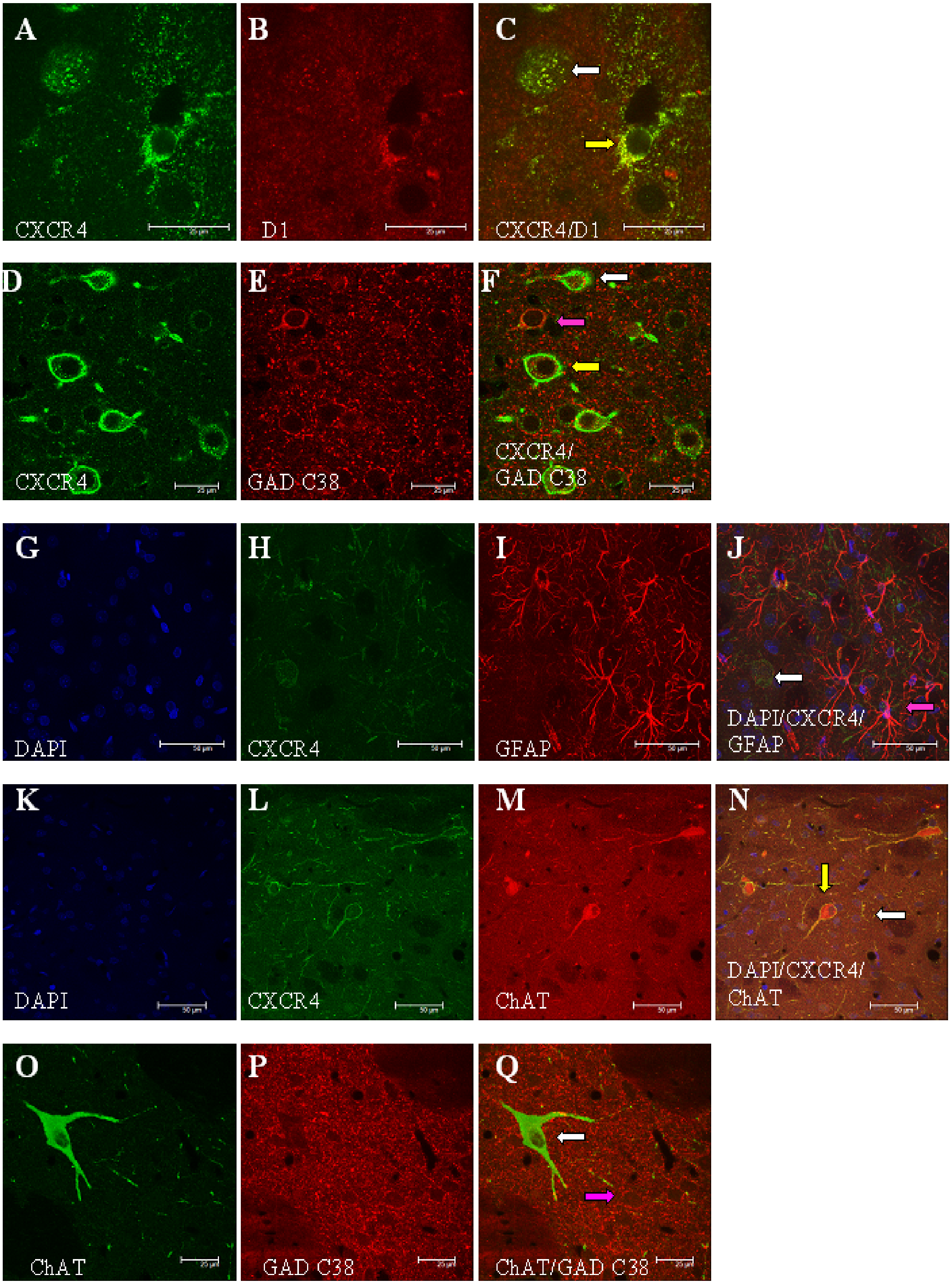
A
CXCR4
B
D1
C
CXCR4/D1
D
CXCR4
E
GAD C38
F
CXCR4/
GAD C38
G
DAPI
H
CXCR4
I
GFAP
J
DAPI/CXCR4/
GFAP
K
DAPI
L
CXCR4
M
ChAT
N
DAPI/CXCR4/
ChAT
O
ChAT
P
GAD C38
Q
ChAT/GAD C38

Figure 3.4 Confocal Images of Rat Caudate Putamen Sections Double Labeled with CXCR4 and D1R, GAD C38, GFAP or ChAT Antiserum.

A. CXCR4-ir cells. B. D1-positive cells. C. A merged image of A and B in which a population of cells are only CXCR4-positive (white arrow) while others express both CXCR4 and D1 (yellow arrow). D. CXCR4-ir cells. E. GAD C38-positive cells. F. A merged image if D and E showing cells which are only CXCR4 positive (white arrow), only GAD C38-positve (pink arrow) or co-express CXCR4 and GAD C38 (yellow arrow). G. DAPI staining for nuclei. H. CXCR4-ir cells. I. GFAP-positive cells. J. A merged image of G, H and I showing cells that are only CXCR4-positive (white arrow) and only GFAP positive (pink arrow) demonstrating that CXCR4 is not expressed in naïve astrocytes. K. DAPI staining for nuclei. L. CXCR4-ir cells. M. ChAT-positive cells. N. A merged image of K, L and M showing cells which are only CXCR4 positive (white arrow) or co-express CXCR4 and ChAT (yellow arrow). O. CXCR4-ir cell. P. GAD C38-positive cells. Q. A merged image of O and P showing no overlap between cholinergic neurons (ChAT, white arrow) and GABAergic neurons (GAD C38, pink arrow). *Scale bar*, A-F, O-Q, 25 µm; G-N, 50 µm.

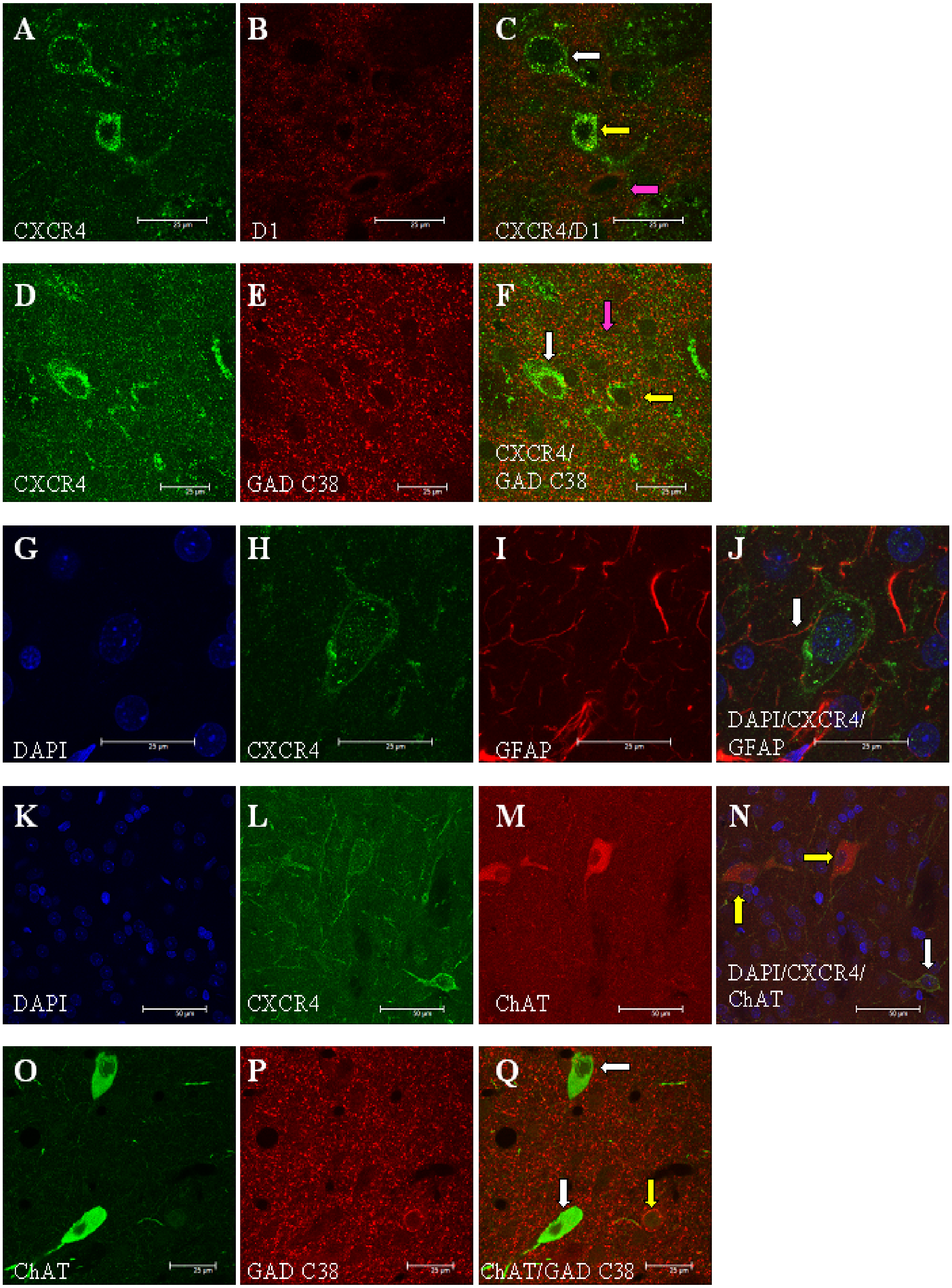
A
CXCR4
B
D1
C
CXCR4/D1
D
CXCR4
E
GAD C38
F
CXCR4/
GAD C38
G
DAPI
H
CXCR4
I
GFAP
J
DAPI/CXCR4/
GFAP
K
DAPI
L
CXCR4
M
ChAT
N
DAPI/CXCR4/
ChAT
O
ChAT
P
GAD C38
Q
ChAT/GAD C38

Figure 3.5 Confocal Images of Rat Lateral Accumbens Shell Sections Double Labeled with CXCR4 and D1R, GAD C38, GFAP or ChAT Antiserum.

A. CXCR4-ir cells. B. D1-positive cells. C. A merged image showing cells which are only CXCR4 positive (white arrow), only D1R-positve (pink arrow) or co-express CXCR4 and D1R (yellow arrow). D. CXCR4-ir cells. E. GAD C38-positive cells. F. A merged image if D and E showing cells which are only CXCR4 positive (white arrow), only GAD C38-positve (pink arrow) or co-express CXCR4 and GAD C38 (yellow arrow). G. DAPI staining for nuclei. H. CXCR4-ir cell. I. GFAP-positive cells. J. A merged image of G, H and I showing CXCR4 is not co-expressed on naïve astrocytes. Processes of adjacent astrocytes are shown extending towards a CXCR4 positive cell (white arrow). K. DAPI staining for nuclei. L. CXCR4-ir cells. M. ChAT-positive cells. N. A merged image of K, L and M showing cells which are only CXCR4 positive (white arrows) or co-express CXCR4 and ChAT (yellow arrow). O. CXCR4-ir cell. P. GAD C38-positive cells. Q. A merged image of O and P showing no overlap between cholinergic neurons (ChAT, white arrows) and GABAergic neurons (GAD C38, pink arrow). *Scale bar*, A-J, O-Q, 25 µm; K-N, 50 µm.

C38 antibody revealed cells positive for CXCR4 only, cells positive for both CXCR4 and GAD C38, and cells positive for GAD C38 only (Fig. 3.4F, 3.5F). Examination of sections incubated with anti-CXCR4 and anti-GFAP antibody showed no profiles positive for both markers, demonstrating that CXCR4 is not expressed on naïve astrocytes (Fig. 3.4J, 3.5J). To determine if CXCR4 is expressed by cholinergic interneurons of the caudate putamen and lateral shell of the accumbens, sections were incubated with CXCR4 and ChAT antiserum. Results demonstrated that cholinergic interneurons expressed CXCR4 on the cell surface and their processes (Fig. 3.4N, 3.5N). Some CXCR4 positive cells did not stain for ChAT (Fig. 3.4N, 3.5N). Lastly, as another control, adjacent sections were incubated with ChAT and GAD C38 to ensure that staining was not overlapping (Fig. 3.4Q, 3.5Q). No cells were positive for both ChAT and GAD C38-ir.

Discussion

This study demonstrates the co-expression of CXCR4 on cholinergic and GABAergic neurons within the caudate putamen and lateral shell of the nucleus accumbens. Results show CXCR4 was co-expressed with D1 dopamine receptors on GABAergic medium spiny neurons in the striatum. Results also found CXCR4 to be expressed with GAD C38, a marker for GABAergic neurons, and with ChAT, a marker for cholinergic neurons. CXCR4-ir was not observed on astrocytes, which is consistent with previous findings showing that CXCR4 is expressed by astrocytes under conditions of inflammation (Stumm et al. 2002).

Previous research has demonstrated that CXCR4 is involved in CXCL12-mediated dopamine release (Skrzydelski et al. 2007). Administration of CXCL12 into the substantia nigra causes an increase in extracellular dopamine in the caudate putamen and increases locomotor activity (Skrzydelski et al. 2007). Research has demonstrated that CXCL12 can exert multiple pre- and postsynaptic effects on dopaminergic neurons, including an increase in the amplitude of total high voltage-activated calcium currents through CXCR4 activation and an increase in the frequency of spontaneous $GABA_A$ postsynaptic currents (Guyon and Nahon 2007). Studies from our laboratory have demonstrated that CXCL12 can modulate the locomotor-stimulating properties of cocaine and its effects are dependent upon the brain region where CXCL12 is administered. Injection of CXCL12 into either the VTA or the caudate putamen potentiated locomotor and stereotypic activity respectively, whereas CXCL12 administered into the lateral shell of the accumbens inhibited locomotor activity (Trecki and Unterwald 2009). These results prompted an investigation into the localization of CXCR4 within the forebrain in an attempt to further identify the cellular phenotypes involved in CXCR4 signaling.

The cell bodies of dopaminergic neurons in the mesolimbic dopamine system originate in the VTA and project primarily to the nucleus accumbens (Koob 1992). The nucleus accumbens contains populations of GABAergic and cholinergic interneurons, as well as a large number of GABAergic medium spiny neurons (Meredith et al. 1993). The activity of the medium spiny neurons is regulated partially by glutamatergic afferents arising from the VTA. Medium spiny neurons have been shown to project to specific brain regions; to the substantia nigra from the core of the nucleus accumbens and the caudate putamen, and to the VTA, hypothalamus and amygdala from the shell of the nucleus accumbens (Pennartz et al. 1992). The activity of these medium spiny neurons are regulated locally by neuropeptides and transcription factors expressed in the nucleus accumbens (Nestler et al. 2002).

Cholinergic interneurons in the caudate putamen and nucleus accumbens provide an ongoing acetylcholine signal via tonically firing action potentials. A high concentration of acetylcholinesterase in the striatum rapidly terminates the acetylcholine signal thus preventing desensitization of nicotinic acetylcholine receptors. The ongoing nicotinic activity has been shown to increase striatal dopamine release (Zhou et al. 2002). Since cholinergic interneurons perform excitatory regulation of dopamine release in the nucleus accumbens, the inhibitory effects of GABA on acetylcholine release inhibits further dopamine release (Shirayama and Chaki 2006).

Results from the present study demonstrate the expression of CXCR4 on GABAergic and cholinergic cell types in the caudate putamen and lateral accumbens shell. These data can be used to further hypothesize the mechanism by which CXCR4 is interacting with the mesolimbic and nigrostriatal dopamine systems. Signaling pathways activated by CXCL12 are mediated by pertussis toxin-sensitive $G_{\alpha i}$ and G_q components which give rise to two distinct signaling pathways. One pathway, involving phosphatidylinositol-3 (PI-3) kinase and extracellular signal regulated kinase (ERK 1/2), have been shown to be activated by CXCL12 in rodent astrocytes, neuronal progenitors and cortical neurons (Bacon and Harrison 2000; Lazarini et al. 2000; Bajetto et al. 2001; Bonavia et al. 2003). IP_3 binds its specific receptor in the endoplasmic reticulum and releases calcium from intracellular stores. The calcium acts as a second messenger is involved in ligand-induced chemokine receptor activation (Boutet et al. 2001; Gillard et al. 2002). A second pathway involves phospholipase Cβ whose activation leads to an increase in the intracellular calcium in astrocytes, neurons, microglia and cerebellar granule cells (Klein et al. 1999; Zheng et al. 1999; Bajetto et al. 1999a). CXCR4 stimulation has been shown to modulate high threshold calcium channels (Zheng et al. 1999; Guyon and Nahon 2007) which can stimulate intracellular calcium release and proline-rich tyrosine kinase activation (Lazarini et al. 2003). In addition, CXCR4 can inhibit cAMP production through G_i (Liu et al. 2003).

In the mid-brain, CXCR4 has been shown to be expressed by dopaminergic neurons in the substantia nigra (Banisadr et al. 2002) and GABAergic neurons of the substantia nigra pars reticulata (Guyon et al. 2006). One mechanism by which CXCL12 administration in the VTA or substantia nigra could be causing extracellular dopamine release in the striatum and increasing locomotion (Skrzydelski et al. 2007; Trecki and Unterwald 2009) is by a disinhibition of dopaminergic neurons. Activation of CXCR4, expressed on GABAergic interneurons in the substantia nigra, inhibit GABA release, thus prompting the disinhibition of dopaminergic neurons. Alternatively, CXCL12 has been shown to stimulate a CXCR4-mediated increase in intracellular calcium via N-type Ca currents (Zheng et al. 1999; Guyon and Nahon 2007). This suggests CXCR4 activation on dopaminergic cell bodies could be partially responsible for the increase in extracellular dopamine release.

The caudate putamen and the nucleus accumbens share the same neuronal cell types, as was demonstrated by immunofluorescent staining in this study. Striatal output GABAergic medium spiny neurons represent 95% of the striatal neurons and can be divided into two groups: the striato-nigral neurons which express the D1-like receptors, and the striato-pallidal neurons which express the D2 like receptor (Le Moine and Bloch 1995; Yung et al. 1995; David et al. 2005). Some medium spiny neurons have also been shown to express both the D1 and D2 like receptors (Surmeier et al. 2007). The remaining striatal cell types include cholinergic or GABAergic interneurons (Kawaguchi 1997). Increases in extracellular dopamine in the nucleus accumbens results from a disinhibition of dopamine cell firing in the VTA through the activation of μ-opioid receptors located on inhibitory GABAergic interneurons (Matthews and German 1984). The reduction of GABAergic neurotransmission in the VTA would lead to an increase of the firing rate of dopaminergic VTA neurons (Szabo et al. 2002). CXCR4 expression in the forebrain could also modulate dopamine release in the caudate putamen and lateral shell of the accumbens by inhibiting GABAergic neurons in the striatum that project to the midbrain and inhibit dopaminergic neurons.

This study identified cellular markers that are expressed in conjunction with CXCR4 in individual neurons in the forebrain of the adult rat. Specifically, CXCR4 was shown to be present on GABAergic and cholinergic neurons. Furthermore, CXCR4 and the D1R were co-expressed by a sub-population of striatal neurons. These results suggest that CXCR4 could be involved in the modulation of neuronal subtypes of the adult rat brain, specifically GABAergic and cholinergic neurons that are responsible for regulating extracellular dopamine release in the striatum.

CHAPTER 4

Measurement of Extracellular Dopamine and Glutamate in the Nucleus Accumbens Following Administration of CXCL12 into the Ventral Tegmental Area

Introduction

The chemokine, CXCL12, and its receptor, CXCR4, have been shown to act as a lymphocyte chemoattractant (Bleul et al. 1996b), induce cellular migration (Tachibana et al. 1998) and apoptosis (Khan et al. 2005), and more recently, act as neuromodulators of the CNS (Adler et al. 2005). CXCL12 and CXCR4 have been found in the VTA, the substantia nigra and the caudate putamen (Banisadr et al. 2002; Banisadr et al. 2003). Both CXCL12 and CXCR4 are constitutively expressed on various cell types including endothelial cells, glia, and neurons (Ohtani et al. 1998; Bajetto et al. 1999a; Bajetto et al. 1999b).

Cocaine is a stimulant of the CNS and inhibits the reuptake of dopamine, serotonin and norepinepherine into presynaptic terminals by blocking DAT, the serotonin transporter (SERT) and the norepinepherine transporter (NET) (Heikkila et al. 1975; Ritz et al. 1987; Nicolaysen and Justice 1988). Studies have identified the inhibition of dopamine reuptake as playing a vital role in the reinforcing properties of cocaine (Ritz et al. 1987). The stimulant and rewarding properties of cocaine are mediated partially by the increase in extracellular dopamine in the synapse released from neurons originating in the VTA and project to the medial shell of the nucleus accumbens (Hoebel 1985; Wise and Bozarth 1985; Koob 1992). CXCR4 is localized on both dopaminergic and GABAergic neurons within the VTA and the substantia nigra (Banisadr et al. 2002; Guyon et al. 2006). CXCL12 administration into the substantia nigra results in increased extracellular dopamine in the striatum and increases in locomotor activity (Skrzydelski et al.

2007). CXCL12 also increases glutamate synaptic activities in the lateral hypothalamus (Guyon et al. 2005), hippocampus (Zheng et al. 1999), cerebellum (Limatola et al. 2000) and substantia nigra (Guyon et al. 2006). Furthermore, an increase in extracellular glutamate in the nucleus accumbens has been observed following acute cocaine administration (Smith et al. 1995; Reid et al. 1997). As described in chapter 1, we found that an acute injection of CXCL12 into the VTA was able to potentiate cocaine-induced locomotion (Trecki and Unterwald 2009). Since locomotor activity is dependent upon dopamine and can be modified by glutamate, we sought to investigate potential changes in extracellular dopamine and glutamate concentrations following CXCL12 administration.

Microdialysis is a technique used to sample the extracellular fluid of tissues in the body. A microdialysis probe contains a small tube made of a semi-permeable membrane. This tube, when implanted into brain tissue and perfused with artificial cerebrospinal fluid, allows chemicals in the brain to pass down a concentration gradient and be collected as dialysate. The present study used *in vivo* microdialysis to assess extracellular changes in dopamine and glutamate within the medial shell of the nucleus accumbens following administration of CXCL12 into the VTA both alone and in combination with an acute i.p. injection of cocaine. We hypothesize that administration of CXCL12 into the VTA will cause an increase in extracellular levels of dopamine and glutamate in the medial shell of the nucleus accumbens based on the results of our behavioral studies showing a potentiation in cocaine-induced behavior following CXCL12 administration.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 325-350g were used in this study. Animals were housed in groups of four per cage on a 12 h light-dark cycle (7 AM – 7 PM) with ad libitum access to food and water. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Temple University School of Medicine.

Drugs

Telazol (tiletamine/zolazepam) was purchased from Fort Dodge Animal Health (Overland Park, KS, USA). Recombinant mouse CXCL12/SDF-1α was obtained from R&D Systems (Minneapolis, MN, USA, 10 μg). Cocaine hydrochloride was generously supplied by the National Institute of Drug Abuse (Bethesda, MD, USA). Cocaine (20 mg/kg) and CXCL12 (25 ng/dose) were dissolved in sterile normal saline (0.9%) before use.

Surgery

Rats were anesthetized with Telazol (40 mg/kg I.P.) and placed in a Stoelting stereotaxic apparatus (Wood Dale, IL). Intracranial injection cannulae were constructed of stainless steel (22 GA guide, Plastics One, Roanoke, VA) and microdialysis probes were made of polyethylene plastic (CMA 12, 1 mm, CMA Microdialysis, North Chelmsford, MA). A unilateral intracranial injection cannula was inserted into the right ventral tegmental area (A/P -6.0, M/L 0.7, V -8.0) and a microdialysis probe was positioned into the ipsilateral shell of the nucleus accumbens (A/P 1.7, M/L ±0.8, V -6.4). The incisor bar was adjusted to achieve a flat skull position (-3.9 ± 0.5) as determined according to the atlas of Paxinos and Watson (Paxinos and Watson 2007). The cannulae were secured with dental acrylic anchored to a stainless steel surgical screw inserted into the skull. Stainless steel stylets, 1.0 mm longer than the intracranial injection cannula, and microdialysis guide cannulae, were inserted to maintain patency. Animals were allowed to recuperate for five days following surgery. Rats were used for a single trial and euthanized at the conclusion of the experiment to determine cannula placement.

Microdialysis

Microdialysis guide cannula were removed and replaced by a CMA 12 microdialysis probe. CNS perfusion fluid (NaCl 147 mmol/L, KCl 12.7 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 0.85 mmol/L, CMA Microdialysis, North Chelmsford, MA, USA) was pumped through the microdialysis probe overnight at a rate of 0.3 µl/min. Food pellets and Hydrogel, a gelatinous water source, were added to the microdialysis bowls and animals allowed ad libitum access. Following 12 hours of habituation, flow rate was increased to 1.3 µl/min for two hours prior to initial baseline sample collection. Treatment groups were as follows: saline (VTA)/saline (i.p.), saline (VTA /cocaine (i.p.), CXCL12 (VTA)/saline (i.p.) and CXCL12 (VTA)/cocaine (i.p.). The first three dialysate samples were taken each 20 minutes and used to define basal extracellular dopamine levels. During the third baseline sample collection, either saline or CXCL12 (25 ng/0.5 µl) was administered through an intracranial cannula into the VTA. Solutions were injected by hand at a rate of 1 µl/min with a Hamilton syringe connected to the cannula by polyethylene tubing. Rats were not restrained during injections. Fifteen minutes later, cocaine (20 mg/kg) or normal saline (1 mL/kg) was administered i.p. Dialysate samples from the medial shell of the nucleus accumbens were collected every 20 minutes. Following dialysate collection, samples were split into two aliquots: 5 µl to be used for glutamate analysis and 25 µl for dopamine analysis. Samples were immediately frozen on dry ice and stored at -80°C.

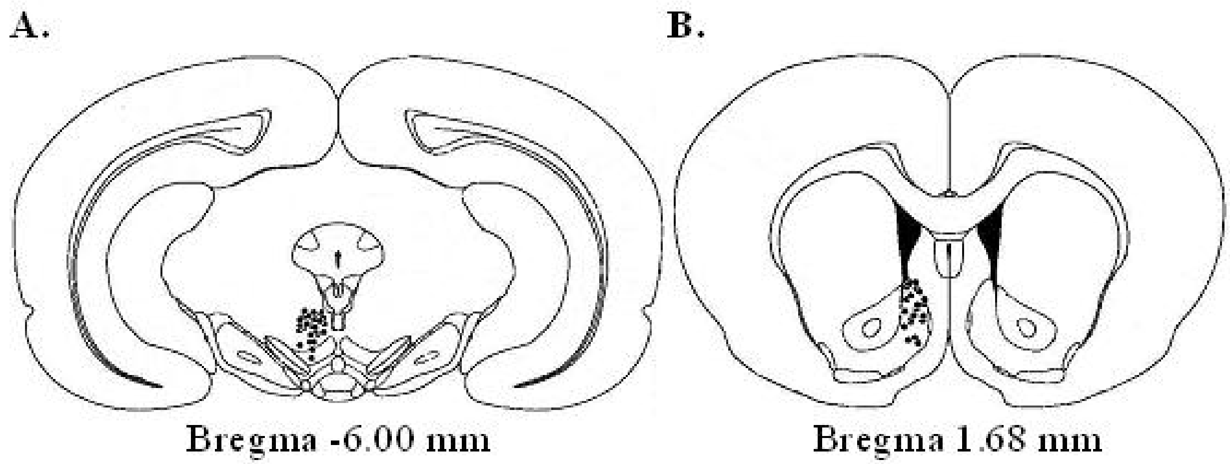

Figure 4.1 Cannula Placements.

A. Injection sites of CXCL12 in the ventral tegmental area. B. Microdialysis probe location in the medial shell of the nucleus accumbens. Locations of the tip of the cannulae are indicated by black circles. Figures reproduced from Paxinos and Watson (2007) with permission.

High Pressure Liquid Chromatography Analysis of Dopamine

Microdialysis samples were assayed for dopamine by high-pressure liquid chromatography (HPLC) with electrochemical detection. HPLC analysis was completed at the laboratory of Bryan Yamamoto Ph.D. in the Department of Neuroscience, University of Toledo School of Medicine (Toledo, OH). Due to low basal dopamine levels detected in the microdialysis samples, not all animals completed in the trial were able to be analyzed for statistical purposes. Original sample sizes per treatment group were as follows: Sal/Sal (n=6), CXCL12/Sal (n=7), Sal/Coc (n=8), CXCL12/Coc (n=8). Animals with retrievable extracellular dopamine levels are indicated in Fig. 4.2. Separation was achieved with a C18 column (100 x 2.0MM, 3μm particle size; Phenomenex, Torrance, CA, USA). The mobile phase for detection of dopamine (pH 4.2) consisted of 32mM citric acid, 54.3 mM sodium acetate, 0.074 mM EDTA, 0.215 mM octyl sodium sulfate and 3% methanol. Detection was accomplished with a DECADE or Intro^R electrochemical detector (Antec Leyden; GBC Scientific Equipment Inc., Hubbardston, MA) and peaks were analyzed with EZChrom^R Scientific Software Inc. (Pleasanton, CA).

Capillary Electrophoresis

Microdialysis samples were assayed for glutamate using capillary electrophoresis in the laboratory of Jonathan Shackman Ph.D. in the Department of Chemistry, Temple University (Philadelphia, PA).

Chemicals and Reagents

Reagent grade L-amino acids (aspartic acid, Asp; glutamic acid, Glu; abbreviations refer to the fluorescently-labeled species), ethanolamine (ETA), citric acid, and boric acid were obtained from Sigma (St. Louis, MO). 5-carboxyfluorescien, succinimidyl ester (FAM, SE) single isomer was purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA). All solutions were made using Milli-Q (Millipore, Bedford, MA) ≥ 18 MΩ cm deionized (DI) water. Leading electrolyte (LE) was 50 mM citrate balanced to pH 9.9 with ETA. Trailing electrolyte (TE) was 125 mM borate balanced to pH 9.9 with ETA.

Stock solutions of amino acids were 10, 0.5, and 0.25 μM in artificial cerebrospinal fluid (aCSF) consisting of (in mM) 147 NaCl, 2.7 KCl, 0.85 $MgCl_2$, and 1.2 $CaCl_2$. FAM, SE was 0.1 M in dimethyl sulfoxide. For labeling, 4 μL of 37.5 mM borax buffer (pH 9.5) was vortex mixed with 5 μL of amino acid stock to which 1 μL of FAM, SE stock was added. The resulting mixture solution was vortexed to mix and incubated in the dark for 12 h at room temperature before being stored at 4 °C. For analysis, 1:100 dilutions of labeled amino acids were made in TE without removal of free dye. Each amino acid was labeled separately to allow for peak identification. For calibration, Glu and Asp standard mixtures were labeled at the same time as the microdialysis samples. The degree of labeling was not characterized. Un-reacted and side products of FAM, SE conjugation were identified by performing the labeling reaction described above without amino acids.

Instrumentation and Operation

Methods were supplied by the laboratory of Dr. Jonathan Shackman at Temple University (Davis et al. 2009). 11-cm fused-silica capillary (25 μm i.d.; 360 μm o.d.; Polymicro Technologies, Phoenix, AZ) with a 2-mm optical window (Microsolv Window Maker, Eatontown, NJ) located 6 cm from the inlet was inserted into a machined 100-μL Derlin sample reservoir (McMaster-Carr, Robbinsville, NJ) with a 400-μm diameter hole sealed with a Teflon-backed silicon septum (Sigma). High voltage (CZE 3000, Spellman High Voltage Electronics, Hauppauge, NY) was applied to the sample reservoir by platinum electrode at a constant field strength of -600V/cm. Solution switching was accomplished by micropipette. For GEITP-CZE, the sample solution was removed, the reservoir washed sequentially with DI water and LE, followed by replacement with LE; the complete cycle was completed in 60 s. The capillary outlet was connected to a 750-µL polymethyl methacrylate machined buffer reservoir (McMaster-Carr) *via* an Upchurch Nanoport (N-124S; Oak Harbor, WA); a grounding platinum electrode was also coupled to this reservoir via a Nanoport.

The buffer reservoir was connected to a ± 69 kPa (10 psi) precision pressure controller (Series 600, Mensor, San Marcos, TX) for hydrodynamic flow regulation through a removable Nylon Swagelok union (Penn Fluidic System Technologies, Huntingdon Valley, PA), which also was the access for fluid exchange. Gradient elution isotachophoresis was initiated at 2200 Pa (adjusted daily between 1700 to 2600 Pa until maximal current was reached). A pressure gradient of -30 Pa/s was maintained for 80 s prior to beginning capillary electrophoresis. Pressure applied during capillary electrophoresis was between -1100 and -1550 Pa (adjusted daily for baseline resolution of analytes).

Detection was performed using an in-house modified Axio Observer A1 upright fluorescence microscope (Carl Zeiss, Thornwood, NY). Due to low basal glutamate levels detected in the microdialysis samples, not all animals completed in the trial were able to be analyzed for statistical purposes. Original sample sizes per treatment group were as follows: Sal/Sal (n=6), CXCL12/Sal (n=7), Sal/Coc (n=8), CXCL12/Coc (n=8). Animals with retrievable extracellular glutamate levels are indicated in Fig. 4.3. The arc-lamp optical train was removed and the output of a 30-mW diode-pumped solid state 488-nm laser (Melles Griot, Carlsbad, CA) was directed onto a fluorescein-specific optical block containing a 500-nm longpass dichroic and 510-560-nm emission bandpass filter (QMAX Green series, Omega Optical, Battleboro, VT).

Laser excitation was focused through a 20× objective (0.4 numerical aperture, N.A., Carl Zeiss) into the capillary. Fluorescence emission was collected through the same objective and detected through a 3-mm spatial filter with a Hamamatsu (Bridgewater, NJ, USA) H5784 photosensor module and C7169 power supply.

All instrument control and data acquisition was performed using LabVIEW software (National Instruments, Austin, TX) written in-house. Digitization of the PMT output utilized a USB-6221 module (National Instruments) recording at 100 Hz. Data analysis was performed using Cutter software with 1 Hz low-pass filtering of raw data.

Operational Parameters

Capillary GEITP-CZE analysis was carried out at a constant field strength of -600V/cm LE: 50mM citrate balanced with ETA to pH 9.91; TE: 125 mM borate balanced with ETA to pH 9.93.

Histological Analysis of Cannula Placements

All rats were killed by rapid decapitation immediately following microdialysis sample collection. Brains were excised and frozen at -80°C. Brains were sectioned on a cryostat (-18°C) and probe placements determined by visual inspection and recorded. Animals with probe placements within the medial shell of the nucleus accumbens and injection cannulae correctly positioned in the VTA were used for data analysis (Fig. 4.1).

Statistical Analysis

For each treatment group, ten dialysate samples were collected and aliquoted for dopamine and glutamate analysis. The first three samples were used to establish baseline levels of either dopamine or glutamate. These three samples were averaged and used to determine a separate percent of baseline for the either dopamine or glutamate. Data are expressed as the mean

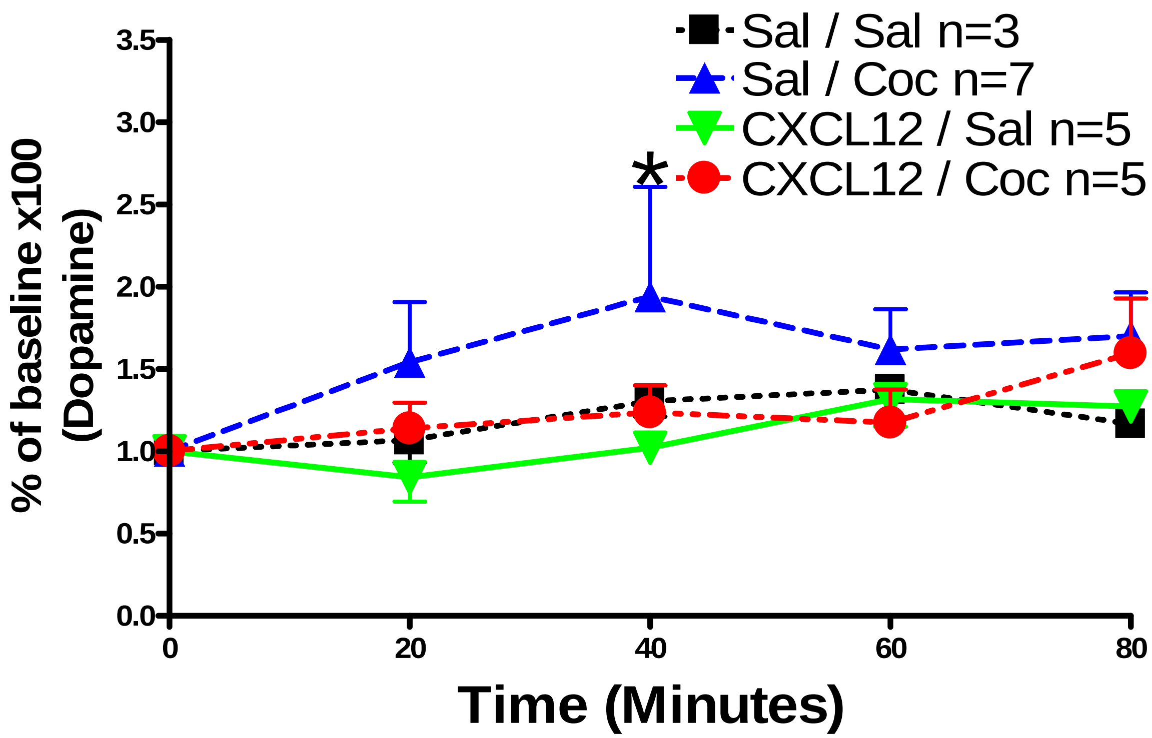

Figure 4.2 CXCL12 Administration into the Ventral Tegmental Area Does Not Affect Extracellular Dopamine Levels within the Medial Shell of the Nucleus Accumbens.

Administration of CXCL12 (25 ng/0.5 µl) into the ventral tegmental area 15 minutes prior to cocaine (20 mg/kg i.p.) did not significantly alter extracellular dopamine concentrations in the medial shell of the nucleus accumbens as compared to either saline or cocaine alone (CXCL12/Coc vs. Sal/Sal; CXCL12/Coc vs. Sal/Coc, $p>0.05$). Animals receiving cocaine (20 mg/kg, i.p.) alone had a significant increase in extracellular dopamine levels compared to animals receiving CXCL12 (VTA) and saline (Sal/Coc vs. CXCL12/Sal, *$p<0.05$). Data are expressed as mean ± SEM percent change from baseline. N= 3-7 per group as indicated for each.

± S.E.M. Data were examined for significance by repeated measures ANOVA followed by Bonferroni post-tests (GraphPad Prism V.4). Statistical significance was achieved when $p<0.05$.

Results

Effects of CXCL12 administration into the VTA on extracellular dopamine concentrations in the medial shell of the nucleus accumbens

Extracellular concentrations of dopamine in the medial shell of the nucleus accumbens (% of baseline) are reported in Fig. 4.2. Dialysate samples (20 µl) were manually injected into a HPLC detection system and analyzed for concentrations of dopamine. Flow rate during HPLC analysis was maintained at 0.8 mL/min with control studies detecting dopamine elution from the column at 7.5 minutes. Repeated measures ANOVA of dopamine levels over time showed there was a significant difference in extracellular dopamine between treatment groups ($F(3,23)=4.075$, $p<0.05$). Results demonstrated a 70% increase in extracellular dopamine at the 40 minute time point following cocaine administration. Post hoc analysis showed extracellular dopamine levels following administration of saline (VTA) and cocaine (i.p.) were significantly different in animals as compared to those receiving CXCL12 (VTA) and saline (i.p.) (CXCL12/Sal vs. Sal/Coc; *$p<0.05$). Unilateral administration of CXCL12 (25 ng/0.5 µl) into the VTA 15 minutes prior to cocaine (20 mg/kg i.p.) did not alter extracellular dopamine levels as compared to saline (VTA) plus systemic injections of either saline or cocaine (CXCL12/Coc vs. Sal/Sal, CXCL12/Coc vs. Sal/Coc, $p>0.05$). Animals injected systemically with cocaine (Sal/Coc) showed a 63% increase from baseline in extracellular dopamine as compared to animals administered saline (Sal/Sal), however the differences were not significant (Sal/Coc vs. Sal/Sal, $p=0.0518$).

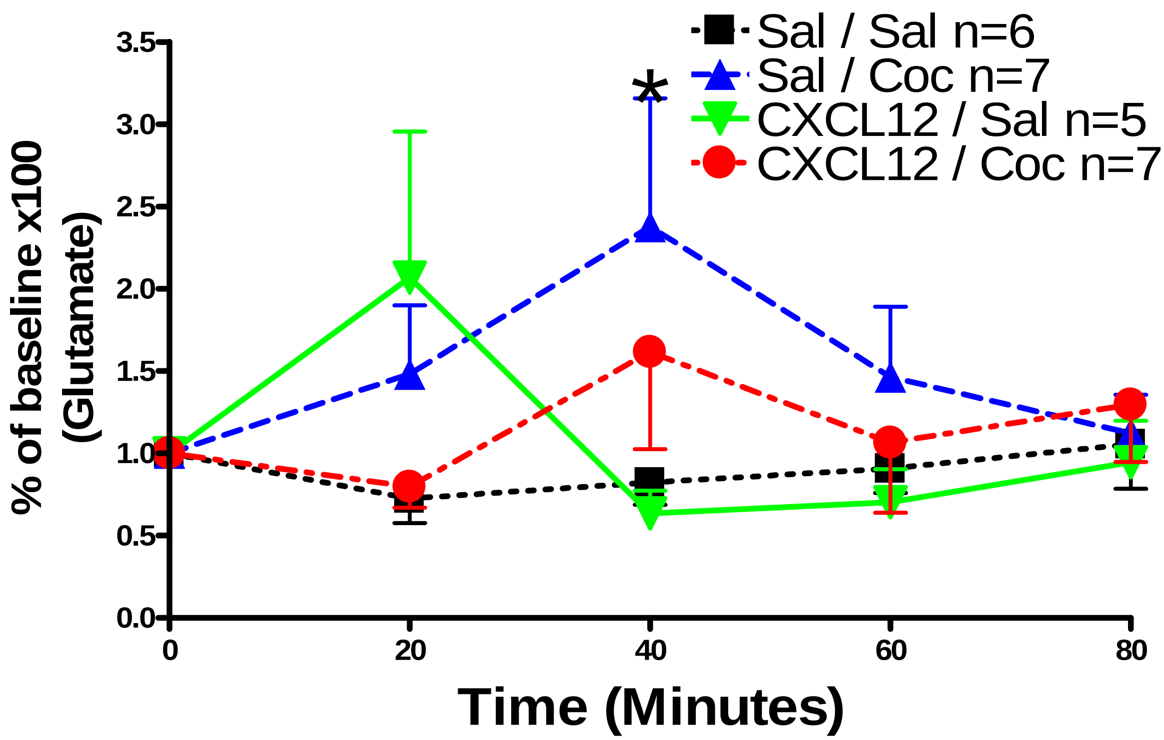

Figure 4.3 CXCL12 Administration into the Ventral Tegmental Area Does Not Affect Extracellular Glutamate Levels within the Medial Shell of the Nucleus Accumbens.

Administration of CXCL12 (25 ng/0.5 µl) into the ventral tegmental area 15 minutes prior to cocaine (20 mg/kg i.p.) did not significantly alter extracellular glutamate concentrations in the medial shell of the nucleus accumbens as compared to either saline or cocaine alone (CXCL12/Coc vs. Sal/Sal; CXCL12/Coc vs. Sal/Coc, $p>0.05$). Animals receiving cocaine (20 mg/kg, i.p.) showed a significant increase in extracellular glutamate as compared to animals receiving saline alone (Sal/Sal vs. Sal/Coc, *$p<0.05$). Data are expressed as mean ± SEM percent change from baseline. N= 5-7 per group as indicated for each.

Effects of CXCL12 administration into the VTA on extracellular glutamate concentrations in the medial shell of the nucleus accumbens

A 5 µl aliquot of each dialysate sample was analyzed using capillary electrophoresis to determine the extracellular concentration of glutamate (Fig. 4.3). Repeated measures ANOVA showed there was a significant difference in extracellular glutamate between groups ($F(3,23)=3.598$, $p<0.05$). Bonferroni post hoc analyses showed that extracellular glutamate levels following administration of cocaine (20 mg/kg i.p.) were significantly different than those in the saline control group (Sal/Sal vs. Sal/Coc; *$p<0.05$). Forty minutes after receiving an acute injection of cocaine (20 mg/kg i.p.), extracellular glutamate levels were increased by 155% as compared to saline treated animals. Unilateral administration of CXCL12 (25 ng) into the VTA increased extracellular glutamate by 135%, however results were not significantly different from saline controls (Sal/Sal vs. CXCL12/Sal, $p>0.05$). Glutamate levels were increased by 80% in animals administered CXCL12 (25 ng) prior to an injection of cocaine (20 mg/kg i.p.) but results were not significantly different from those of saline controls (Sal/Sal vs. CXCL12/Coc, $p>0.05$).

Discussion

This study investigated the modulation of extracellular levels of dopamine and glutamate in the medial shell of the nucleus accumbens following administration of CXCL12 into the VTA. In a similar manner, a previous study examined the effect of CXCL12 injected into the substantia nigra on extracellular dopamine levels in the striatum (Skrzydelski et al. 2007). Their results demonstrate an increase of extracellular dopamine in the caudate putamen following an acute injection of CXCL12 into the substantia nigra and an increase in locomotor behavior (Skrzydelski et al. 2007). Results of our studies described in Chapter 1 of this thesis have shown that an acute injection of CXCL12 into the VTA potentiated the locomotor activity produced by cocaine (Trecki and Unterwald 2009). Based on this finding, we hypothesized that CXCL12 administered in the VTA would result in an increase of extracellular dopamine in the medial shell of the nucleus accumbens. Similarly, it was hypothesized that administration of CXCL12 (VTA) concomitantly with cocaine (i.p.) would result in a greater increase in extracellular dopamine as compared to that produced by cocaine alone.

As shown in the figures above, neither extracellular dopamine (Fig. 4.2) or glutamate (Fig. 4.3) levels were significantly increased following an acute intra-VTA injection of CXCL12. Extracellular dopamine levels doubled following an i.p. injection of cocaine alone, similar to a previous report (Pettit et al. 1990). Glutamate levels were also increased following an acute injection of cocaine. Neither dopamine nor glutamate levels were significantly different from baseline following concomitant administration of CXCL12 and cocaine.

Results of this study suggest that CXCL12 administered into the VTA did not have a significant effect on extracellular dopamine concentrations within the medial shell of the nucleus accumbens. Surprisingly, animals treated with CXCL12 and cocaine did not demonstrate a change in extracellular dopamine as compared to saline controls. Since CXCL12 has previously been shown to cause an increase in locomotion (Skrzydelski et al. 2007) and potentiate the locomotor effects of cocaine (Trecki and Unterwald 2009), it would seem unlikely that CXCL12 and concomitant cocaine administration would result in lower extracellular dopamine levels as compared to animals receiving cocaine alone (Fig. 4.2). Upon visual inspection of the animals receiving CXCL12 and cocaine, animals were observed as having an increased locomotor response as previously reported (Trecki and Unterwald 2009). The placement of all probes and injection cannula were verified at the conclusion of the experiments. Based upon HPLC and capillary electrophoresis results, there is no current explanation as to why animals receiving CXCL12 and cocaine failed to show a change in extracellular dopamine concentrations as compared to animals receiving cocaine alone.

Cocaine inhibits the presynaptic reuptake of dopamine, serotonin and norepinephrine (Ross and Renyi 1967; Heikkila et al. 1975; Koe 1976). Furthermore, it is well established using microdialysis that cocaine causes an increase in the extracellular concentration of dopamine in the nucleus accumbens (Di Chiara and Imperato 1988; Carboni et al. 1989; Hurd et al. 1989; Pettit and Justice 1989). The cell bodies of dopaminergic neurons in the mesolimbic dopamine system that originate in the VTA and project primarily to the nucleus accumbens are involved in the reinforcing properties of cocaine (Dahlstrom and Fuxe 1964; Koob 1992). Alterations in dopamine levels in the nucleus accumbens have been implicated in motivational and rewarding processes (Heimer et al. 1991). Studies have demonstrated that an acute injection of cocaine is sufficient for a significant increase in extracellular dopamine in the shell of the nucleus accumbens (Hedou et al. 1999; Gerrits et al. 2002). Reported basal levels of extracellular dopamine in the shell of the nucleus accumbens range from 2 pg/μl (Hedout et al. 1999) to 4 ng/μl (Morgan and Dewey 1998). Following acute systemic administration of cocaine (20 mg/kg), extracellular dopamine has been shown to be increased in the nucleus accumbens shell in the range of 200% (Fontana et al. 1993; Hedou et al. 1999; Gerrits et al. 2002) up to 400% of baseline (Morgan and Dewey 1998; Frantz et al. 2007).

In the current study, an acute injection of cocaine increased extracellular dopamine within the medial shell of the nucleus accumbens by approx 70% of baseline. Due to the low number of animals in the saline/saline control group that produced viable dialysate samples (n=3), statistical significance was not achieved when comparing data from the saline/saline group to the saline/cocaine group. We believe that if more animals had been included in the saline control group, statistical significance would have been achieved. A power analysis was conducted following the conclusion of the experiment to determine the number of animals per treatment group needed to achieve statistical significance. Using the average standard deviation from each time-point included in the dopamine analysis (SD = 0.55), the power analysis results suggest that 11 animals per group would be required to achieve statistical significance ($p<0.05$) between the saline/saline and saline/cocaine treatment groups.

Previous research has shown glutamatergic inputs to the nucleus accumbens from the hippocampus, amygdala and prefrontal cortex (Kalivas 2004). Extracellular glutamate levels are increased in the nucleus accumbens following cocaine administration (Crespo et al. 2002; Sutton et al. 2003). Microdialysis studies have demonstrated that acute i.p. administration of cocaine enhances extracellular glutamate in the nucleus accumbens (Smith et al. 1995; Reid et al. 1997). Repeated cocaine administration increases the potential for a subsequent lower dose of cocaine to increase extracellular glutamate levels in the nucleus accumbens (Pierce et al. 1996; Reid and Berger 1996; Kalivas and Duffy 1998). In agreement with previous findings, results from this study demonstrated a significant increase in extracellular glutamate within the medial shell of the nucleus accumbens following an acute i.p. injection of cocaine. Administration of CXCL12 alone showed increase in extracellular glutamate, however results were not significant. Using the average standard deviation from each time-point included in the dopamine analysis (SD = 0.74), the power analysis results suggest that 9 animals per group would be required to achieve statistical significance ($p<0.05$) between the saline/saline control animals and those administered CXCL12 alone (CXCL12/saline). Similarly, animals administered concomitant CXCL12 and cocaine showed a small increase in levels of extracellular glutamate as compared to baseline samples, however results did not achieve statistical significance. A power analysis for this group suggested that 15 animals per group are required to achieve statistical significance ($p<0.05$) between the saline/saline control animals and animals administered concomitant CXCL12 and cocaine. Results presented in this study suggest that CXCL12 alone might increase glutamate; however statistical significance was not achieved. Since administration of cocaine alone significantly increased extracellular glutamate within the medial shell of the nucleus accumbens, it is interesting that, at the very least, an additive effect was not observed in samples collected from rats receiving concomitant CXCL12 and cocaine. Similarly, results from dopamine analysis also showed no significant change in animals receiving CXCL12 concomitantly with cocaine, suggesting that there could have been a potential technical problem with CXCL12/cocaine treatment group. Contrary to this assumption, experimental conditions remained unchanged throughout all testing. Microdialysis was conducted on two animals per day and the two animals were from separate treatment groups. Therefore, there is no indication of why this one treatment group would have been compromised.

CXCR4 expression by astrocytes, glia and neurons has been studied extensively. CXCL12 and CXCR4 have been identified in high concentrations in both glial cells (astrocytes and microglia) and in neurons (Asensio and Campbell 1999). Studies have identified the role of CXCL12-stimulated CXCR4 activation and the ability of this chemokine to increase intracellular calcium levels in both astrocytes and neurons (Bezzi et al. 2001; Bonavia et al. 2003). Pretreatment of cell cultures with pertussis toxin inhibited the ability of CXCL12 to stimulate calcium mobilization, serving as evidence that CXCR4 is acting through $G\alpha_{i/o}$ (Guyon et al. 2006). The ability of CXCR4 activation to stimulate calcium mobilization is thought to be a significant factor in regulating neurotransmitter release (Guyon et al. 2008).

Administration of CXCL12 produced a non-significant increase in extracellular glutamate, suggesting that the chemokine could be causing the release of the amino acid. It is possible that CXCL12 alone could be initiating an acute inflammatory response that could result in increased glutamate release. It is also possible that CXCL12-mediated CXCR4 activation could be initiating a calcium influx that could also stimulate the release of glutamate. Studies show that uncontrolled CXCL12-mediated CXCR4 activation results in severe cytotoxic effects including inflammation and apoptosis (Zheng et al. 1999; Kaul et al. 2001). Previous research has shown that administration of CXCL12 results in calcium-dependent CXCR4-mediated glutamate release from astrocytes in mice (Bezzi et al. 2001). Studies also demonstrate the excitotoxicity resulting from accumulated glutamate in the brain from sources of traumatic brain injury, ischemia, HIV/AIDS and enhanced stimulation of CXCR4 (Bezzi et al. 2001; Jiang et al. 2001; Mark et al. 2001). This suggests that the excitotoxic effects of increased extracellular glutamate release following CXCL12 administration in the lateral shell of the accumbens could be responsible for inhibiting the locomotor response observed following an injection of cocaine. However, the inflammatory response induced by CXCL12 and/or glutamate does not explain why animals receiving CXCL12 concomitantly with cocaine failed to illicit similar changes in extracellular dopamine or glutamate concentrations observed by those animals receiving cocaine alone.

Future studies should focus on the administration of CXCL12 and the resulting modulation in extracellular dopamine and glutamate. Microdialysis studies should be conducted in conjunction with an in-line HPLC detection system to minimize dopamine and glutamate loss due to time and freezing of samples. Results from further microdialysis studies could help explain the mechanism by which CXCL12 is potentiating a cocaine-induced locomotor response and further examine extracellular dopamine and glutamate fluctuations in the striatum following chemokine administration.

5. GENERAL DISCUSSION

The studies completed in this dissertation investigated the modulation of cocaine-induced behavior following administration of CXCL12. The research presented in this thesis identified the ability of CXCL12 to modulate cocaine-induced locomotion and stereotypy dependent upon the brain region where it was administered. In addition, immunofluorescent microscopy was utilized to identify cells in the brain that express CXCR4. CXCR4 was shown to be localized on cholinergic and GABAergic neurons in the caudate putamen and lateral shell of the nucleus accumbens. Finally, microdialysis studies were used to quantify levels of extracellular dopamine and glutamate following administration of CXCL12. Studies revealed that CXCL12 administration did not alter extracellular dopamine or glutamate in the medial shell of the nucleus accumbens.

Previous studies have identified the expression of CXCR4 receptors on dopaminergic and GABAergic cell bodies in the substantia nigra and VTA (Banisadr et al. 2002). Research has also shown that administration of CXCL12 into the substantia nigra results in an increase in extracellular dopamine in the caudate putamen as well as an increase in locomotion (Skrzydelski et al. 2007). The studies described in Chapter 1 of this thesis evaluated the site of action where CXCL12 was modulating cocaine-induced behavior. Results demonstrate that intracerebral CXCL12 administration into the VTA or caudate putamen potentiated cocaine-induced locomotion and stereotypy (Trecki and Unterwald 2009). In contrast, CXCL12 administration into the lateral shell of the nucleus accumbens inhibited cocaine-induced locomotion. The studies described in Chapter 2 used immunohistochemistry and immunofluorescent microscopy to demonstrate the expression of CXCR4 in the caudate putamen and lateral shell of the accumbens. Results identified CXCR4 receptors on cholinergic and GABAergic cells in the striatum. Studies also showed the co-expression of CXCR4 with the D1 dopamine receptor. The studies presented in the final chapter of this thesis evaluated extracellular dopamine and glutamate levels in the medial shell of the nucleus accumbens following administration of CXCL12 in the VTA. Results showed an increase in both extracellular dopamine and glutamate following administration of cocaine, however there was no significant change in extracellular dopamine or glutamate levels in animals receiving an intracerebral injection of CXCL12 with or without cocaine. CXCL12 administration produced a small non-significant increase in extracellular glutamate, possibly from activation of CXCR4 receptors located on adjacent glial cells. A power analysis suggested that the addition of four animals in the CXCL12/saline treatment group would have resulted in statistical significance.

Based upon previous research and results obtained through this thesis, it is possible to suggest a possible mechanism of action relating to the CXCL12 modulation of behavioral activity produced by cocaine. As noted previously, CXCR4 is expressed on dopaminergic cell bodies in the substantia nigra (Banisadr et al. 2002), on cholinergic interneurons in the caudate putamen (Banisadr et al. 2002) and on GABAergic interneurons in the substantia nigra and VTA (Guyon et al. 2006). Results from this thesis demonstrate the expression of CXCR4 on cholinergic and GABAergic neurons of the caudate putamen and lateral shell of the nucleus accumbens. Immunofluorescent staining also showed the co-localization of CXCR4 with the D1 receptor, located on GABAergic medium spiny neurons in the striatum. The localization of CXCR4 on select populations of cells can aid in hypothesizing potential mechanisms of action of CXCL12. Identification of the cell types in the striatum and midbrain that express CXCR4 in conjunction with the known properties of CXCR4 can direct future research on downstream signaling pathways and mechanisms of action of CXCL12 and how CXCL12 might interact with classical neurotransmitters that modulate the same neuronal populations.

Throughout this thesis, it became increasingly difficult to analyze dialysate samples for extracellular dopamine concentrations using the available HPLC detector. Using multiple cohorts of animals, results could not be replicated and samples from control animals showed unbelievable values. It was decided by the committee to complete a final trial and use external sources to analyze dialysate samples for dopamine and glutamate. Due to the degree of difficulty in HPLC or capillary electrophoresis detection of dopamine and glutamate, it is thought that results from these experiments are diminished in their significance. While the results do not demonstrate significant changes in extracellular dopamine or glutamate following CXCL12 administration alone, it is my opinion that these results should not be viewed as conclusive evidence that a change is not occurring. Therefore, mechanisms hypothesizing neurotransmitter involvement following CXCL12 administration will be discussed.

To be classified as a chemokine receptor, the receptor must engage in a signaling mechanism upon activation, such as calcium mobilization (Zlotnik and Yoshie 2000; Li and Ransohoff 2008). One mechanism by which CXCL12-mediated CXCR4 activation is thought to increase extracellular dopamine levels in the striatum is through an increase in intracellular calcium (Guyon et al. 2008). Since previous studies have shown CXCR4 to be present on dopaminergic cell bodies, calcium mobilization could be responsible for an increase in neurotransmitter release at the terminal region. Interestingly, it is thought that the increase in intracellular calcium, which can increase neuronal firing, can supersede the inhibitory actions of the CXCR4 receptor, which activates inhibitory G proteins (G_i). It is hypothesized that CXCL12 binding and activation of CXCR4 on a dopaminergic cell body would result in a calcium signaling cascade.

Neurotransmitter release following calcium mobilization has been shown for other neuronal types besides those that release dopamine. Cholinergic neurons have been shown to release acetylcholine following calcium mobilization (Giovannini et al. 2002). CXCR4, localized on cholinergic interneurons in the caudate putamen and lateral shell of the nucleus accumbens, could be causing acetylcholine release that could stimulate nicotinic receptors following CXCL12-mediated activation. This increase in nicotinic receptor activity on dopaminergic terminals stimulates action potentials resulting in increased striatal dopamine release (Zhou et al. 2002).

Besides initiating a calcium signaling cascade following CXCL12 binding to CXCR4, there is a second mechanism by which CXCR4 activation can cause neurotransmitter release. This method is by an indirect pathway, by which CXCR4 activation on adjacent cells, particularly glia, can cause a release of glutamate that can in turn activate surrounding cells (Limatola et al. 2000). One example of this is CXCR4 localized on GABAergic neurons and glia in the cerebellum. CXCR4 activation on glial cells causes a release of glutamate which binds to receptors on the cell bodies of adjacent GABAergic neurons. The activation of glutamatergic receptors causes a subsequent release of GABA. By activating CXCR4 receptors on glial cells, CXCL12 can indirectly cause GABA release (Limatola et al. 2000).

Using the results from this thesis, it is possible to predict how CXCL12-induced CXCR4 activation can result in a modulation of behavioral activity. Administration of CXCL12 into the VTA followed by an i.p. injection of cocaine resulted in an enhancement of locomotor activity compared to that produced by cocaine alone. It is thought that GABAergic interneurons in the

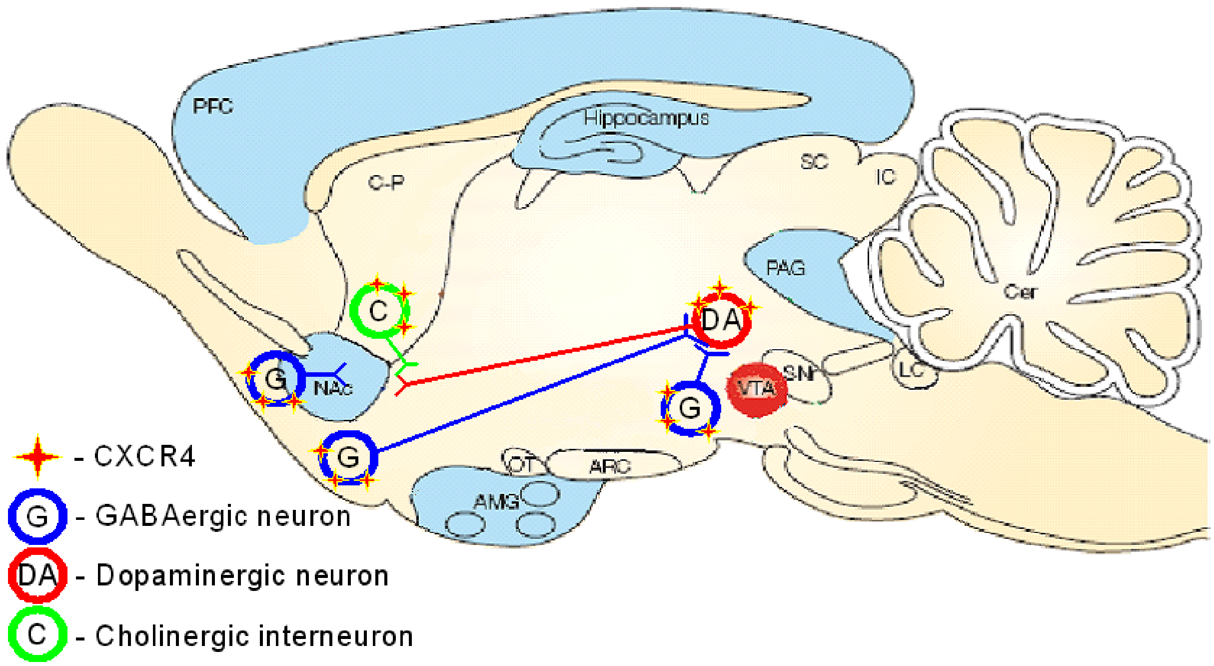

Figure 4.4 Pathways Involved in the Modulation of Cocaine-Induced Behavior by CXCL12

A sagittal rat brain section showing multiple pathways involved in the modulation of cocaine-induced behavior following administration of CXCL12. (Adapted from Nestler EJ et. al., 2001) midbrain are inhibited by CXCR4 activation, allowing dopaminergic neurons to function with no inhibitory control (Fig. 4.4). It is hypothesized that the presence of CXCR4 on dopaminergic cell bodies allows calcium mobilization resulting in a subsequent increased release of dopamine in the forebrain, specifically in the caudate putamen and nucleus accumbens. This mechanism could result in an increase of extracellular dopamine that could be responsible for the increase in behavioral activity observed following CXCL12 administration into the VTA concomitantly with cocaine. A previous study showed that administration of CXCL12 (50 ng/dose) into the substantia nigra resulted in an increase in locomotion (Skrzydelski et al. 2007). Results presented in Chapter 1 of this thesis showed that administration of CXCL12 (25 ng/dose) in the VTA alone did not produce any significant increase in behavioral activity. Administration of CXCL12 into the caudate putamen followed by an i.p. injection of cocaine resulted in a potentiation of stereotypic activity. Results of this thesis demonstrated that CXCR4 is co-expressed with the dopamine D1 receptor in the caudate putamen, which are located on GABAergic medium spiny neurons. One hypothesis to explain the potentiation of cocaine-induced activity by CXCL12 involves CXCR4-mediated inhibition of the medium spiny neurons (Fig. 4.4). GABAergic medium spiny neurons located in the striatum project back to the areas of the midbrain including the substantia nigra and VTA. Inhibition of GABAergic medium spiny neurons allows an overall disinhibition of dopaminergic neurons in the midbrain, thus increasing dopaminergic neurotransmission and increasing dopamine release. The presence of CXCR4 on cholinergic interneurons in the caudate putamen suggests an added stimulus to dopaminergic terminals. Nicotinic receptors are localized on presynaptic dopaminergic nerve terminals and have been shown to cause dopamine release following stimulation by acetylcholine (Zhou et al. 2002). CXCR4 activation stimulates calcium influx which supersedes the inhibitory effects of the chemokine receptor and results in an increase in acetylcholine release. The combination of activated nicotinic receptors with the inhibition of GABAergic medium spiny neurons is hypothesized to cause an overall increase in extracellular dopamine, which is responsible for the potentiation of stereotypic activity following CXCL12 administration into the caudate putamen concomitantly with systemic cocaine.

Possibly the most interesting result discussed in this thesis involved the administration of CXCL12 in the lateral shell of the accumbens followed by an i.p. injection of cocaine. Results demonstrated that following this concomitant administration, locomotor activity was significantly inhibited as compared to animals receiving cocaine alone. These results are also the most difficult to explain. The region of the lateral shell of the nucleus accumbens contains similar cellular phenotypes as those in the caudate putamen (Fig. 4.4). Both areas contain a small percentage of GABAergic and cholinergic interneurons. The remaining 95% of neurons in the caudate putamen and nucleus accumbens are GABAergic medium spiny neurons. Most of these GABAergic medium spiny neurons project back to midbrain areas and exert an inhibitory tone. It is hypothesized that GABAergic neurons in the lateral shell of the accumbens are working thought a different mechanism than GABAergic neurons in the caudate putamen. Previous results have demonstrated that CXCR4 on similar cell types but in different brain regions can have different effects. For example, CXCR4-expressing GABAergic interneurons in the cerebellum are stimulated indirectly via CXCR4. CXCR4 receptors cause a calcium influx in adjacent glial cells, of which the released glutamate is responsible for activating the GABAergic neurons (Limatola et al. 2000). Alternatively, GABAergic interneurons in the substantia nigra have been shown to have a direct CXCR4-mediated GABA release following CXCL12 binding (Guyon et al. 2006). It is possible that glutamatergic afferents to the lateral shell of the nucleus accumbens could be a contributing factor in the inhibition of locomotor activity observed following concomitant CXCL12 and cocaine administration. Glutamatergic inputs into the nucleus accumbens originate from the hippocampus, amygdala and prefrontal cortex (Kalivas 2004). An inhibition of dopaminergic neurons originating in the midbrain is hypothesized to result from stimulation of glutamatergic afferents to GABAergic medium spiny neurons in the nucleus accumbens. In addition, CXCR4 activation on GABAergic interneurons and medium spiny neurons can also contribute to an overall inhibition of midbrain dopaminergic neurons. Inhibition of dopaminergic neuronal activation results in a decrease in extracellular dopamine in the nucleus accumbens and caudate putamen, thus resulting in a diminished locomotor response following cocaine administration.

Previous studies investigating the bi-directional heterologous desensitization of chemokine and opioid receptors prompted us to study the interaction of chemokines and dopamine receptors. As outlined in the Introduction, administration of chemokines prior to a mu-opioid receptor agonist resulted in the attenuation of an analgesic response (Szabo et al. 2002). Similarly, administration of opioids prior to administration of a chemokine inhibited a chemokine response (Szabo et al. 2002; Finley et al. 2008). At the initiation of this thesis project, it was hypothesized that administration of CXCL12 would inhibit the behavioral response observed following an injection of cocaine. It was thought that CXCL12 administration might desensitize dopamine receptors resulting in an attenuation of locomotor and stereotypic activity. As previously discussed in the Introduction, CXCR4 activates inhibitory G proteins and by definition, inhibits the production of cAMP from ATP. Inhibition of cAMP production results in decreased activity of cAMP-dependent protein kinase (PKA), which is responsible for phosphorylating other proteins, including the D1 dopamine receptor. The results hypothesized included inhibition of dopamine receptors and a decrease in cAMP production, ultimately resulting in a decrease in calcium flux throughout the cell. The decrease in calcium flux could result in a net decrease in neurotransmitter release and, in regards to extracellular dopamine, an overall decrease in behavioral activity and reward. This was, in fact, the effect that was seen when the chemokine was administered into the lateral shell of the nucleus accumbens. Overall, the results demonstrate that CXCL12 can modulate cocaine-induced behavior; however the direction of the modulation is dependent upon the site of administration, suggesting that various mechanisms of actions are possible depending upon the cellular phenotypes involved.

Another possibility as to how CXCR4 activation can affect locomotion involves potential cross-talk between signaling pathways activated by CXCR4 and dopamine receptors located post-synaptically in the caudate putamen and nucleus accumbens. Contrary to previous interpretations based upon views that CXCL12 causes dopamine release, possibilities exist that CXCR4 activation could be occurring through CXCL12 binding post-synaptically. Binding of CXCL12 to CXCR4 receptors on GABergic medium spiny neurons or cholinergic interneurons in the striatum could cause a signaling cascade resulting in GABA or acetylcholine release. Previous research has also shown an increase in locomotor activity following administration of D1 receptor agonists into the medial shell of the nucleus accumbens (Ikemoto 2002). CXCR4 activation on D1 receptor-containing medium spiny neurons could be enhancing similar signaling pathways resulting in an increase in behavioral activity. In contrast, activation of CXCR4 on D1 receptor positive neurons could be inhibiting the signaling of the dopamine receptors, thus reducing activity. Previous research has shown that acute administration of a dopamine D1 receptor agonist results in activation of ERK in the nucleus accumbens without activation in the dorsal striatum (Kim et al. 2006). Similarly, it has been reported that beta-arrestins-2 and -3 enhance CXCR4-mediated ERK activation (Luttrell et al. 1999). Cross-talk between CXCR4 receptors and glycogen synthase kinase-3β (GSK3β) could also be affecting locomotor activity following CXCL12 and cocaine administration. Results from our laboratory have demonstrated that inhibition of GSK3 resulted in the inhibition of the acute behavioral response to cocaine (Miller et al. 2009). Similarly, the inhibition of GSK3 has been shown to increase CXCR4 expression in CD34+ cell cultures (Holmes et al. 2008). This downstream signaling could be contributing to the modulation of behavioral activity of cocaine following CXCL12-mediated CXCR4 activation.

Finally, an issue commonly raised in response to this research involves the clinical relevance of the modulation of cocaine-induced activity by CXCL12. The ability of CXCL12 to potentiate the locomotor affects of cocaine following intracerebral administration into the VTA and caudate putamen may suggest that there is a connection between cocaine addicted patients and those that suffer from neuroinflammatory pathologies including MS, HIV/AIDS and trauma. To date, no studies have been published which evaluate the potential for patients with neurodegenerative conditions or trauma to abuse cocaine to a greater or lesser extent than those persons with no previous neurological conditions. Studies have shown chemokine levels are increased in the CSF of patients with neurological conditions (ref?). It is possible that persons with elevated chemokine levels could suffer an increased addiction to cocaine due to the increased basal levels of chemokines. Interestingly, CXCL12 inhibited the locomotor properties of cocaine when it was administered into the lateral shell of the accumbens. These results might suggest that patients with neurological conditions would be less likely to abuse cocaine or to develop an addiction. More research is needed to fully appreciate the implications of the findings presented herein in regards to the clinical aspects of addiction.

The research presented hypothesizes the molecular mechanisms of how CXCL12-induced CXCR4 activation could be influencing dopaminergic, GABAergic and cholinergic pathways in the adult rat brain. This research is clinically relevant based upon the involvement of CXCR4 in a variety of diseases including HIV/AIDS and MS. Results from this thesis also suggest the involvement of CXCL12-induced CXCR4 activation in the pathology of cocaine addiction. Further understanding of the molecular mechanisms of CXCR4 in the brain could lead to novel treatments in neurodegenerative and neuroinflammatory conditions.

Potential Future Directions

The research presented in this thesis extends the knowledge concerning the modulation of cocaine-induced behavior following CXCL12 administration in the brain while concurrently generating new questions for future studies. The behavioral studies answered the question of what would occur following administration of CXCL12 into specific brain regions. In the future, studies should be designed to identify the pathways in which CXCL12 activation of CXCR4 is modulating behavioral activity. Previous studies have used the neurotoxin 6-hydroxydopamine (6-OHDA) to selectively lesion dopaminergic and noradrenergic neurons. In the General Discussion of this thesis, mechanisms of how CXCL12 could be modulating cocaine-induced behavior were discussed. An injection of 6-OHDA would destroy dopaminergic neurons in a specific brain region. If CXCL12-mediated CXCR4 activation was involved in a pathway lesioned by 6-OHDA, it is hypothesized that previous behavioral effects observed would be abolished. This would suggest that the lesioned brain region was partially responsible for the behavioral modification of cocaine-induced locomotor activity following CXCL12 administration.

Another technique that could further investigate the pathways of the brain involved in CXCR4-mediated behavioral modification is retrograde labeling. Hydroxystilbamidine, also known as fluoro-gold, is a widely used retrograde neuronal tracer that allows researchers to view labeled neurons using fluorescence microscopy. An additional benefit of using fluoro-gold is the extended period of time in which it remains in the neuron. The extended duration of fluoro-gold immunoreactivity in the neuron enables the completion of long-term studies. The use of fluoro-gold in combination with CXCR4 immunofluorescent labeling enables studies to evaluate the retrograde tracing of CXCR4-positive neuronal projections from other regions of the brain. Long-term studies using fluoro-gold would allow the observation of CXCR4-positive neurons during naïve states as well as following CXCL12 administration.

Previous studies have shown CXCL12 (0.01-10 nm) increased the amplitude of total high-voltage-activated calcium currents through CXCR4 activation by using whole-cell patch clamp studies in substantia nigra slices (Guyon et al. 2008). Future studies would include evaluation of striatal sections to determine the conductivity of calcium currents following CXCL12 administration. Of particular interest would be GABAergic cell bodies in the lateral shell of the nucleus accumbens due to behavioral data showing an inhibition of cocaine-mediated activity following CXCL12 administration.

Based upon immunohistochemical results presented in this thesis, it would also be important to evaluate the effect of GABA and cholinergic agonists into regions where behavior was modulated. With results demonstrating the presence of CXCR4 receptors on GABAergic medium spiny neurons and cholinergic interneurons, it is possible that administration of CXCL12 and cocaine could be resulting in an increase of neurotransmitters other than dopamine which could be mediating the behavioral effects observed following concomitant administration.

In addition to agonists at GABAergic and cholinergic receptors, administration of compounds that inhibit GABAergic or cholinergic activity should also be investigated. Morphine administration has shown to induce hyperactivity by inhibiting GABAergic neurons, thus resulting in a disinhibition of dopaminergic neurons in the midbrain. While the administration of opioid agonists concomitantly result in a bi-diretctional heterologous desensitization in the peri-aquaductal grey, studies have not demonstrated their possible interaction when administered into mid-brain regions including the VTA or substantia nigra. Since both morphine and CXCL12 are thought to exert their actions via GABAergic neurons in the VTA and substantia nigra, studies evaluating concomitant administration in these areas could help further explain how CXCL12 is mediating cocaine-induced locomotion and stereotypic activity.

Lastly, this thesis has suggested the possible involvement of glutamate in the effects of CXCL12. Future studies should use updated microdialysis, HPLC and capillary electrophoresis to further evaluate extracellular glutamate levels in the nucleus accumbens. Micro-dialysate collection demonstrated a small increase in extracellular glutamate following CXCL12 administration. It would be interesting to determine if glutamate is increased in the lateral shell of the accumbens following CXCL12 administration, and to determine if glutamate was an important factor in CXCL12-mediated behavior. Administration of a glutamatergic agonist concomitantly with CXCL12 into specific brain regions followed by systemic cocaine could help determine if glutamate is involved in CXCL12-mediated behavior. Brain regions such as the VTA or caudate putamen, areas previously shown to potentiate cocaine-induced behavior, would be of most interest to determine if extracellular glutamate could inhibit behavior as hypothesized in the lateral shell of the nucleus accumbens.

Conclusion

Overall, this thesis has demonstrated that CXCL12-mediated CXCR4 activation can modulate cocaine-induced behavior and has provided evidence of CXCR4 localization with dopaminergic, GABAergic and cholinergic pathways in the brain. I believe this research to be relevant to the current understanding of chemokine activation and neuromodulation in the brain, although studies evaluating the molecular pathways involved in CXCR4 signaling must be investigated. From a clinical standpoint, understanding the mechanisms of action of CXCL12 and CXCR4 could lead to new therapies in neurodegenerative and neuroinflammatory pathologies. The ability of CXCL12 to modulate cocaine-induced behavior also provides the motivation to investigate chemokines in the brain and their possible involvement in addiction. Future studies investigating the neuromodulatory properties of chemokines, specifically CXCL12, could help our understanding of the neuronal pathways of the brain and the pathologies that affect them.

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