ALLOSTERIC MODULATION OF DOPAMINE D2 RECEPTOR

CHARACTERIZATION OF THE MECHANISM OF ACTION FOR NOVEL ALLOSTERIC MODULATORS OF THE DOPAMINE D2 RECEPTOR

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To my family,

With all my love

ABSTRACT

Allosteric modulators are a newly emerging concept in the field of drug discovery which have shown remarkable success in their ability to alter G-protein coupled receptor (GPCR) activity in a precise and subtle manner. A GPCR of particular interest for allosteric targeting is the dopamine D2 receptor. This receptor has repeatedly been implicated in the etiology of complex neurological and neuropsychiatric disorders including Parkinson's disease and schizophrenia. Previous studies from our lab have effectively developed allosteric modulators targeting the D2 receptor based on the pharmacophore of the endogenous tripeptide L-prolyl-L-leucyl-glycinamide (PLG). PLG and its potent peptidomimetics, particularly 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA) (PCT/CA2011/000968), have shown robust preclinical efficacy in treating models of Parkinson's disease, depression, tardive dyskinesia and schizophrenia. These ligands modulate agonist binding to the D2 receptor in a biphasic manner, although further information on their mechanisms of action are currently unknown. Therefore, the overarching objective of this thesis was to enhance our knowledge on the mechanisms of action of the promising D2 allosteric ligands PLG and PAOPA. Results of the studies presented here show PAOPA to cause significant upregulation of D2 regulatory proteins and downstream signaling kinases, as well as cause an increase in D2 internalization. Additionally, the PLG allosteric binding site was narrowed down to be localized between transmembrane domains 5 and 6 on the D2 receptor. The collection of work presented here enhance our understanding of the mechanisms of action of the potentially therapeutic D2 allosteric ligands PLG and

PAOPA, progressing them closer to helping clinically affected populations. The findings of these studies prove globally significant as they highlight the diverse cellular pathways which could be affected by allosteric modulators, and bring to light the importance of studying these candidate ligands for eventual improvements in the treatment of human health.

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6-OHDA	6-hydroxydopamine
ADHD	Attention deficit hyperactivity disorder
ADTN	2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene
AM	Allosteric modulator
ANOVA	Analysis of variance
APD	Antipsychotic drug
Asn	Asparagine
Asp	Aspartate
ATCM	Allosteric ternary complex model
ATP	Adenosine triphosphate
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CF	Cell- free
CNS	Central nervous system
CTP	Cytosine triphosphate
Cys	Cysteine
D2high	High affinity state of the D2 receptor
D2L	D2 long-isoform
D2low	Low affinity state of the D2 receptor
D2S	D2 short-isoform
Da	Daltons
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPM	Decays per minute
E.coli	Escherichia coli
EBSS	Earle's buffered saline solution
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPS	Extrapyramidal symptoms
ERK	Extracellular receptor kinase
eYFP	Enhanced yellow fluorescent protein
FM	Feeding mixture
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanine diphosphate
G_i	Inhibitory G-protein
GPCR	G-protein coupled receptor
GRK	G-protein-coupled receptor kinases
G_s	Stimulatory G-protein
GSK3β	Glycogen synthase kinase 3β

GST	Glutathione-S-transferase
GTP	Guanine triphosphate
HEK 293	Human embryonic kidney cells 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
Inhibitory G-protein	G_i
I.P.	Intraperitoneal
JNK	c-JUN N-terminal kinase
kbp	Kilo base pairs
K_D	Dissociation constant
kDa	Kilodaltons
L-DOPA	L-3, 4 dihydroxyphenylalanine
MAPK	Mitogen activated protein kinase
Met	Methionine
MIF-1	MSH inhibiting factor-1
MK-801	Dizocilpine
MNPA	(R)-2-CH3O-N-n-propylnorapomorphine
MOBIX	McMaster Institute for Molecular Biology and Biotechnology
MPTP	1- methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MWT,	Molecular weight
Na^+	Sodium ions
Nano LC-MS	Nano liquid chromatography-mass spectrometry
Ni-NTA	nickel-nitrilotriacetic acid
NMDA	N-methyl-D-aspartate
NPA	*N*-propylnorapomorphine
PAGE	Polyacrylamide gel electrophoresis
PAOPA	(3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Peptidomimetic 157	3R-[4(S)-(4-azido-2-hydroxy-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2- oxo-1-pyrrolidineacetamide hydrochloride,
PET	Positron emission tomography
Phe	Phenylalanine
PHNO	(+)-4-propyl-9-hydroxynaphthoxazine
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PLC	Phospholipase C

PLG	L-prolyl-L-leucyl-glycinamide
PVDF	Polyvinylidene
RGS	Regulators of G protein signaling
RM	Reaction mixture
SDS	Sodium dodecyl sulfate
Ser	Serine
TBS-T	Tris buffered saline with Tween
T-Rex-293	Tetracycline-regulated expression HEK293cellular line
Trp	Tryptophan
UTP	Uridine triphosphate
Val	Valine
Zn^{2+}	Zinc ion

CHAPTER ONE

INTRODUCTION

G-protein coupled receptors (GPCRs) are encoded by 2-4% of the human genome, and are involved in virtually all essential physiological functions in the body. Ligands targeting these GPCRs account for the largest proportion of currently marketed drugs, and have a significant impact on the treatment of human pathophysiologies. Allosteric modulation is a recently emerging concept, which has shown promise in improving current drug action, by providing a more specific and fine-tuned targeting of GPCRs. The global aim of this thesis was to progress the understanding and development of these allosteric modulators from a crucial neuroscientific perspective. A focus has been placed on elucidating the mechanism of action of allosteric modulators for the dopamine D2 receptor, which have previously shown preclinical efficacy in treating neuropsychiatric disorders, such as schizophrenia. The studies presented in this thesis further our appreciation for the challenges and advantages of using allosteric modulators to treat complex brain disorders, and direct us towards improved treatment for millions suffering from GPCR-related pathologies worldwide.

1.1 G-PROTEIN COUPLED RECEPTORS

GPCRs are a large and diverse superfamily of proteins crucial in eukaryotic signal transduction. The effect of signaling molecules ranging from hormones to neurotransmitters, and even sensory stimuli, are often mediated via GPCRs. These receptors are implicated in a vast variety of cellular functions such as development, proliferation, neuronal function etc. (Rohrer & Kobilka, 1998) There are three major

GPCR subfamilies: the largest subfamily, the rhodopsin/β-adrenergic family (Family A), the vasoactive intestinal polypeptide/secretin family (Family B) and the metabotropic glutamate receptor family (Family C) (Foord et al., 2005) (**Figure 1.1**). The prevalence and importance of these receptors make them an important candidate for scientific research and understanding. As a superfamily, GPCRs share various structural and functional homologies. The seven hydrophobic transmembrane segments of GPCRs often show amino acid sequence conservation and overall folding similarities between the different families of receptors. Increased divergence between the receptor families are observed at the amino -terminal, carboxyl -terminal, the three intracellular and the three extracellular loops. These poorly conserved regions are designed to serve the individual functions of each GPCR, and encode domains involved in ligand interaction, signaling molecule interaction, and cellular trafficking (Rohrer & Kobilka, 1998; Audet & Bouvier, 2012).

The mechanisms utilized by GPCRs to transduce signals show much similarity across this family. These receptors transduce a signal across the plasma membrane by undergoing a conformational change, and changing the activity of an associated G-protein. A G-protein is a heterotrimeric protein consisting of three subunits: α, β and γ. In the inactive state, the α subunit is attached to a guanine diphosphate (GDP) molecule, and the G-protein exists with all three of its subunits intact. As the GPCR is activated by the binding of a ligand, a conformational change is induced in the receptor, which leads to activation of the associated G-protein. In the active state, the G-protein changes conformation, and exchanges its GDP on the α subunit for a guanine triphosphate (GTP)

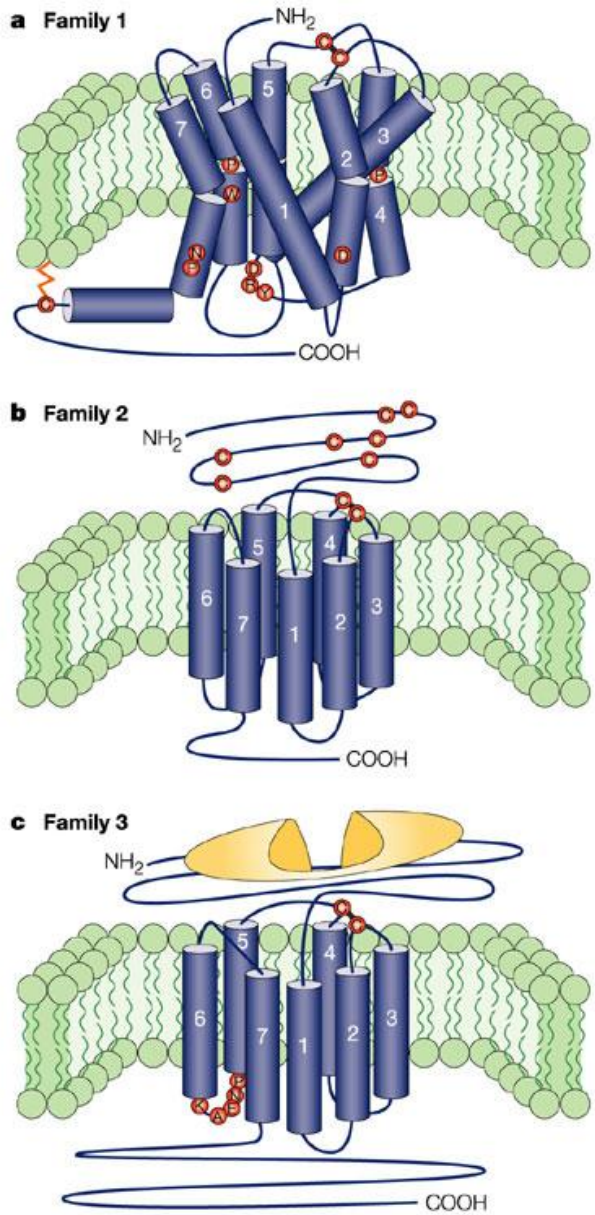

Figure 1.1: Three major G-protein coupled receptor (GPCR) families

Image from The state of GPCR research, (2004)

molecule. The binding of the GTP molecule leads to the dissociation of the α subunit from the β/γ subunits. These dissociated subunits are then able to modulate various signaling pathways within the cell. The activation of a pathway involving a G-protein is terminated by the hydrolysis of the GTP molecule to GDP by an intrinsic GTPase enzyme. This hydrolysis reaction leads to reassociation of the G-protein subunits, and inactivation of the signaling molecule till the next GPCR stimulation (Neer, 1995) **(Figure 1.2)**. Initial studies have demonstrated ligands as interacting at GPCRs almost exclusively via this G-protein dependent, or canonical reaction. Recent studies, however, are revealing the existence of G-protein independent, or non-canonical, signaling pathways, through interactions with other effectors, such as arrestins (DeWire et al., 2007; Premont & Gainetdinov, 2007). Thus these membrane receptors are beginning to conceptually shift towards being termed as seven transmembrane spanning receptors, rather than GPCRs, due to their overall structural organization which is shared between all receptors in this group.

1.2 DOPAMINE AND DOPAMINE RECEPTORS

Dopamine belongs to the family of neurotransmitters called catecholamines. This neurotransmitter is initially synthesized as L-3, 4 dihydroxyphenylalanine (L-DOPA) from tyrosine by the enzyme tyrosine hydroxylase. L-DOPA is subsequently decarboxylized by the enzyme aromatic L-amino acid decarboxylase leading to the formation of the final dopamine product (Siegel et al., 1994) **(Figure 1.3)**. Dopamine has various functions in the central nervous system (CNS), including regulation of locomotor

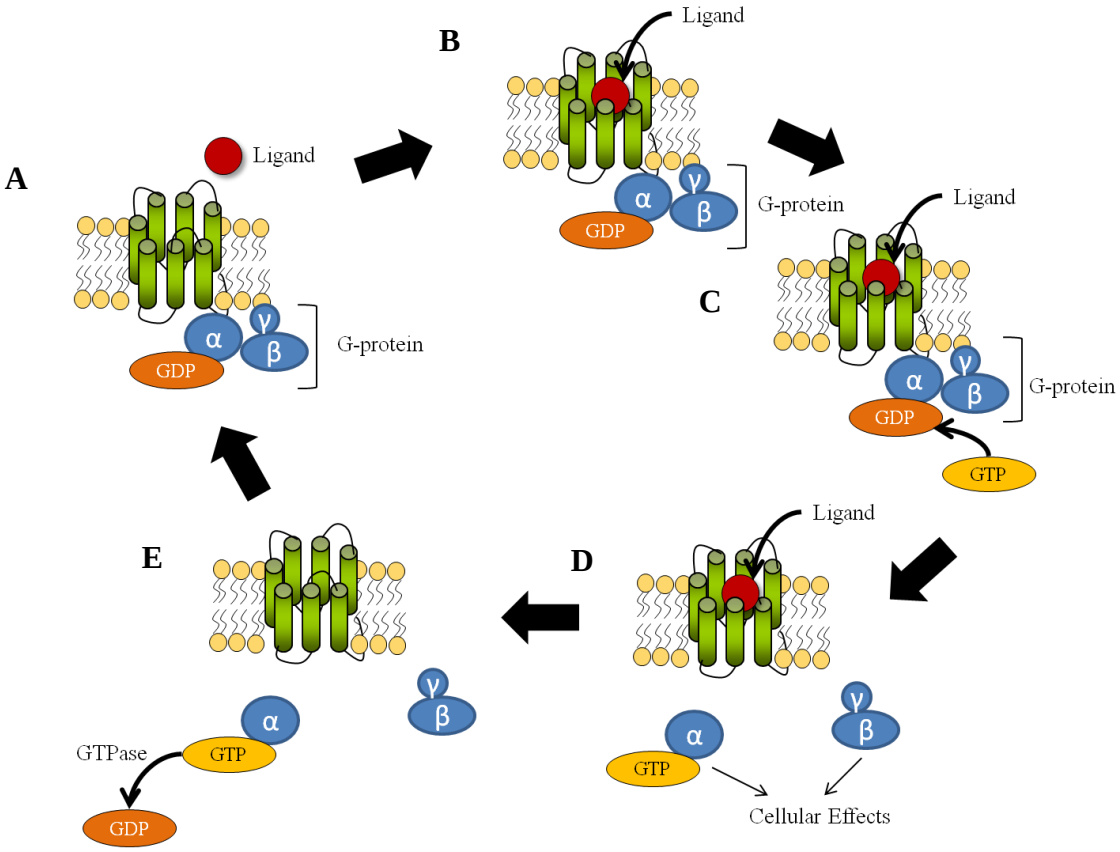

Figure 1.2: G-protein signaling following activation of a G-protein coupled receptor (GPCR). (A) In the inactive state, the α, β and γ subunits are intact and bound to guanine diphosphate (GDP). (B) Activation of GPCR by ligand binding leads to (C) exchanging of GDP for guanine triphosphate (GTP), and (D) dissociation of the G-protein subunits which can have cellular effects. (E) The reaction stops by hydrolysis of GTP to GDP on the G-protein by GTPase activity, which results in (A) reassociation of the subunits in anticipation for subsequent stimulation of the GPCR by ligand binding.

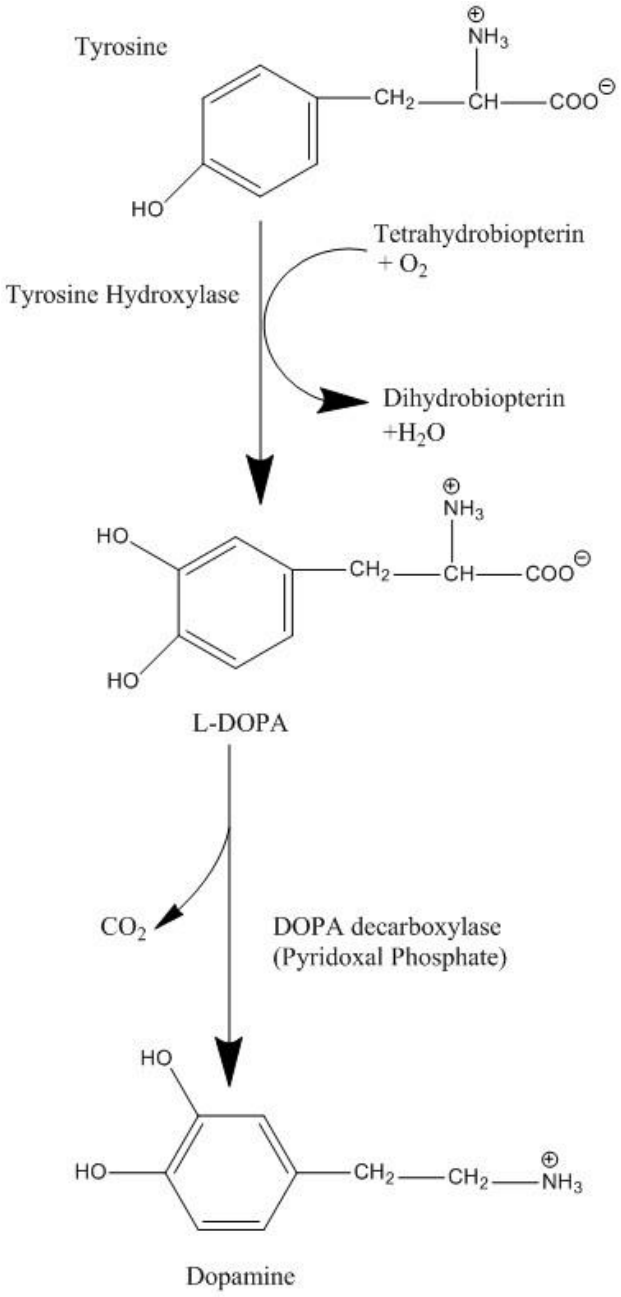

Figure 1.3: Biosynthesis of dopamine from L-tyrosine

activity, cognition/emotion, reward, learning, memory, and endocrine function. Some dopamine molecules also have an effect peripherally where they help to regulate cardiovascular function, hormone secretion, vascular tone, renal function and gastrointestinal motility. Projections originate from the brain areas of dopamine synthesis, giving rise to 4 axonal pathways: i) mesolimbic, ii) mesocortical, iii) nigrostriatal and iv) tuberoinfundibular pathway. The mesolimbic as well as the mesocortical pathway both arise from the ventral tegmental area. The mesolimbic pathway innervates the nucleus accumbens and parts of the limbic system involved in motivational behaviour, whereas the mesocortical pathway innervates regions of the frontal cortex involved in learning and memory. The nigrostriatal pathway arises from the substantia nigra compacta and innervates the striatum, where it is involved in the control of movement. Finally, the tuberoinfundibular pathway originates from the cells of the periventricular and arcuate nuclei of the hypothalamus, reaching the pituitary (Missale et al., 1998; Beaulieu & Gainetdinov, 2011; Vallone et al., 2000; Iversen & Iversen, 2007).

Dopamine exerts its action by binding to specific membrane receptors, which belong to a class of GPCRs in the rhodopsin subfamily. The first evidence for the existence of these receptors in the CNS came in 1972, from studies showing dopamine was able to stimulate the activity of adenylyl cyclase (Kebabian & Calne, 1979). Further studies revealed the existence of dopamine receptors which could be categorized into two groups, and five subgroups: D1-like receptor class (includes D1 and D5) and D2-like receptor class (includes D2, D3 and D4). The various receptor subtypes are hypothesized to have originated as a result of gene duplication, most likely of the opsin gene, followed

by genetic diversification (O'dowd, 1993). The dopamine receptors are classified into their respective subtypes based on the various similarity and differences in their signal transduction pathways, genetic organization, structure, sequence alignment and function. The D1 and D5 receptors are classified as members of the D1-like subfamily as they share 80% amino acid sequence homology within the transmembrane regions, and couple with the stimulatory G-protein (G_s) to stimulate the activity of adenylyl cyclase. Similarly, D2 shares 75% homology with the D3 transmembrane region, and 53% homology with the D4 transmembrane region. Additionally, these receptors couple with the inhibitory G-protein (G_i) to inhibit the activity of adenylyl cyclase, allowing their classification in the D_2-like subfamily (O'dowd, 1993; Civelli et al., 1993; Gingrich & Caron, 1993; Missale et al., 1998).

1.3 Dopamine D2 Receptor

1.3.1 Structure, Function and Localization

The dopamine D2 receptor is encoded by the chromosomal region 11q22-23, using 8 exons of approximately 270-kb in size each. Alternative splicing of an 87-bp exon between introns 4 and 5 results in the formation of two splice variants of this receptor, including the short isoform, D2S, and the long isoform, D2L, which differ by 29 amino acids in the third intracellular loop (Dal Toso et al., 1989). D2S receptors primarily function as autoreceptors on the presynaptic membrane, whereas D2L act as postsynaptic receptors. Aside from this, studies thus far have revealed both receptors appear to have

highly comparable pharmacological profiles, and similar signal transduction cascades (Usiello et al., 2000).

The resulting dopamine D2 protein is a Type A seven transmembrane GPCR, and shares most of the common structural characteristics of this class of proteins. The D2 receptor specifically possesses four glycosylation sites at the amino-terminal, and a long third intracellular loop, to aid in the coupling to the inhibitory G-protein (G_i). The protein core of the D2 receptor is approximately 40-44 kDa, which can increase in size to 94-150 kDa following glycosylation. This receptor also has phosphorylation sites on the third intracellular loop, which is crucial for receptor desensitization. Cysteine residues are present on the carboxyl-terminal, which are hypothesized to act as an anchor for the cytoplasmic tail of the receptor to the cell membrane. Also, disulfide bonds form between cysteines on extracellular loops 2 and 3, in order to stabilize the tertiary structure of the receptor (Missale et al., 1998; Civelli et al., 1993; Gingrich & Caron, 1993; O'dowd, 1993) **(Figure 1.4)**. A few key residues have been identified as being crucial components to form the D2 receptor dopamine binding pocket. The carboxyl group of Asp-114 in transmembrane 3 has been shown to form a tight salt bridge with the amino group of dopamine. Ser-193 and 197 located in transmembrane 5 hydrogen bond to the metahydroxyl and parahydroxyl groups of the catechol ring of dopamine, and therefore, play a key role in recognizing this neurotransmitter. Phe-110, Met-117, Cys-118 (transmembrane 3), Phe-164 (transmembrane 4), Phe-189, Val-190 (transmembrane 5), Trp-386, Phe-390, and His-394 (transmembrane 6) all form a mostly hydrophobic pocket for the binding of dopamine (Goddard, III & Abrol, 2007) **(Figure 1.5)**.

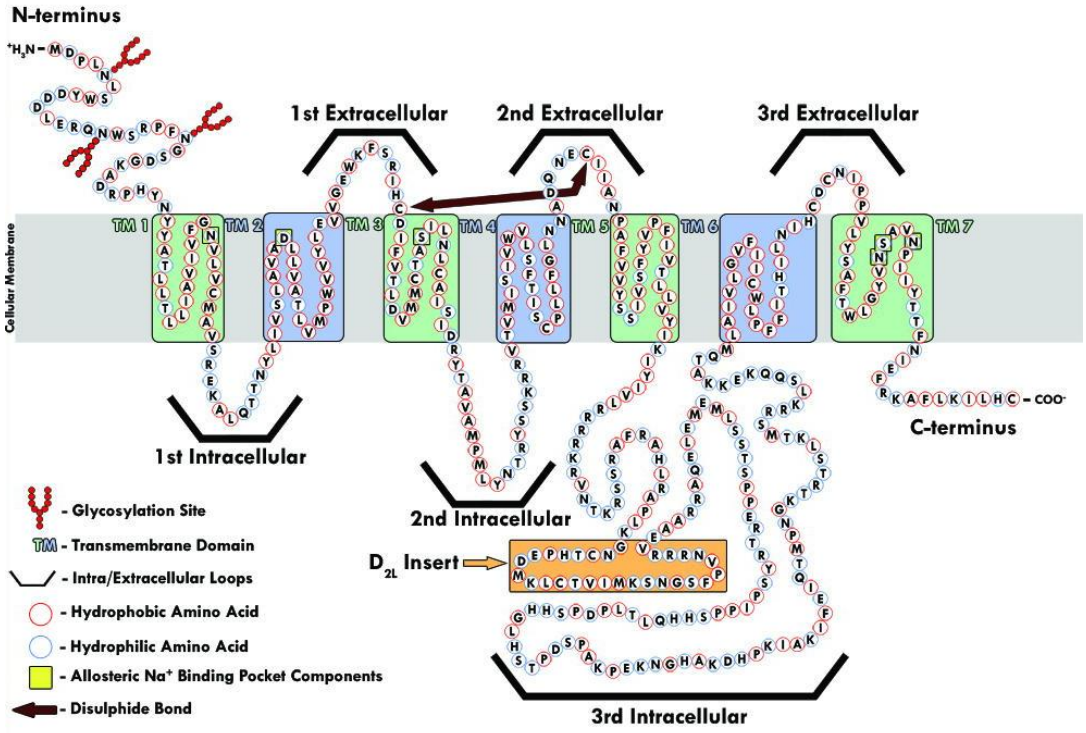

Figure 1.4: Structural features of dopamine D2 receptor

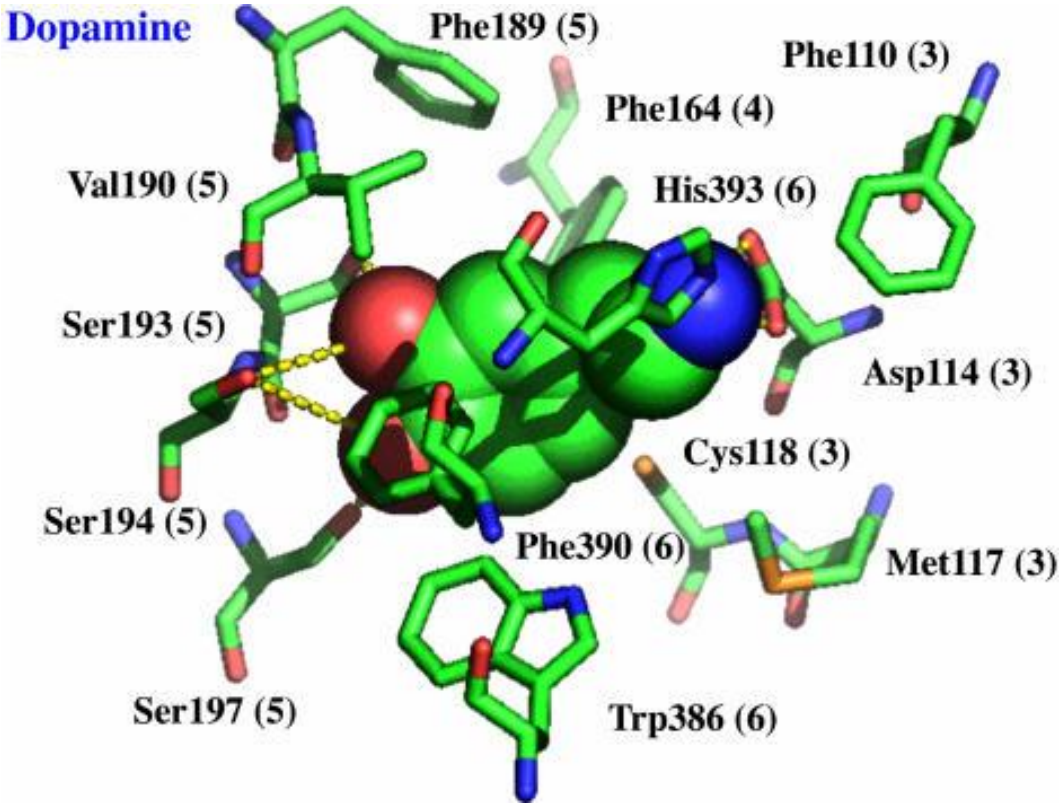

Figure 1.5: Computational modeling of dopamine binding to the D2 receptor, highlighting the crucial amino acids required for this interaction

Image from Goddard, III & Abrol, 2007

The D2 receptor localizes primarily to the striatum, olfactory tubercle and the nucleus accumbens. Within the striatum, D2 receptors are present on the medium spiny neurons, where they are concentrated in the spiny dendrites and spine heads. D2 receptor messenger ribonucleic acid (mRNA) has also been localized to parts of the cortex, including the prefrontal and cingulate regions, as well as in the substantia nigra, ventral tegmental area, hypothalamus, amygdala and hippocampus (Missale et al., 1998; Vallone et al., 2000; Beaulieu & Gainetdinov, 2011).

The D2 receptor has a variety of functions within a physiological system. These receptors have been implicated in controlling locomotor activity, whereby activation of the D2S autoreceptors results in decreased dopamine release, and decreased locomotor activity, whereas activation of the postsynaptic D2L receptors leads to increases in locomotion (Beaulieu & Gainetdinov, 2011). Additionally the D2 receptor has repeatedly been implicated in reward and motivation mechanisms, as well as learning functions, therefore relating this receptor to addictive and compulsive behaviours (Wise, 2004; Johnson & Kenny, 2010). Finally, this receptor has shown to mediate memory functions within hippocampal regions, such as working memory, as well as higher order cognitive, and executive functions, such as attention, planning etc. (Cropley et al., 2006). It is important to note that although these functions implicate the D2 receptor, GPCRs often work synergistically with other receptors (e.g.: D1 receptor), to bring about a full expression of these physiological responses (Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

1.3.2 SIGNAL MECHANISMS

Activation of the D2 receptor leads to a variety of potential cellular responses. This receptor is coupled to G_i, therefore, activation of D2 by ligand binding leads to an inhibition of adenylyl cyclase activity, causing an overall decrease in the levels of cyclic adenosine monophosphate (cAMP), and subsequently, decreases protein kinase A (PKA) activity. Activation of D2 can also have opposing effects on intracellular calcium levels, depending on the cell type. Stimulation of these receptors have shown to increase intracellular calcium via activation of phosphotidyl inositol hydrolysis by phospholipase C (PLC), or decrease intracellular calcium levels by inhibition of inward calcium currents. Additionally, activation of the D2 receptor potentiates the release of arachadonic acid evoked by calcium. Finally, D2 receptor activation increases outward potassium currents, leading to cell hyperpolarization (Missale et al., 1998; Neve et al., 2004). It is noteworthy to mention that although majority of the above mentioned pathways are activated via the α-subunit of the G-protein, certain pathways, such as activation of PLC or potassium channels, occur via the βγ subunits of the G-protein (Lin & Smrcka, 2011).

Initial evidence demonstrated the existence of the above G-protein dependent, or canonical, pathways. However, more recent investigations have also shown the D2 receptor to be involved in non-canonical signaling pathways, which are independent from G-protein activation. This new mode of signaling often involves proteins which have classically been implicated in GPCR desensitization (See section 1.3.3). One of the best studied non-canonical pathways occurs via the molecule, β-arrestin-2, which is also called, arrestin-3. Activation of GPCRs by ligand binding leads to the formation of

signaling scaffolds mediated by arrestin-3, which bring elements of specific signaling pathways into closer proximity. These scaffolds regulate an increasing number of signaling molecules, including mitogen activated protein kinase (MAPK), extracellular receptor kinase (ERK), c-JUN N-terminal kinase (JNK), Akt, phosphoinositide-3 kinase (PI3K) etc. (DeWire et al., 2007) **(Figure 1.6)**.

In addition to canonical and non-canonical signaling, a further level of complexity is added to D2 signaling, due to the existence of homo- or hetero-oligomeric units. Cellular models expressing the D2 receptor, as well as rat and human striatal membranes, have all shown this receptor to exist primarily as dimers, as well as higher order oligomers, such as tetramers (Zawarynski et al., 1998; Gazi et al., 2003; Lee et al., 2000). This homo-oligomerization interaction results in negative cooperativity, whereby ligand binding at one D2 receptor, decreases affinity for further binding to another D2 receptor within the oligomeric complex. This simplistic cooperativity model for this receptor is, however, currently being further investigated to incorporate more complex findings, and is likely to be reconceptualised in the near future (Armstrong & Strange, 2001; Vivo et al., 2006). The D2 receptor has also been shown to hetero-oligomerize with other subtypes of dopamine receptors, particularly, the D1 receptor. These D1/D2 hetero-oligomers can induce novel signaling pathways, including stimulation of the $G_{q/11}$ protein within the striatum, to activate phospholipase C-mediated increase in intracellular calcium signaling (Rashid et al., 2007; Lee et al., 2004). Experimental evidence has also reported the formation of D2 into a heteromeric complex with the adenosine A2A receptor within the basal ganglia, leading to an antagonistic interaction at the membrane,

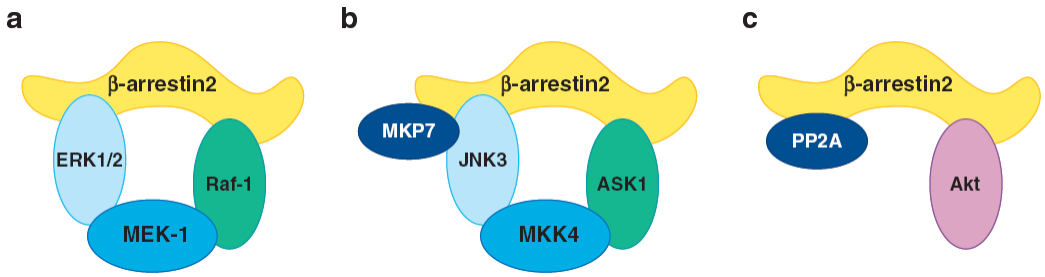

Figure 1.6: Three major scaffolds mediated by β-arrestin-2, also known as arrestin-3

(A) Arrestin-3 scaffold for extracellular receptor kinase (ERK) activation, (B) Arrestin-3 scaffold for c-Jun N-terminal kinase (JNK) 3 activation and (C) Arrestin-3 scaffold for Akt activation.

Image from DeWire et al., 2007

whereby stimulation of the A2A receptor by ligands, decreases agonist affinity for the D2 receptor (Kamiya et al., 2003; Ferre et al., 1991). Other similar oligomeric interactions of the D2 receptor remain to be investigated in future studies.

The dopamine D2 subtype is a receptor strongly implicated in various human physiological functions, which result from the activation of the complex signaling layers characterizing this receptor. Advanced understanding of these signaling pathways can lead to the development of novel methods to target the downstream molecules, resulting in improved treatment methods for D2-related pathologies.

1.3.3 RECEPTOR REGULATION

GPCR signaling is regulated by a number of key intricate processes. The trafficking of GPCRs has been shown to be regulated by a number of regulatory proteins, including G-protein-coupled receptor kinases (GRK) and arrestins. Receptor overstimulation by exposure to agonists leads to receptor phosphorylation by GRKs, followed by recruitment of arrestins, which could subsequently lead to downregulation in receptor activity by clathrin-mediated internalization. Internalized receptors can be recycled back to the plasma membrane, or targeted for lysosomal degradation (Gainetdinov et al., 2004; Zhang et al., 1997) (**Figure 1.7**). The human genome encodes for seven different GRKs (GRK1-7), and four different arrestins (arrestin-1-4). GRK1 and 7, as well as arrestin-1 and 4, are all exclusively expressed in the visual system. The remaining GRKs and arrestins are found widely expressed through the neuronal regions, and studies are currently in progress to delineate which GRKs and arrestins, regulate the

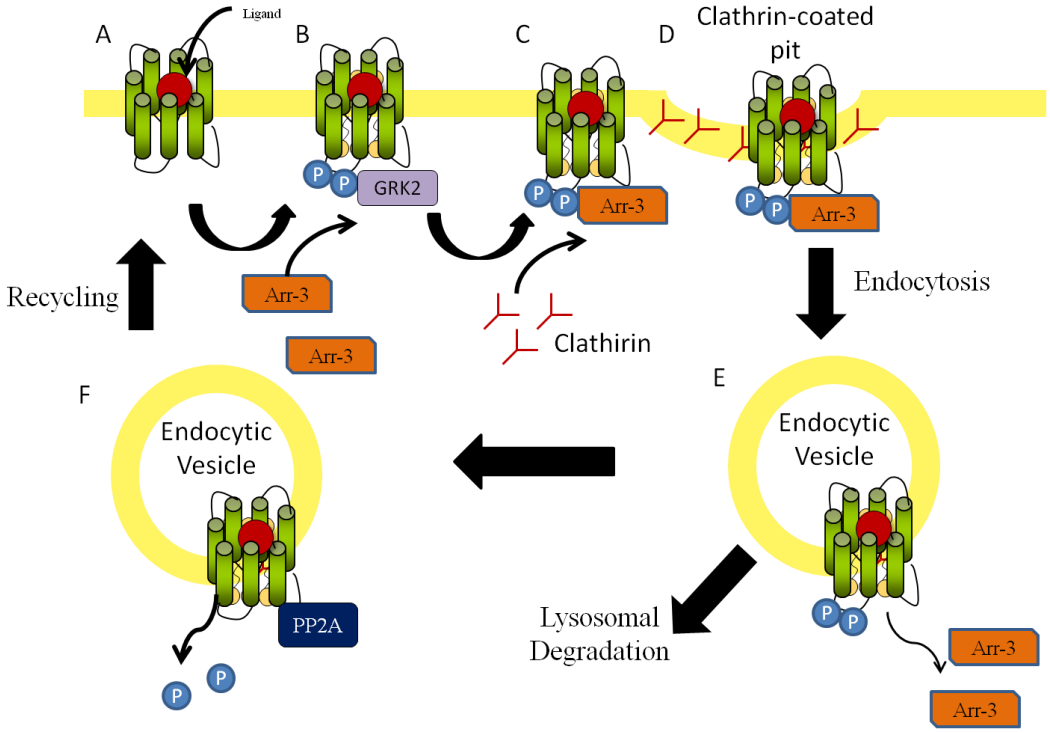

Figure 1.7: Dopamine D2 receptor downregulation by clathrin-mediated internalization. (A) Persistent agonist stimulation of the D2 receptor leads to (B) phosphorylation of the receptor by G-protein receptor kinase 2 (GRK2), which leads to (C) recruitment of arrestin-3 and clathrin. This results in (D) formation of clathrin-coated pits and (E) endocytosis of the D2 receptor. Internalized receptor can be degraded in the lysosome, or (F) dephosphorylated by protein phosphatase 2A and recycled back to the cell membrane.

activity of which subtype of the dopamine receptor. GRK2 is widely expressed in primary dopaminergic areas within the brain, and has been shown to phosphorylate to regulate the activity of D1, D2 and D3 receptors (Iwata et al., 1999; Kim et al., 2001). Previous studies have demonstrated the dominant-negative mutations of GRK2 in cellular systems to abolish any D2 receptor internalization, evincing the importance of this protein in the downregulation of this receptor (Ito et al., 1999). *In vivo* studies with GRK2 deletion have proven challenging to conduct, as mice expressing such deletions are not viable. Therefore, *in vivo* studies have utilized mice heterozygous for this mutation. These mice have shown minor D2 related locomotor supersensitivity only to cocaine administration, but not to amphetamine or D2 agonist apomorphine, even though all ligands increase D2 receptor activity (Premont & Gainetdinov, 2007; Beaulieu & Gainetdinov, 2011; Gainetdinov et al., 2004). Future studies will need to develop methods to accomplish a more global knockdown of the GRK2 protein *in vivo,* to further study its effect on D2 receptor regulation. In addition to GRK2, GRK6 is another subtype shown as being implicated in D2 receptor regulation, with supersensitivity observed in *in vivo* deletion models, when challenged with dopaminergic psychostimulant drugs (Beaulieu & Gainetdinov, 2011; Gainetdinov et al., 2004; Premont & Gainetdinov, 2007). In the case of arrestins, arrestin-3 appears to be the isoform which has shown, in past studies, to translocate preferentially to the D2 receptor (Kim et al., 2001). In fact, mice with arrestin-3 knockout have shown significant attenuation of agonist induced internalization of the D2 receptor in striatal tissue slices, proving the importance of this arrestin subtype in D2 receptor regulation (Skinbjerg et al., 2009a). Additional mechanisms for regulating D2

receptor signaling implicate the GTPase-activating proteins from the family of regulators of G protein signaling (RGS). This family is composed of 37 members, all of which function as negative modulators of G-protein signaling, by accelerating GTP hydrolysis at the Gα subunit, and terminating the signal. Biochemical and *in vivo* mice studies have revealed the specific importance of RGS9-2 specific, in the regulation of the D2 receptor (Rahman et al., 2003). Overexpression of this RGS subtype *in vivo* decreased locomotor response to D2 stimulants, where as knockout of this gene led to heightened response to these same stimulants.

In addition to GRKs, arrestins and RGS molecules, a further level of receptor regulation occurs by a process termed heterologous desensitization. This process involves the broad desensitization of receptors in a cell, following prolonged activation of other GPCRs within that cell. Heterologous desensitization occurs using a negative feedback mechanism mediated by second-messenger activated kinases. For the dopamine D2 receptor, protein kinase C (PKC) has been shown to mediate the phosphorylation and subsequent heterologous desensitization of these receptors (Namkung & Sibley, 2004; Thibault et al., 2011).

Scientific studies are revealing the importance of progressing from targeting GPCRs directly, to modulating post receptor mechanisms as a more fine-tuned method of receptor activity modulation (Beaulieu & Gainetdinov, 2011; Beaulieu et al., 2009; Freyberg et al., 2010). Receptor regulatory proteins and signaling mechanisms are therefore generating growing interests as novel targets to exert modulatory effects on

GPCR activity, which can eventually be developed into improved treatment for human disorders.

1.3.4 CLINICAL RELEVANCE

The extensive studies on D2 receptor structure, signaling and function have largely been motivated by convincing research implicating this receptor in a number of neurological and neuropsychiatric disorders. Disorders implicating the D2 receptor include Parkinson's disease, schizophrenia, bipolar disorder, major depression, Huntington's disease, attention deficit hyperactivity disorder (ADHD), Tourette's syndrome and drug abuse (Missale et al., 1998; Beaulieu & Gainetdinov, 2011). The improved knowledge of the physiological properties of the D2 receptor can ultimately result in improved understanding and treatment for these disorders, and other related ones.

A large number of studies have revealed one of the most robust clinical implications of the dopamine D2 receptor to be in the pathophysiology of schizophrenia (Ginovart & Kapur, 2012; Howes & Kapur, 2009; Seeman, 2010; Seeman, 2011; Seeman & Kapur, 2000). Schizophrenia is a chronic debilitating mental disorder inflicting about 1% of the world's population. Clinically, this disorder manifests as a range of symptoms, which can mostly be divided into three categories of positive, negative and cognitive symptoms. Positive symptoms of schizophrenia reflect those which present as a distortion of normal function, including hallucinations and delusions. Negative symptoms manifest as a loss of normal function, and include social withdrawal, affective blunting, poverty of speech,

anhedonia and avolition. Cognitive symptoms represent abnormal thought processes, and can include deficits in attention, working memory and executive functioning. Current studies have yet to identify a single causative agent for the development of schizophrenia, revealing the highly complex and heterogeneous nature of this disorder. Schizophrenia is a result of genetic factors combined with abnormalities in early neuronal development, which can often result in aberrant synaptic connectivity and neurotransmission. These physiological issues confer disease vulnerability to an individual, which can be triggered by subsequent environmental cues, to develop into the full blown clinical manifestation of schizophrenia (Walker et al., 2004; Roth et al., 2009).

Despite the complexity presented by the etiology of this disorder, studies over the past few decades have repeatedly shown dysregulation of the dopaminergic system as a characteristic feature of schizophrenia. The dopamine hypothesis of schizophrenia was initially proposed by van Rossum, which attributes schizophrenia to hyperactive dopaminergic signaling (van Rossum, 1966). This hypothesis has been revised since its first proposal, and evolved to incorporate the novel pieces of collected evidence. Studies looking at positron emission tomography (PET) imaging of dopaminergic neurotransmission have repeatedly shown increased dopamine synthesis, which could be observed even at the prodromal stage, prior to the onset of symptoms (Reith et al., 1994; Hietala et al., 1995; Meyer-Lindenberg et al., 2002; Howes et al., 2009). Additionally, evidence has also demonstrated exaggerated dopamine release in the striatum of patients with schizophrenia, at both baseline, and amphetamine challenged conditions (Abi-Dargham et al., 2000; Abi-Dargham et al., 1998; Bertolino et al., 2000). In contrast to

these presynaptic abnormalities, evidence has been more conflicting regarding postsynaptic changes in patients with schizophrenia. Imaging studies in drug naïve patients have been inconclusive, with select studies revealing an increase in striatal D2 receptor density, and others showing no such observed changes (Hietala et al., 1994; Pilowsky et al., 1994; Tune et al., 1993; Lomena et al., 2004; Talvik et al., 2006; Yang et al., 2004). A recent meta-analyses of previous imaging studies have revealed a modest increase, of 13-14%, of striatal D2 expression (Kestler et al., 2001; Laruelle, 1998; Zakzanis & Hansen, 1998). Studies have accounted for some of these conflicting results, by demonstrating the existence of a functionally active, GDP bound, high-affinity state of the D2 receptor, called D2high. In studies by Seeman *et al.*, these D2high receptor species have been observed to exist in higher proportion in all animal models of schizophrenia, including lesioned-models, drug sensitized models (amphetamine, phencyclidine, cocaine), social isolation models and gene deletion models (Seeman, 2011; Seeman et al., 2002; Seeman et al., 2005; Seeman et al., 2007; Seeman et al., 2009; Briand et al., 2008; King et al., 2009). Therefore, although there may little to no change in D2 receptor density, there could be an increased proportion of D2high receptors within the striatum of patients with schizophrenia. Imaging studies have not been able to demonstrate the existence of increased D2high levels in human patients with schizophrenia (Graff-Guerrero et al., 2009). A study by Seeman *et al.* has accounted for this discrepancy by demonstrating currently used radio-agonists, (+)-4-propyl-9-hydroxynaphthoxazine (PHNO) and (R)-2-CH3O-N-n-propylnorapomorphine (MNPA), as not being selective for D2high receptors, and having equal affinity to the functionally inactive D2low state as

well (Seeman, 2012). Future studies will need to focus on the development of radiotracers capable of discerning in human patients the two affinity states of the D2 receptor. In addition to increased dopaminergic activity within striatal regions, metabolite findings and imaging studies looking at extrastriatal regions have shown decreased dopaminergic activity, also termed "hypofrontality", within the frontal cortex of animal models, as well as human patients with schizophrenia (Davis et al., 1991; Howes & Kapur, 2009). Therefore, based on an accumulation of all evidence to date, the reconceptualized dopamine hypothesis introduces the element of cortical-subcortical interplay. This modified hypothesis relates the positive symptoms of schizophrenia to striatal hyperdopaminergia, and the negative symptoms to cortical hypodopaminergia, emphasizing the importance of synaptic connectivity, and region-specific changes in the pathophysiology of this disorder (Howes & Kapur, 2009; Davis et al., 1991).

The most convincing evidence in support of the dopamine hypothesis emerge from the mechanism of action of antipsychotic drugs (APD) used to treat schizophrenia, all of which target and block activity at the dopamine D2 receptor. In fact, early studies established a relationship between the ability of a older generation drugs to block the D2 receptor, and its clinical potency in the treatment of schizophrenia (Creese et al., 1976; Seeman & Lee, 1975). This has recently been proven true for newer generation of APDs as well (Kapur et al., 1998; Nordstrom et al., 1998). APDs can be divided into typical and atypical, based on their mode of action. Typical APDs, including haloperidol and chlorpromazine, block and antagonize the D2 receptor to decrease hyperdopaminergia. The strong D2 antagonism, however, results in a variety of movement-related adverse

events, termed extrapyramidal symptoms (EPS), including acute incidences of Parkinsonism, akathisia and dystonia, and later-onset of tardive dyskinesia, as well as an increase in prolactin levels (Ginovart & Kapur, 2012; Dayalu & Chou, 2008; Casey & Keepers, 1988). In addition to side effects, these APDs have an improved effect mostly on the positive symptoms of schizophrenia, and a large proportion (about 20-40%) of affected patients do not even respond to these medications (Hellewell, 1999). Atypical APDs, including olanzapine and clozapine, are a heterogeneous group, which, in addition to antagonizing the D2 receptor, can also affect serotonin, acetylcholine and adrenero-receptors (Deutch et al., 1991; Meltzer, 2004). This class of APDs have shown, in select studies, to have lower risk of EPS, and increased efficacy against the negative symptoms of the disorder, although both these properties have shown to be highly dependent on the specific drug being used (Arvanitis & Miller, 1997; Goldstein, 2000; Lemmens et al., 1999). The major drawback in the use of atypical APDs is their ability to induce metabolic side effects in patients, including weight gain, increased risk of type II diabetes and cardiovascular disease (Nasrallah, 2008). Amongst the available atypical APDs, the one deemed most effective has been clozapine, although this drug is prescribed with a great deal of caution along with regular clinical monitoring, due to its strong propensity to cause agranulocytosis in patients (Opgen-Rhein & Dettling, 2008). In the case of both typical and atypical APDs, neuroimaging studies have confirmed an optimal therapeutic striatal D2 receptor occupancy to range from 65-78% (Farde et al., 1992; Kapur et al., 2000; Kapur et al., 1998; Kapur et al., 1999; Nordstrom et al., 1998). Occupancies exceeding this tight therapeutic window are characterized by the emergence of EPS in

patients. In addition to antagonism of the D2 receptor, a newer generation of APDs, such as aripiprazole, reduces dopaminergic neurotransmission by acting as D2 partial agonists. D2 partial agonists activate the D2 receptor, but to a lesser extent when compared to the intrinsic activation by the endogenous ligand, dopamine. These ligands can thus restore the cortical-subcortical imbalance observed in schizophrenia, as they can attenuate activity in regions with hyperactive D2 receptor (by competing with endogenous dopamine), and can enhance signaling in regions with D2 hypoactivity (with its agonistic effects) (Lieberman, 2004; Tamminga & Carlsson, 2002). Additionally, due to its partial agonistic properties, these ligands can block 85-95% of D2 receptors without adverse effects, allowing for a much bigger therapeutic window and safer drug profile, when compared to D2 antagonizing APDs (Grunder et al., 2008; Mamo et al., 2007). In addition to partial agonistic effects at the postsynaptic D2L receptor, studies are currently underway to determine its agonistic properties at the presynaptic D2S receptor, which would lead to decreased dopamine release (Tamminga & Carlsson, 2002). Finally, studies are also currently elucidating the non-canonical pathways, and functional selective properties of this novel drug, including its cell-dependent signal pathway activations (Shapiro et al., 2003; Urban et al., 2007). Although promising, it is worthy to note that long term clinical studies are currently not available for aripirazole, and a very recent clinical review revealed, though inducing fewer metabolic side effects, aripiprazole is hardly absent of adverse events (Khanna et al., 2013).

Studies on all types of APDs for the treatment of schizophrenia are clearly convergent on the crucial role played by the D2 receptor in attenuation of the symptoms

of this disease. Current APDs appear to inadequately treat schizophrenia, as they often induce distressing movement and metabolic side effects. However, research on the new generation of APDs, such as the ones focusing on aripiprazole, highlight the importance of focusing future investigations on the development of pharmacological ligands which, rather than completely blocking the D2 receptor, modulate these receptors in a fine-tuned, and subtle manner.

1.4 ALLOSTERISM

Recent studies in drug development have shown remarkable advances in the use of allosteric modulators to alter receptor activity. The term "allo" means "other" in Greek, and was first coined by Monod, Wyman, Changeux and colleagues (Monod et al., 1965; Monod et al., 1963). Conceptually, the definition of "allosteric modulator" has evolved to refer to ligands which interact with a site on a receptor topographically distinct from the orthosteric site (Christopoulos & Kenakin, 2002; Keov et al., 2011; May et al., 2007). These modulators can affect binding or signaling properties at the orthosteric site, and/or activate their own signaling pathways **(Figure 1.8)**. A few models have been developed to create a framework for our understanding of allosteric interactions. The simplest model is referred to as the allosteric ternary complex model (ATCM) (Ehlert, 1988). This model suggests that the orthosteric ligand (A) or allosteric ligand (B) can bind to the receptor (R) to form binary species, or these ligands can both interact with the receptor to form a ternary complex (ARB). The cooperativity factor (α) incorporated into this model denotes the change in affinity of A or B to the receptor when the complementary binary species

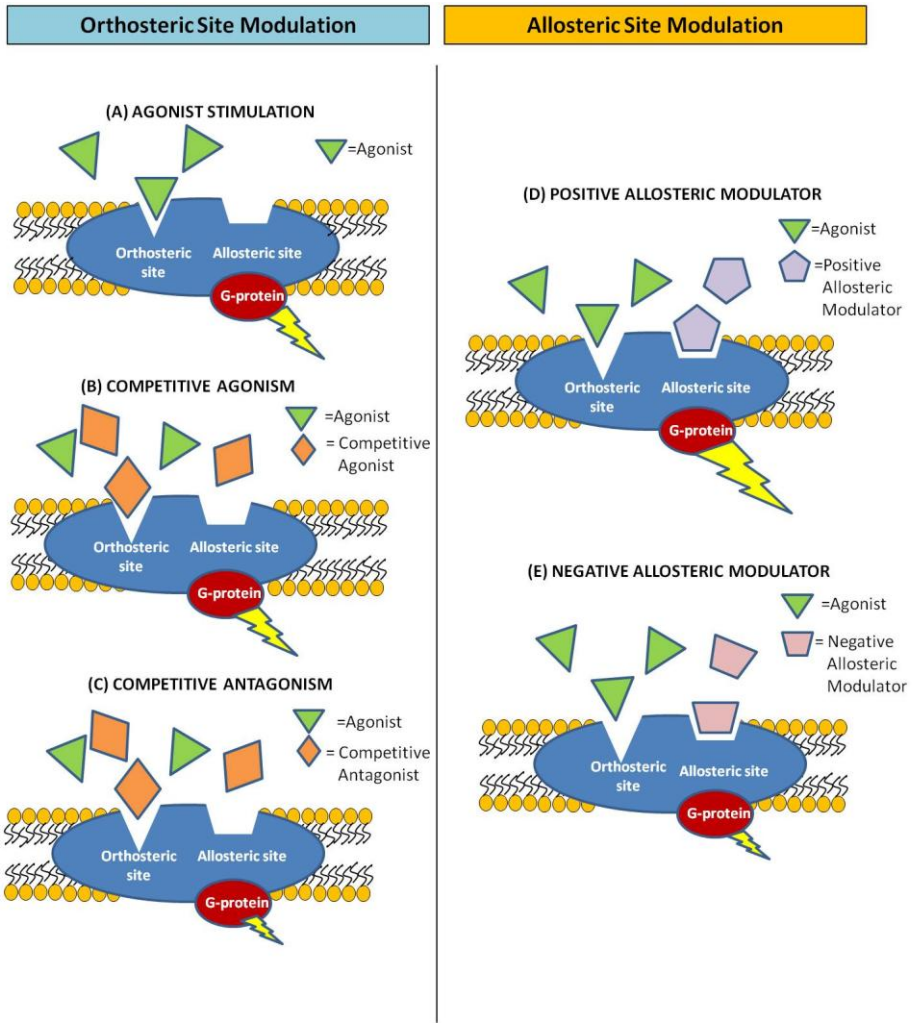

Figure 1.8: Comparison of G-protein coupled receptor (GPCR) orthosteric site and allosteric site modulation. (A) Depiction of agonist binding to the orthosteric site. (B) Competitive agonism showing two agonists competing for the orthosteric site (C) Competitive antagonism showing an antagonist competing with the agonist thereby preventing activation of the GPCR. (D) Positive allosteric modulation entails binding of a modulator to the allosteric site resulting in potentiation of the orthosteric agonist activity, whereas (E) Negative allosteric modulation entails binding of a modulator to the orthosteric site leading to an attenuation of orthosteric agonist activity.

is formed. This ATCM model was further modified into a two state model (Hall, 2000). The two state model accounts for receptor isomerisation, and includes within its framework, the existence of inactive (R) and active (R*) receptor conformations. Ligands (orthosteric or allosteric) have altered affinities for R compared to R*, and therefore, have different dissociation constants, which are accounted for within this mathematical model. In addition to these models, there have been proposed higher complexity level models to account for the range of observed allosteric interaction, although a review on these topics are beyond the scope of this thesis (Christopoulos & Kenakin, 2002; Keov et al., 2011; May et al., 2007). The development of these models demonstrates the layer of diversity in modulation which can be added to GPCR signaling by introducing the concept of allosteric modulation. The mechanisms of action allow allosteric modulators to have significant advantages over orthosteric ligands for therapeutic drug design. Orthosteric sites face the evolutionary pressure for all subtypes (e.g.: dopamine D1-D5) to recognize and interact with one endogenous ligand (e.g.: dopamine). Allosteric sites, on the other hand, can exhibit a much greater sequence divergence, allowing for the design of highly receptor subtype specific ligands. Orthosteric ligands often block or activate receptors, overriding intrinsic cycles in neurotransmission, which can result in toxicity or long term up/downregulation of crucial proteins. Allosteric modulators, on the other hand, can be quiescent by themselves, and only work to enhance or attenuate endogenous receptor activity, thus presenting a spatial and temporal modulation of the natural physiological tone. This allows these types of ligands to be designed into much safer therapeutic options. Progress in this field of drug development has therefore identified allosteric

modulators as promising molecules that can be researched and developed into better research tools and therapeutic drugs for the near future (Christopoulos, 2002; Christopoulos & Kenakin, 2002; Conn et al., 2009; May et al., 2007).

1.5 ALLOSTERIC MODULATION OF DOPAMINE D2 RECEPTOR

The dopamine D2 receptors have a few endogenous allosteric modulators which have been identified. The first modulator, and usually the best studied one for most receptors, is sodium ion (Na^+). Na^+ at millimolar concentration has been shown to convert D2 receptors from high to low affinity receptors. The binding pocket for this modulator is shaped like a pyramid, compromised of Asp52, Ser93, Asn390, Ser391 and His394 (Schetz, 2005). A second allosteric modulator affecting D2 receptors is the zinc ion, Zn^{2+}. This ion robustly inhibits antagonist binding to the receptor, and recent studies have narrowed down its allosteric binding site to His394 and His399 on the D2 receptor (Liu et al., 2006). In addition to Na^+ and Zn^{2+}, the D2 receptor is allosterically modulated by amiloride (3,5-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide) and its derivatives such as methylisobutylamiloride and benzamil. These modulators inhibit antagonist binding by a competitive and allosteric interaction (i.e.: they facilitate their own binding to the orthosteric site by binding to the allosteric site) (Schetz, 2005). There is, however, very little information available on the allosteric binding site for this class of D2 modulators. These currently studied allosteric modulators of the D2 receptor have not shown any therapeutic effects. However, their mechanistic concept of allosterism can be

used to design ligands which modulate D2 receptor activity, to bring about changes which can be physiologically significant.

1.6 PROLYL-LEUCYL-GLYCINAMIDE AND PEPTIDOMIMETICS

The neuropeptide L-prolyl-L-leucyl-glycinamide (PLG) has been a primary focus of studies for our lab due to its ability to allosterically interact with the dopamine D2 receptor. PLG was first isolated in 1971 from bovine hypothalamic tissue by Nair, Kastin and Schally (Nair et al., 1971). It was initially found to inhibit melanocyte-stimulating hormone (MSH) release, thereby leading to its alternate name of MSH inhibiting factor-1 (MIF-1), and was shown to be synthesized by exopeptidase dependent C-terminal cleavage of oxytocin (Celis et al., 1971) **(Figure 1.9)**. More recent studies in mice brain, using mass spectrometry techniques with higher sensitivity, have also localized PLG to extra-hypothalamic regions, including the putamen and globus pallidus of the striatum, the hippocampus, and the cerebral cortex (Kheterpal et al., 2009). These studies have suggested additional mechanisms of PLG synthesis in the extra-hypothalamic regions, which may not necessitate oxytocin as a precursor, with future studies required to clarify these findings (Kheterpal et al., 2009; Burbach et al., 1980; Pan & Kastin, 2007). It is highly worthy to note that investigations with PLG have shown its remarkable stability in human plasma, with 50% degradation requiring a lengthy 5 days (Kastin et al., 1994). Additional studies have also identified a saturable transporter to be present in the blood brain barrier, which would allow transport of this neuropeptide into the brain

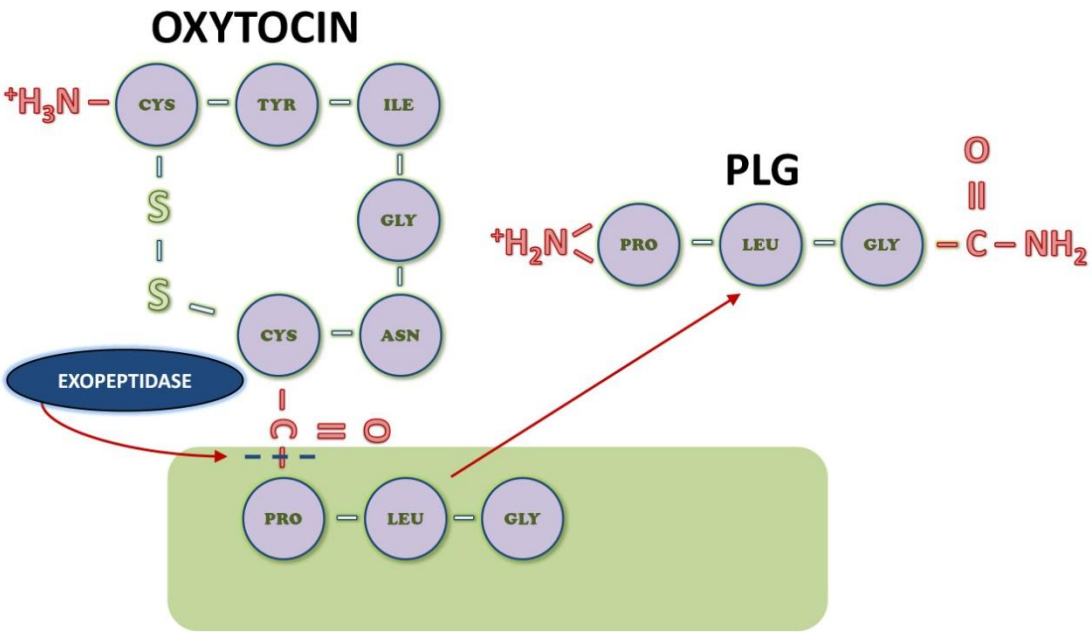

Figure 1.9: Synthesis of tripeptide L-prolyl-L-leucyl-glycinamide (PLG). PLG is synthesized by exopeptidase cleavage at a site close to the C-terminal of oxytocin.

(Banks & Kastin, 1994). These characteristics substantiate further research into PLG, owing to its possible development into a stable, distributable drug.

In addition to its highly favourable characteristics, PLG has proven to be an important candidate for further research, due to its observed therapeutic effects in human clinical disorders. Between 1972 and 1979, a number of pilot studies were conducted in human populations, with majority of these studies evincing the robust effects of PLG in acting as an anti-Parkinsonian agent, whereby this ligand was capable of attenuating a range of movement disorders, or potentiating the effect of the currently prescribed drug, L-DOPA (Barbeau, 1975; Kastin & Barbeau, 1972; Caraceni et al., 1979). Additionally, human trials also demonstrated PLG's therapeutic effect as an anti-depressant in patients suffering from clinical mental depression (Ehrensing & Kastin, 1974; Ehrensing & Kastin, 1978). These initial promising therapeutic results were the motivation for our lab to further investigate, and develop PLG as an allosteric ligand for the D2 receptor.

Over 200 peptidomimetics of PLG have been developed and screened to date in our lab, with a collaboration with Dr. R. L. Johnson from the University of Minnesota (Baures et al., 1999; Bhagwanth et al., 2012; Dolbeare et al., 2003b; Dolbeare et al., 2003a; Evans et al., 1999; Fisher et al., 2006; Genin et al., 1993; Johnson et al., 1986a; Johnson et al., 1990; Khalil et al., 1999; Raghavan et al., 2009; Sreenivasan et al., 1993; Subasinghe et al., 1993; Vartak et al., 2007; Yu et al., 1988). The most potent of these analogues was identified to be the conformationally constrained (3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA). PAOPA is constrained from rotational movements by utilizing the methyl groups in the leucine of

PLG, and constricting it to form a ring structure. The constrained nature of this peptidomimetic has led it to be 100-1000 times more potent than PLG, allowing it be considered as the more clinically relevant analogue (Verma et al., 2005). Additionally, previous studies have also identified a photoaffinity peptidomimetic 157, 3R-[4(S)-(4-azido-2-hydroxy-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2- oxo-1-pyrrolidineacetamide hydrochloride, which has been developed for radioiodination of the PLG compound (Fisher et al., 2006). This compound binds to the exact same allosteric site on the D2 receptor as PLG, and thus can be used for autoradiography and radioligand displacement assays to identify the nature of the PLG allosteric binding site (Mann et al., 2010) **(Figure 1.10)**.

1.6.1 PRECLINICAL EFFECTS

PLG and PAOPA have both shown robust preclinical therapeutic effects, in a range of pathophysiologies which implicate the dopamine D2 receptor. A combination of studies from both, our lab, and others, have shown PLG to be effective in attenuating Parkinson's related symptoms in drug induced preclinical models of this disorder, including oxotremorine (Plotnikoff et al., 1972), and 1- methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models (Marcotte et al., 1998). Additionally, our lab has also found PLG to potentiate the effects of D2 receptor agonists apomorphine and L-DOPA in 6-hydroxydopamine (6-OHDA) lesioned rat models of Parkinson's disease, suggesting its potential to treat the hypodopaminergic state characterizing this disorder(Mishra et al., 1997). In certain paradigms of preclinical depression models, PLG has shown to be

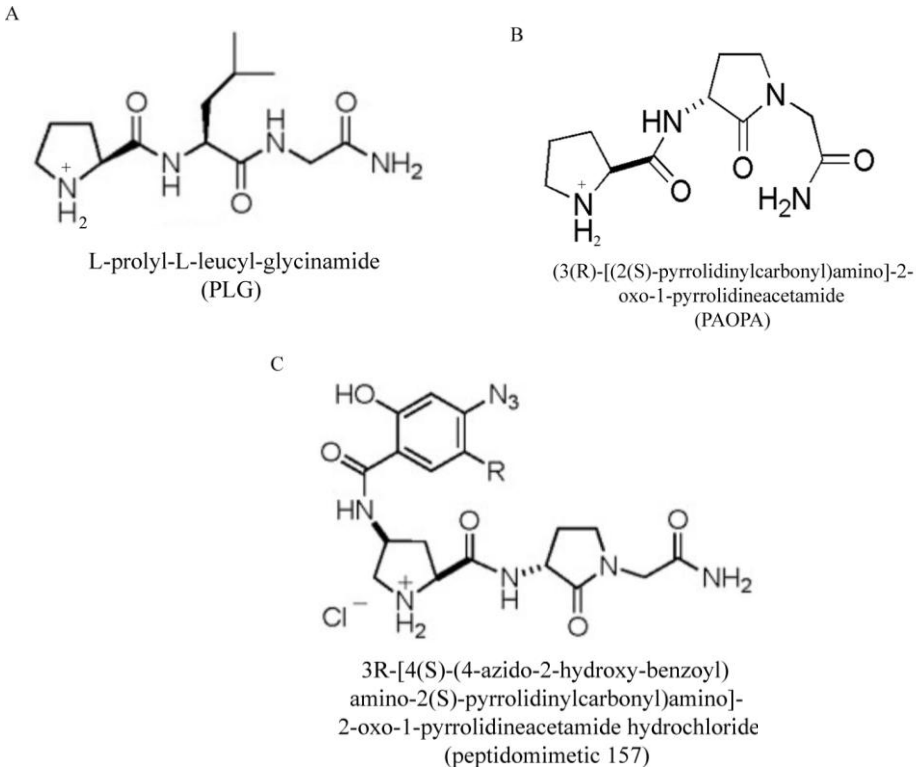

Figure 1.10: Chemical structures of tripeptide L-prolyl-L-leucyl-glycinamide (PLG) and its peptidomimetics. (A) Chemical structure of parent compound, PLG. (B) Chemical structure of (3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA), a chemically constrained peptidomimetic of PLG with a closed ring at the leucyl group, leading this compound to be more potent. (C) Chemical structure of 3R-[4(S)-(4-azido-2-hydroxy-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2- oxo-1-pyrrolidineacetamide hydrochloride (peptidomimetic 157), a peptidomimetic of PLG which can be radioiodinated at the "R" site (R= H in unaltered compound, and R= ^{125}[I] in radiodiodinated compound).

effective in reversing behaviours (Kastin et al., 1978), or potentiating the action of tricyclic antidepressants (Kostowski et al., 1991). Further studies, primarily conducted by our lab, have also shown PLG to antagonize APD (haloperidol) induced vacuous chewing movements in rats, a well accepted model of tardive dyskinesia, demonstrating its effectiveness in treating dyskinesias and other such related movement disorders (Castellano et al., 2007; Bhargava, 1984; Chiu et al., 1985; Chiu et al., 1981; Rajakumar et al., 1987; Hara & Kastin, 1986; Costain et al., 1999).

The more potent peptidomimetic synthesized from the pharmacophore of PLG, is PAOPA, and similar to PLG, this potent analogue has also shown therapeutic effectiveness in Parkinson's disease (Marcotte et al., 1998), as well as tardive dyskinesia models (Castellano et al., 2007). However, in much more recent studies, PAOPA has also shown to have very promising therapeutic effects in preclinical models of schizophrenia. This D2 allosteric modulator has shown to successfully prevent and reverse schizophrenia-like behavioural abnormalities in the amphetamine-sensitized animal model, including deficits in sensory motor gating, as measured by pre-pulse inhibition, deficits in social interaction, and hyperlocomotor activity (Beyaert et al., 2013). In addition, PAOPA has also been shown to prevent deficits in social interaction, a negative symptom associated with schizophrenia, observed in rats treated with the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 (Dyck et al., 2011). Further characterization of the preclinical pharmacokinetic parameters and toxicological profile of PAOPA has also shown favourable results (Tan et al., 2013). These results have demonstrated the metabolism of PAOPA to be comparable to current market APDs.

Necropsy and histopathological analyses of the examined organs showed a lack of tissue abnormalities, as well as an absence of movement, hematological and metabolic adverse events with acute and chronic *in vivo* treatment with PAOPA. This study revealed the significantly improved safety profile of PAOPA compared to current therapeutic options, mitigating its progression towards further investigations.

The specific, fine-tuned allosteric modulation exerted by administration of PLG and PAOPA appear to induce safe attenuation of preclinical symptoms in a range of neurological and neuropsychiatric disorders, including Parkinson's disease, tardive dyskinesia and especially schizophrenia. Characterizing the mechanism of action of these D2 allosteric modulators could provide key insights into understanding the pathophysiology of these diseases, and provide novel therapeutic pathways which could be targeted.

1.6.2 PHARMACOLOGICAL EFFECTS

The use of allosteric modulators can provide significant advantages for therapeutic treatment, as they can activate in a precise and subtle manner, novel and diverse pathways when compared to orthosteric ligands. Understanding of these pathways can improve the treatment of various GPCR-related disorders. The therapeutic effects of PLG and PAOPA can be understood by characterizing the mechanisms of action of these ligands at a receptor level. A range of radioligand binding studies conducted with PLG and PAOPA have developed a pharmacological profile for these tripeptides. Various studies have repeatedly confirmed both tripeptides to affect dopaminergic neurotransmission,

specifically by targeting the D2 and D4 receptor subtypes, and potentiating agonist binding to these receptors (Verma et al., 2005). This was shown with the radioligand agonists apomorphine (Chiu et al., 1981; Mycroft et al., 1987), *N*-propylnorapomorphine (NPA) (Srivastava et al., 1988) and/ or 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) (Johnson et al., 1986b). Scatchard analysis of these studies demonstrated a significant decrease in the dissociation constant (K_D), with no significant change in B_{max} (Srivastava et al., 1988), thus suggesting that PLG potentiates agonist binding by increasing the percentage of D2 receptors in the high affinity state, and not by increasing the total number of receptors. These studies found no significant binding changes at other receptors such as the dopamine D1 and D3 receptor subtypes (Verma et al., 2005), α_2-adrenoreceptors (Srivastava et al., 1988), γ-aminobutyric acid (GABA) receptors (Miller & Kastin, 1990), and serotonin receptors (Gulati & Bhargava, 1990), and PLG was also ineffective in modulating antagonist binding (Srivastava et al., 1988). PAOPA was observed in various studies as presenting with a very similar pharmacological profile as PLG, but with a potency of 100 to 1000 times greater, owing to its conformationally constrained nature (Verma et al., 2005; Yu et al., 1988). An interesting pharmacological feature common to both PLG and PAOPA seen in all these studies, was an inverted "U-shaped" or "biphasic" dose response curve, whereby with higher concentrations of the ligands, there was initially an increase in agonist binding, followed by a slow decrease (Srivastava et al., 1988; Verma et al., 2005) **(Figure 1.11)**. This interesting phenomenon has not only been observed in *in vitro* cellular studies,

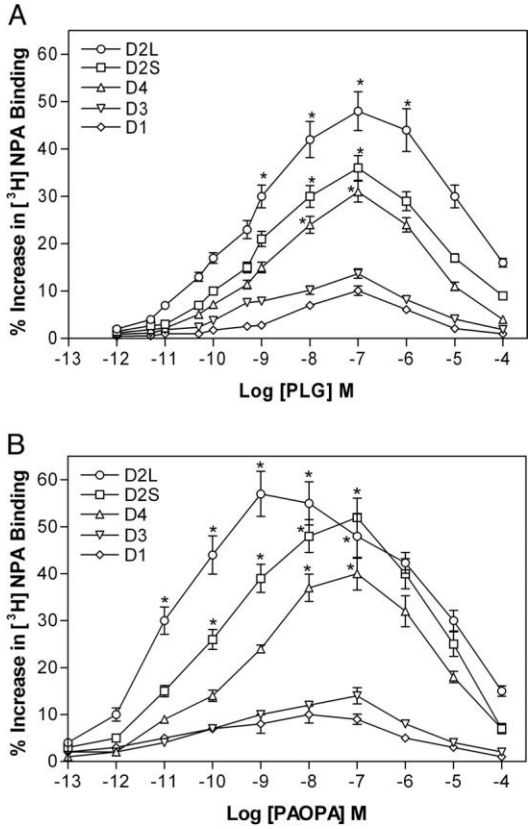

Figure 1.11: Representative graph depicting the biphasic dose response curve observed with treatment using D2 allosteric modulators PLG and PAOPA. This graph shows agonist binding to dopamine receptor subtypes in transfected cells. Agonist binding significantly affects the D2 and D4 receptors, but not the D1or D3 receptors. The most robust modulation occurs specifically at the D2 receptors (D2S and D2L), with an increase in agonist binding observed at lower PLG/ PAOPA concentrations, and a decrease in agonist binding observed at higher ligand concentrations, leading to the inverted "U-shaped" or "biphasic" dose response curve.

(Image from (Verma et al., 2005)

but has also repeatedly been demonstrated in *in vivo* preclinical studies, as well as human trials (Pan & Kastin, 2007), suggesting it to be a crucial commonality between all therapeutic levels, and therefore, worthy of further investigations.

Past studies have generated a framework implicating PLG and PAOPA to both have a significant effect on the dopaminergic system, by particularly modulating the D2 receptor. This effect provides a basic explanation for the efficacy of PLG and PAOPA in D2-related pathologies, such as Parkinson's disease and schizophrenia. However, to further develop these allosteric modulators towards improved treatment for such disorders, much remains to be elucidated regarding their mechanisms of action at the cellular and receptor level.

1.7 STUDY HYPOTHESES

The strong therapeutic implications of the allosteric modulators PLG and PAOPA have led to the design of the studies proposed in this thesis, which help progress our understanding of the mechanisms of actions for these novel potential drugs. The diversity and complexity of allosteric signaling requires us to narrow down proposed studies to focus on specific aspects of the PLG/PAOPA mechanism of action which include exploring answers to the following two critical questions: **1.** Which mechanistic factors contribute to the biphasic dose response ubiquitiously observed in *in vitro* and *in vivo* studies with PLG and PAOPA? **2.** What is the nature of the PLG allosteric binding site on the D2 receptor? Elucidation of these essential questions can help enhance our knowledge on a possible mechanism by which PLG/ PAOPA are therapeutic in dopaminergic

disorders, and allow for the design of improved ligands targeted towards this allosteric site.

As mentioned above, previous studies have shown the repeated existence of a biphasic dose response curve in the case of both PLG and PAOPA. Similar doses response curves have been observed in various other D2 receptor modulators, collectively known as a group of dopaminergic stabilizers. Dopaminergic stabilizers can be defined as drugs that stimulate or inhibit dopaminergic signaling depending on the dopaminergic tone. At elevated dopamine concentrations, these modulators cause dopaminergic inhibition, and during low dopamine signaling, an opposite effect is seen (Rung et al., 2008b). Such pharmacological agents are particularly useful in neuropsychiatric disorders such as schizophrenia, which, as mentioned above, is characterized by spatial differences in dopaminergic activity (Howes & Kapur, 2009). Previous studies with other dopaminergic stabilizers have revealed two potential hypotheses to explain the biphasic-curve phenomenon: **1.** At lower concentrations, the ligands act on the allosteric site, where it has higher affinity, to increase agonist binding, but at higher concentrations, the ligands act on the orthosteric site, where it has lower affinity, to antagonize dopamine binding (Rung et al., 2008a; Lahti et al., 2007); **2.** Increased dopaminergic agonist stimulation resulting from the presence of higher concentrations of the ligands causes increased agonist-induced D2 receptor internalization, thereby decreasing overall receptor activity (Guo et al., 2010; Marchese et al., 2008; Macey et al., 2004; Skinbjerg et al., 2009b). Our previous work with PLG and PAOPA has never observed a complete antagonistic effect (i.e.: zero agonist binding) of these ligands on D2 receptor binding, as

well as, these modulators have never shown displacement of orthosteric ligands, even with the highest concentrations of PLG or PAOPA. This leads to the rejection of the first hypothesis suggesting these modulators at act as orthosteric antagonists at higher concentrations. On the other hand, the second hypothesis appears more plausible, as it suggests agonist-induced internalization as an explanation for the biphasic curve and the partial decrease in agonist binding observed with PLG/ PAOPA treatment. **Therefore, our first hypothesis suggests one mechanism of PLG and PAOPA contributing to the biphasic dose response curve is mediated by these ligands causing dopamine D2 receptor internalization and downregulation at higher concentrations.**

Despite the strong pharmacological evidence that has been generated for PLG and its analogues very little is known about the exact nature of the allosteric binding site used by these tripeptides to bind the D2 receptor. Elucidating the exact sequence of the PLG/D2 allosteric binding site will allow for the systematic design of compounds that are more potent and site-specific (Jacobson & Costanzi, 2012). The pKa of PLG can be predicted to be around 9.4-10.0, which is based on the known pKa values for other similar pyrrolidine-based compounds,. Thus, at a physiological pH of 7.4, the Henderson-Hasselbach equation suggests that approximately 99% of the tripeptide will be in a protonated state and unable to cross the hydrophobic plasma membrane by diffusion. Currently, there are no known PLG transporters located on the cell membrane, suggesting the allosteric binding site should involve parts of the receptor exposed to the extracellular environment. **Therefore, our second hypothesis suggests PLG allosterically bind to**

the D2 receptor at an extracellular or transmembrane region exposed to the cell's extracellular space.

The investigation of the above mentioned hypotheses will lend to a significantly heightened understanding of novel mechanisms of action which could potentially be used by PLG and PAOPA to induce a therapeutic effect in the preclinical models of D2-related pathologies. Additionally, it will guide us to utilize the nature of the allosteric binding site to lead to enhanced drug designs.

1.8 STUDY OBJECTIVES

OBJECTIVE 1: Determine the *in vitro* effects of PAOPA on D2 receptor internalization using cellular models.

OBJECTIVE 2: Determine the *in vivo* effects of chronic PAOPA administration on the expression of D2- internalization related proteins and related downstream proteins using rodent models

OBJECTIVE 3: Express and purify the dopamine D2 receptor to use as a tool to study the PLG putative allosteric binding site using cell-free protein synthesis techniques.

OBJECTIVE 4: Identify the PLG putative allosteric binding site using expressed D2 receptor protein.

CHAPTER TWO

Effects of Dopamine D2 Allosteric Modulator, PAOPA, on the Expression of GRK2, Arrestin-3, ERK1/2, and on Receptor Internalization

AUTHOR'S PREFACE:

The objective of this study was to determine the *in vivo* effects of the allosteric modulator, PAOPA, on cellular proteins required for the internalization of the dopamine D2 receptor, as well as effects on downstream signaling kinases. Additionally, a cellular model was used to study the effects of this ligand on D2 receptor internalization. The results of this study improve our understanding on the possible mechanisms of action of this allosteric modulator, including enhancing our understanding of the biphasic dose response curve associated with PAOPA use.

I was responsible for study design, data collection, analysis, and writing of the manuscript. Graduate student, Jayant Bhandari, and undergraduate student Patricia Hui, helped with daily treatment of rats with investigated drugs. Undergraduate student Yuxin Tian aided in western blotting protocols, and Jian Ru Jiang assisted with sulpiride assay data collection. We are grateful for our collaboration with Dr. Johnson, who has worked with us to design the allosteric D2 ligand, PAOPA. Additionally, the expertise of Dr. Mishra and Dr. Johnson was invaluable for study design, data analysis, and editing of this manuscript. This manuscript has been accepted and published in the journal PLOS One.

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2.1 ABSTRACT:

The activity of G protein-coupled receptors (GPCRs) is intricately regulated by a range of intracellular proteins, including G protein-coupled kinases (GRKs) and arrestins. Understanding the effects of ligands on these signaling pathways could provide insights into disease pathophysiologies and treatment. The dopamine D2 receptor is a GPCR strongly implicated in the pathophysiology of a range of neurological and neuropsychiatric disorders, particularly schizophrenia. Previous studies from our lab have shown the preclinical efficacy of a novel allosteric drug, 3(R)- [(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA), in attenuating schizophrenia-like behavioural abnormalities in rodent models of the disease. As an allosteric modulator, PAOPA binds to a site on the D2 receptor, which is distinct from the endogenous ligand-binding site, in order to modulate the binding of the D2 receptor ligand, dopamine. The exact signaling pathways affected by this allosteric modulator are currently unknown. The objectives of this study were to decipher the *in vivo* effects, in rats, of chronic PAOPA administration on D2 receptor regulatory and downstream molecules, including GRK2, arrestin-3 and extracellular receptor kinase (ERK) 1/2. Additionally, an *in vitro* cellular model was also used to study PAOPA's effects on D2 receptor internalization. Results from western immunoblots showed that chronic PAOPA treatment increased the striatal expression of GRK2 by 41%, arrestin-3 by 34%, phospho-ERK1 by 51% and phospho-ERK2 by 36%. Results also showed that the addition of PAOPA to agonist treatment in cells increased D2 receptor internalization by 33%. This study provides the foundational evidence of putative signaling pathways, and changes in

receptor localization, affected by treatment with PAOPA. It improves our understanding on the diverse mechanisms of action of allosteric modulators, while advancing PAOPA's development into a novel drug for the improved treatment of schizophrenia.

Abbreviations:

G-protein-coupled receptor (GPCR), G-protein-coupled kinase (GRK), 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA), extracellular receptor kinase (ERK), L-prolyl-L-leucyl-glycinamide (PLG), dizocilpine (MK-801), mitogen activating protein kinase (MAPK), intraperitoneal (I.P.), phosphate-buffered saline (PBS), sodium dodecyl sulfate (SDS), polyvinylidene (PVDF), enhanced chemiluminescence (ECL), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), horseradish peroxidase (HRP), tetracycline-regulated expression cellular line (T-Rex-293), enhanced yellow fluorescent protein (eYFP), Dulbecco's modified Eagle's medium (DMEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Earle's buffered saline solution (EBSS), decays per minute (DPM), analysis of variance (ANOVA), glycogen synthase kinase-3 (GSK-3), epidermal growth factor (EGF).

2.2 INTRODUCTION:

Modulation of G protein-coupled receptor (GPCR) signaling represents an important mechanism of action for a range of therapeutics. Consequently, recent scientific studies have focussed on modulating GPCR activity with the use of a novel range of allosteric modulators. These ligands bind to a site distinct from that of the endogenous ligand, thereby activating novel pathways to modulate receptor signaling. Allosteric sites display a much higher sequence divergence compared to orthosteric sites, allowing for the design of highly receptor-specific ligands. Additionally, they provide a unique method to subtly modify receptor activity to varying degrees, allowing for a more fine-tuned control of signaling events[1–5].

Previous studies from our lab have developed novel allosteric ligands targeting the dopamine D2 receptor, based on the endogenous tripeptide, L-prolyl-L-leucyl-glycinamide (PLG)[6–19]. The D2 GPCR is expressed in various neuronal regions, including the striatum, nucleus accumbens and substantia nigra, and is highly implicated in human psychiatric and neurological disorders, including schizophrenia and Parkinson's disease[20–23]. The allosteric ligand demonstrating the highest preclinical efficacy in our studies is the conformationally constrained PLG peptidomimetic, 3(R)- [(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA) (**Figure 2.1**)[24,25]. At the preclinical level, PAOPA has been shown to be effective in attenuating behavioural abnormalities in rodent models of schizophrenia, including the amphetamine-sensitized and dizocilpine (MK-801)-sensitized disease models[26,27].

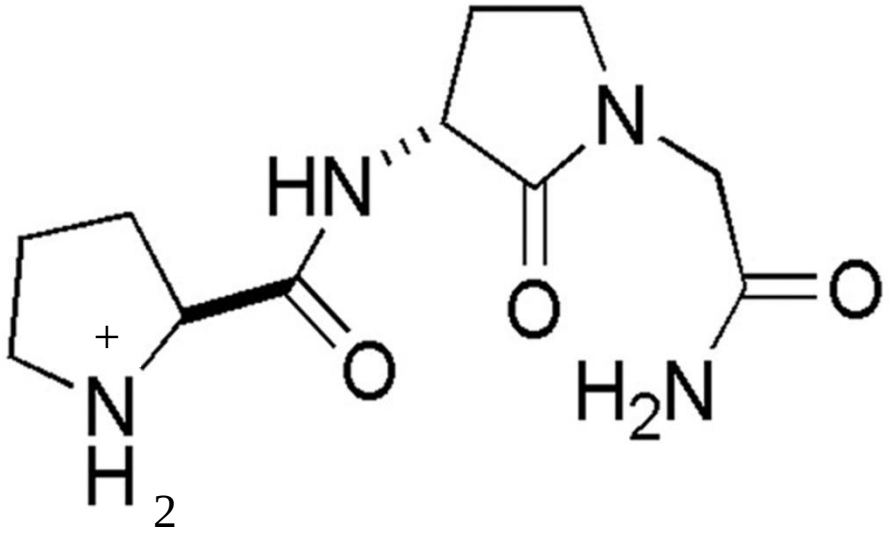

Figure 2.1: Chemical structure of the dopamine D2 receptor allosteric modulator 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA)

Previous studies have shown PAOPA to interact with a unique allosteric site on the dopamine D2 receptor, to facilitate agonist binding by increasing the proportion of D2 receptors in the high affinity state[24,28]. However, the precise mechanism of action for this drug is currently unknown.

GPCR trafficking and signaling is controlled by a number of key regulatory proteins, including G protein-coupled receptor kinases (GRK), arrestins, dynamin and clathrin. Receptor overstimulation by exposure to agonists leads to receptor phosphorylation by GRKs, followed by recruitment of arrestins, and could lead to downregulation in activity by receptor internalization. Previous studies have demonstrated the dominant-negative mutations of GRK2 to abolish any D2 receptor internalization, evincing the importance of this protein in the downregulation of this receptor[29]. Moreover, arrestin-3 appears to be the isoform which when knocked out in mice models, has shown to inhibit the internalization of D2 receptors in striatal cell cultures[30]. Internalized receptors can be recycled back to the plasma membrane, or targeted for lysosomal degradation. This process plays a key role in regulating the activity level, and downstream effects of crucial GPCRs.

Pathophysiological states, as well as chronic drug treatments, can alter the expression of regulatory GRK and arrestin proteins, which can significantly affect GPCR signaling and the resulting downstream effects[31–34]. Measurement of post-mortem cortical levels has shown decreased mRNA of GRKs and arrestins, and decreased GRK3 protein levels in patients suffering from schizophrenia[35]. Chronic treatment of schizophrenia with the widely prescribed antipsychotic drugs haloperidol and clozapine,

have also shown to have significant, regional-specific effects on GRK and arrestin expression[34]. These modulations can affect receptor trafficking and density, thereby having a significant effect on the downstream signaling pathways.

The objectives of this study were to examine changes in protein expression of D2 receptor-related regulatory proteins GRK2 and arrestin3 in the dopaminergic-rich region of the striatum, following chronic PAOPA treatment in rodents. To detect changes in downstream signaling associated with altered expression of the above regulatory proteins, this study also examined changes in phosphorylation levels of the effector molecule belonging to the mitogen-activated protein kinase (MAPK) family, specifically the extracellular signal regulated kinase (ERK) 1/2 molecule. Finally, this study also examined the effects of these expression changes on D2 receptor internalization in a cellular model. This study was able to demonstrate that chronic PAOPA treatment caused an *in vivo* increase in the striatal expression of GRK2, arrestin-3 and phospho-ERK1/ERK2, while also enhancing D2 receptor internalization in *in vitro* models. Understanding the chronic effects of PAOPA on these signaling proteins and on D2 receptor trafficking can provide a better understanding of its mechanism of action, as well as improve our knowledge on the etiology and future treatment of schizophrenia.

2.3 Materials and Methods:

2.3.1 Ethics statement:

All studies were approved by the McMaster Animal Research Ethics Board and were in compliance with the guidelines of the Canadian Council on Animal Care (Animal

utilization protocol # 10-08-59). All cell culture work was approved by the Presidential Biosafety Advisory Committee (Permit # 2011-079).

2.3.2 Animals, drug regimen and tissue collection:

Twelve adult male Sprague Dawley rats (250 g) were obtained from Charles River Laboratories (Wilmington, MA) and individually housed at the McMaster Central Animal Facility. Animals were maintained under constant temperature and humidity, with a reverse 12:12 light/ dark cycle, and *ad libitum* access to food and water. The dopamine D2 allosteric peptide PAOPA was synthesized in the laboratory of Dr. Rodney L. Johnson (University of Minnesota, MN) as described previously[25]. Fresh peptide solutions were prepared daily at a 1 mg/mL concentration. Rats (n= 6/group) were treated daily, for 45 days, with a chronic administration of PAOPA via intraperitoneal (I.P.) route at a dose of 1 mg/kg. One hour following the final drug administration, rats were anaesthetized with isofluorane, and sacrificed by decapitation. Rat brains were removed, the striatum and cerebellum dissected over ice, and stored at -80°C until use.

2.3.3 Western Immunoblotting:

Collected rat striata and cerebellum were homogenized using a pestle in phosphate-buffered saline (PBS) with Complete Mini, EDTA-free protease inhibitor tablet (Hoffmann La-Roche, Mississauga, ON) and PhosStop phosphatase inhibitor cocktail tablet (Hoffmann La-Roche, Mississauga, ON). Resulting homogenate was sonicated on ice three times, for 10 seconds each, and a Bradford protein assay was used to quantify

the sample protein concentration. 15 µg of sample was resuspended in 2X sodium dodecyl sulfate (SDS) sample buffer, and separated by electrophoresis on 10% or 12% acrylamide gels, using protocols as previously described by our lab[36]. Briefly, separated proteins were transferred to a 0.45 µM polyvinylidene (PVDF) membrane, blocked for 1 hour in 5% skim milk, and exposed to primary antibody overnight. Primary antibody-bound membranes were exposed to secondary antibody for 1.5 hours at room temperature, and visualized using enhanced chemiluminescence (ECL) substrates. GRK2 was detected using monoclonal anti-GRK2 antibody produced in rabbit (1:2000; Abcam, Cambridge, MA), and arrestin-3 was detected using monoclonal anti-ARRB2 antibody produced in mouse (1:1000; Sigma, St. Louis, MO). Phosphorylation of downstream molecule ERK1/2 was measured using polyclonal phospho-specific anti- p44/42 MAPK (ERK1/2) (Thr202/Tyr404) produced in rabbit (1:2000; Cell signaling, Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize changes in protein expression and was detected using anti-GAPDH antibody produced in mouse (1:10000; Millipore, Billerica, MA). The anti-rabbit exposed membranes were incubated with anti-rabbit IgG horseradish peroxidase (HRP)- linked whole secondary antibody from donkey (1:5000, Sigma Aldrich, St. Louis, MO), and anti-mouse exposed membranes were incubated with anti-mouse IgG HRP-linked whole secondary antibody from sheep (1:5000; Sigma Aldrich, St. Louis, MO).

2.3.4 Live cell microscope imaging:

A cellular model was kindly provided by Dr. J. Javitch (Columbia University, New York) to study D2 receptor internalization, with details provided in a previous paper[37]. Briefly, this model was a tetracycline-regulated expression cellular line (T-REx-293) (Invitrogen, Carlsbad, CA), expressing bovine GRK2 in pcDNA4/TO vector, rat arrestin-3 in pcDNA5/TO vector, and human D2 receptor with a FLAG epitope and fused with enhanced yellow fluorescent protein (eYFP) in pCIN4 vector (Invitrogen, Carlsbad, CA). The co-expression of GRK2 and arrestin-3 was to allow for the availability of adequate machinery in order to internalize the overexpressed D2/eYFP. Cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 5 mg/mL blasticidin. The positive stable expression in the T-REx-293 cellular system of all three proteins, including GRK2, arrestin-3 and D2/eYFP, was confirmed by immunoblotting in our lab (data not shown). To visualize D2 receptor internalization using live cell imaging, cells were seeded on 25 mm poly-lysine coated coverslips, and allowed to grow for 48 hours (Neuvitro, El Monte, CA). 24 hours prior to microscope visualization, cells were treated with 1 µg/mL tetracycline to induce the overexpression of GRK2 and arrestin-3. Images were acquired by a 63X glycerol immersion objective using a confocal microscope Leica DMI 6000B (Wetzlar, Germany), equipped with a Hamamatsu C9100-12 back-thinned EMCCD camera. To maintain cell viability, images were captured in a Neue LiveCell2 whole microscope chamber, which maintained a well-regulated environmental temperature of 37°C and CO_2 of 5%. Drug treatments included 30 µM quinpirole (Sigma, St. Louis, MO), and 10 µM PAOPA. These were diluted in

DMEM and perfused immediately at the start of visualization onto the cells, by mounting the coverslip with the cells in an Attoflour cell chamber (Invitrogen, Carslbad, CA). Cell images were acquired every 2 minutes for 30 minutes using the Volocity 4 acquisition software.

2.3.5 [^{3}H]-sulpiride binding assay:

[^{3}H]-sulpiride assays have previously been conducted by other labs as a method to quantify D2 receptor internalization in cellular models[29,38–40]. In this study, these assays were conducted by seeding the triple transfected T-REx-293 cells expressing GRK2, arrestin-3 and D2/eYFP, onto poly-lysine coated 24-well plates at a density of $2X10^5$ cells/well, and allowing them to grow for 48 hours. 24 hours prior to the assay, cells were induced to overexpress GRK2 and arrestin-3 by adding a final concentration of 1 µg/mL tetracycline. Cells were treated for 1.5 hours in DMEM media, with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.2 mM sodium metabisulfite. Treatments included combinations of 30 µM quinpirole (Sigma, St. Louis, MO), and/or PAOPA, as written in the results section. Drug treatments were ceased by rinsing the cells gently three times with ice-cold Earle's buffered saline solution (EBSS). Cells were then bound to 6 nM of [^{3}H]-sulpiride (PerkinElmer Life, Waltham, MA) diluted in EBSS, for 3.5 hours at 4°C. [^{3}H]-sulpiride is a D2 receptor-like selective antagonist, with maximal inhibition observed at D2 and D3 receptors [41,42]. Additionally, as a highly hydrophilic compound, sulpiride only interacts and tags membrane receptors, disregarding any cytoplasmic internalized receptor, thereby

allowing for a quantification of receptor internalization. Following radioligand binding, cells were once again rinsed three times with ice-cold EBSS, to remove any unbound radioactivity. Bound, intact cells were lifted using 1% Triton-X, placed into a scintillation vial with cocktail and counted for radioactivity using a scintillation counter Model LS5KTA (Beckman Coulter, Mississauga, ON). Percent D2 receptor internalization was calculated using the decays per minute (DPM) count, comparing control cells to the treated cells.

2.3.6 Data Analysis

Intensity of protein bands was quantified using ImageJ 1.43M (National Institutes of Health). Student's t-test was used to compare differences in protein expression between control and treated groups for immunoblot assays. One-way analysis of variance (ANOVA) was used in combination with Tukey's post-hoc analysis to compare differences between treatment groups for the [^{3}H]-sulpiride binding assays. An expression change was considered significant at $p < 0.05$. Data are presented as the percent change compared to control ± SEM.

2.4 RESULTS

2.4.1 Effect of PAOPA on the expression of GRK2 and arrestin-3 in the striatum:

GPCR signaling is regulated by key molecules, including GRKs and arrestins, the expressions of which can be affected by long-term ligand modulation[31–34]. To determine whether protracted treatment with PAOPA results in changes in the expression

of these molecules, rats were chronically treated with this D2 allosteric modulator. Chronic treatment of rodent models with the conformationally-constrained PLG peptidomimetic, PAOPA, caused a significant increase of GRK2 expression in the striatum, by 41%, and arrestin-3, by 34% **(Figure 2.2)**. There was no change in the expression of the housekeeping protein GAPDH, which was used to normalize the results (data not shown).

2.4.2 Effect of PAOPA on the expression of ERK1/2 in the striatum:

Ligands affecting GRK and arrestin protein expression have also been shown to have effects on the mitogen-activated protein kinase (MAPK) signaling pathway[43,44]. The activation of members of the MAPK pathways, including ERK1/2, is dependent on the phosphorylation of these proteins[45]. Therefore, only the activated levels of these proteins were measured in this study, using phospho-specific antibodies for the Thr202 and Tyr404 sites for ERK1 and ERK2 respectively. In this study, PAOPA was observed to have significant effects in activating ERK function within the striatum of treated animals, leading to an increase in levels of phospho-ERK1 by 51% and phospho-ERK2 by 36% **(Figure 2.3)**. Expression of the housekeeping GAPDH protein was again analyzed within the same samples to normalize the expression of ERK1/2, and was shown to not change with PAOPA treatment (data not shown).

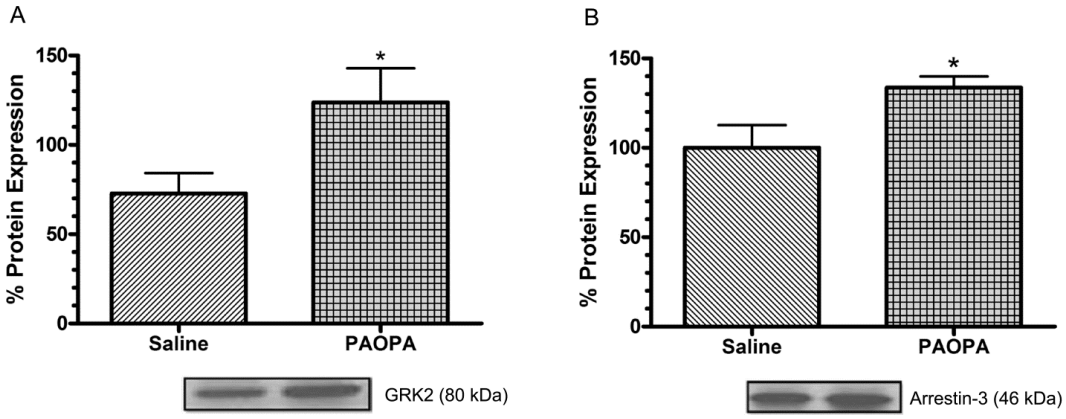

Figure 2.2. Effect of chronic PAOPA administration on expression of GPCR regulatory proteins in rat striatum. PAOPA (1mg/kg) was chronically administered to rats, and immunoblotting was used to quantify changes in expression of proteins involved in the downregulation process of the dopamine D2 receptor. Chronic PAOPA treatment increased the expression within the striatum of (A) G protein-coupled receptor kinase 2 (GRK2) by 41% and of (B) arrestin-3 by 34%. Data are shown with representative immunoblots, and expressed as a percentage of control ± SEM where *$p < 0.05$ (Student's t-test).

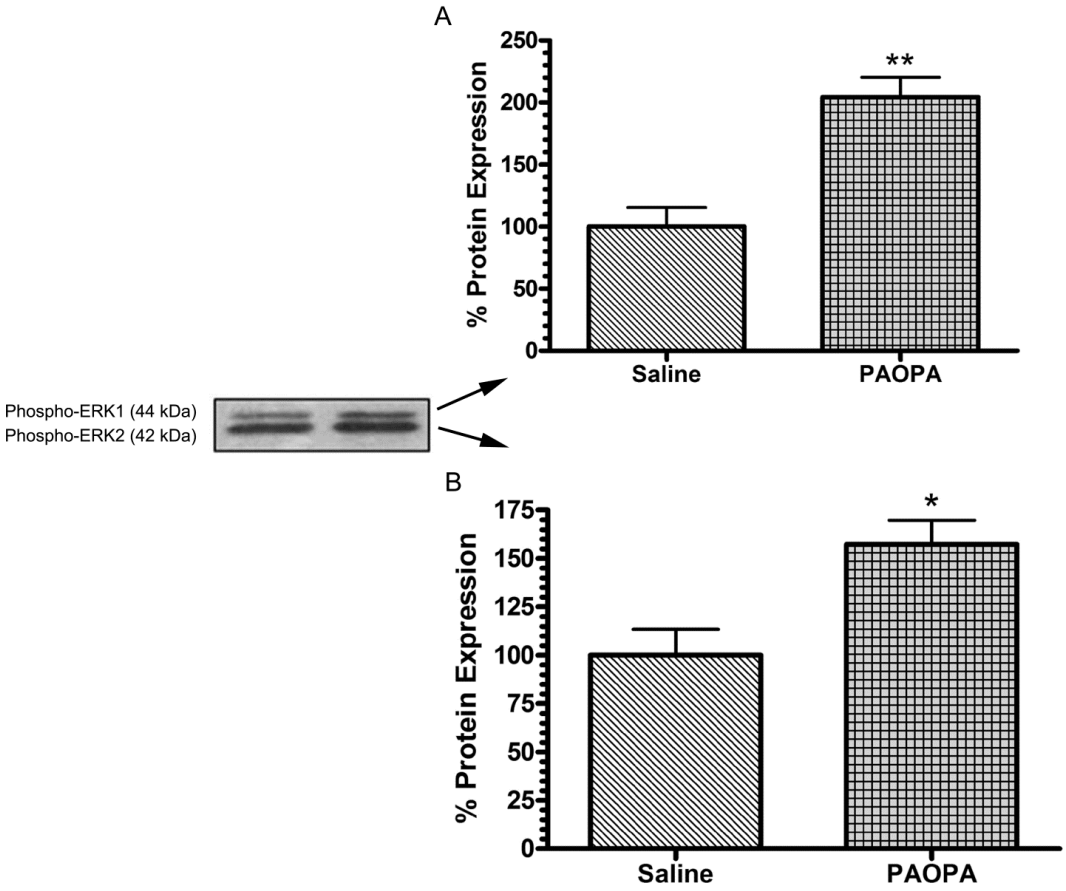

Figure 2.3. Effect of chronic PAOPA administration on expression of downstream molecules in rat striatum. PAOPA (1mg/kg) was chronically administered to rats, and immunoblotting was used to quantify changes in expression of dopamine D2 receptor-related downstream signaling proteins extracellular receptor kinase 1/2 (ERK1/2). Chronic PAOPA treatment increased the expression within the striatum of (A) ERK1 by 51% and of (B) ERK2 by 36%. Data are presented with representative immunoblots, and as a percentage of control ± SEM where *$p < 0.05$ and **$p<0.01$ (Student's t-test).

2.4.3 Effect of PAOPA on the expression of GRK2, arrestin-3 and ERK1/2 in the cerebellum:

Studies have often used the cerebellum as a region of reference to study D2 receptor-related effects, as this region is considered to have a negligible density of this receptor[46–48]. Thus, in order to relate the observed changes in protein expression in the striatum caused by PAOPA to modulation of the dopaminergic system, changes in protein expression of GRK2, arrestin-3 and phospho-ERK1/2 were measured in the cerebellum. Results, as shown in **Figure 2.4**, demonstrate that no significant alteration in the expression of these proteins was observed in the cerebellum of chronically treated rat models. These expression levels were also normalized to expression of the housekeeping protein GAPDH, which did not show any significant changes with PAOPA treatment (data not shown).

2.4.4 Effect of PAOPA on D2 receptor internalization in a cellular model:

GPCR stimulation by a ligand can often result in changes in receptor localization, leading to the movement of receptors from the cell membrane to internal cellular regions, causing a downregulation of overall activity. PAOPA acts as a positive allosteric modulator of the dopaminergic system, potentiating agonist binding to the D2 receptor, and therefore, could have an effect on the internalization of this receptor[24]. Cells triply transfected with GRK2, arrestin-3 and D2/eYFP, were treated with DMEM (control), PAOPA, a D2 agonist (quinpirole) or a combination of both PAOPA and quinpirole. Confocal microscopy results in **Figure 2.5A**, show the control cells (left panel) to express

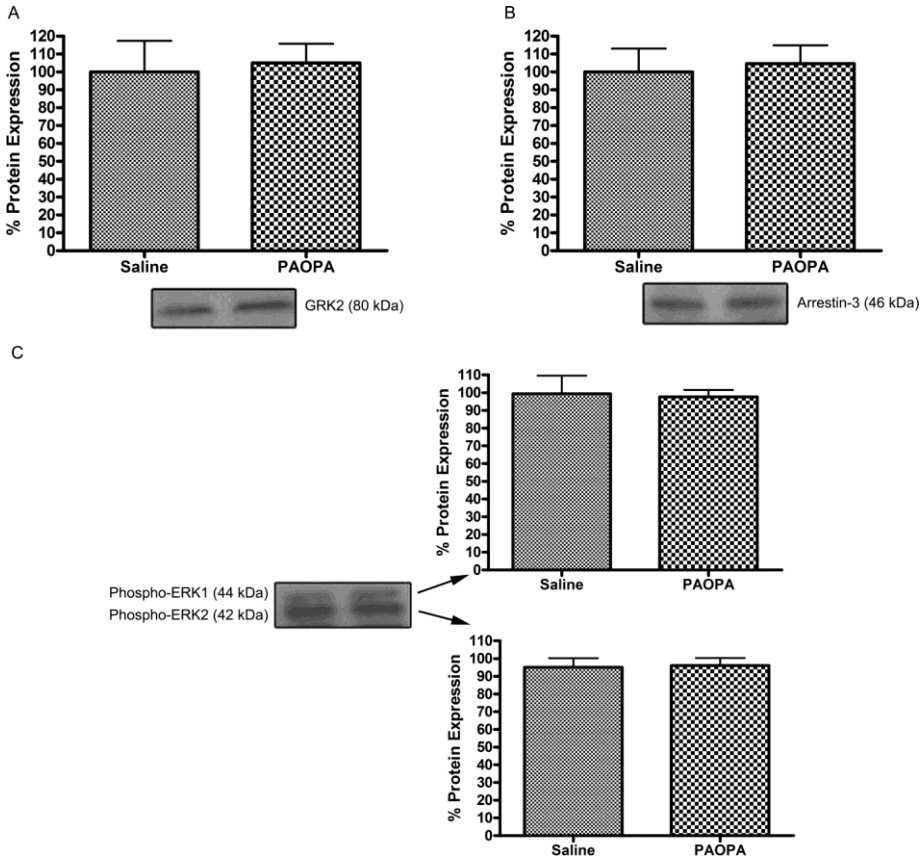

Figure 2.4. Effect of chronic PAOPA administration on expression of GPCR regulatory and downstream molecules in rat cerebellum. PAOPA (1 mg/kg) was chronically administered to rats, and immunoblotting was used to quantify changes in expression of dopamine D2 receptor-related signaling proteins G protein-coupled receptor kinase 2 (GRK2), arrestin-3 and extracellular receptor kinase 1/2 (ERK1/2). PAOPA treatment caused no significant differences in the expression within the cerebellum of (A) GRK2, (B) arrestin-3 and (C) ERK1/2. Data are presented with representative immunoblots, and as a percentage of control ± SEM.

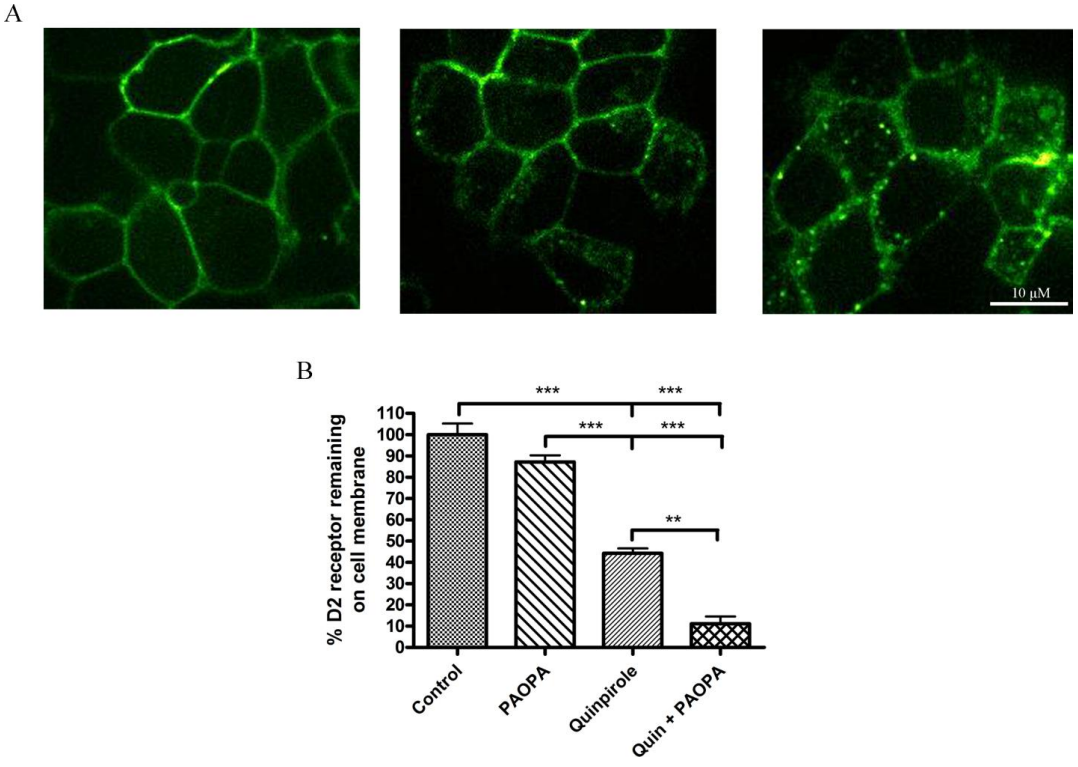

Figure 2.5. Effect of PAOPA on dopamine D2 receptor internalization in a cellular model. TREx-293 cells were stably transfected with D2/enhanced yellow fluorescence protein (eYFP), G protein-coupled receptor kinase 2 (GRK2) and arrestin-3. (A) Live cell confocal microscopy imaging of this cellular model treated as control (left panel), D2 agonist quinpirole (30 µM) (middle panel), and quinpirole with PAOPA (10 µM) (right panel) are shown. The green fluorescence represents the dopamine D2 receptor, which appears to be primarily present on the cell membrane in the control cells, and locating to intracellular regions with quinpirole, and quinpirole with PAOPA treatment. (B) [^{3}H]-sulpiride binding assays were used to quantify D2 receptor internalization, and this graph shows the percent of D2 receptors remaining on the cell membranes following given treatments of quinpirole (30 µM), PAOPA (10 µM) and quinpirole with PAOPA. Results show the addition of PAOPA to increase dopamine D2 receptor internalization by ~33%.

Data are presented as a percentage of control ± SEM where *** $p < 0.001$, ** $p < 0.01$ (one-way analysis of variance).

the D2/eYFP primarily on the cell membrane. Treatment with quinpirole (middle panel) illustrates D2/eYFP receptor internalization, with a certain amount of the green fluorescence appearing within the cytoplasmic region of the cell. Finally, the addition of PAOPA to the quinpirole treatment (right panel) shows increased levels of punctate D2/eYFP receptors located in the intracellular region, suggesting a possible enhancement in receptor internalization with the addition of the allosteric modulator, PAOPA. We found it challenging to quantify the level of D2/eYFP internalization, as no reliable software could be attained to automate the fluorescence counting separately for the membrane compared to the cytoplasmic region. Therefore, to supplement the visualization of D2 receptor internalization, quantification of D2 receptor internalization with PAOPA treatment was conducted using [^{3}H]-sulpiride assays. Results, displayed in **Figure 2.5B**, show that 1.5 hours following treatment, quinpirole causes ~56% internalization. The addition of PAOPA to quinpirole treatment increased internalization of the D2 receptor by ~33%, leading to ~89 % internalization. PAOPA administration by itself had no significant effect on internalization, as is expected, since an allosteric modulator is defined as being quiescent in the absence of its endogenous ligand. Overall, the addition of PAOPA to an agonist treatment potentiated the ability of this agonist to cause D2 receptor internalization.

2.5 DISCUSSION

This study was able to demonstrate that the chronic administration of PAOPA increases protein expression of GRK2, arrestin-3, and activates, by phosphorylation, the downstream molecules ERK1 and ERK2, within the striatum. Enhanced agonist stimulation of GPCRs, as occurs with chronic PAOPA administration, have previously shown to have unique effects on the expression of the cell's regulatory machinery of GRKs and arrestins, as well on downstream kinase molecules [49]. For example, chronic infusion with β-adrenergic agonist or antagonist leads to increases and decreases in levels of GRK2 mRNA and protein expression respectively[49]. Furthermore, previous studies have clearly demonstrated that agonist-induced activation of D2 receptors increases ERK1/2 phosphorylation, including within the striatum[50–52]. The observed increases in protein expression in our studies ranged from 34-51%. This level of change in GPCR regulatory proteins, though modest, can have functionally important effects on GPCR signaling, which can manifest as physiologically significant responses[53]. Studies in cellular systems have shown overexpression of either GRK2 or arrestin-3 to enhance agonist-induced phosphorylation and sequestration of the D2 receptor, which is further enhanced by co-expression of both GRK2 and arrestin-3[54]. Additionally, hemizygous knockouts expressing 50% less GRK proteins in mice models leads to phenotypes similar to complete knockouts[53]. Collectively, these studies show that chronic drug treatments can have an impact on the expression of GPCR trafficking and downstream molecules, which can result in significant phenotypic changes.

The current understanding of the GPCR trafficking pathway posits that chronic activation by ligand binding leads to phosphorylation of the receptor by a GRK, allowing for arrestin binding and attenuation of further G protein signaling[53]. Activation of the GRK/arrestin system can have dual effects on physiological systems. This system can suppress G protein signaling by promoting receptor desensitization, but can also enhance non-G protein signaling via the arrestin-3-dependent pathways[43,53]. Therefore, drugs which alter the expression of these regulatory proteins can not only suppress D2 receptor activity, but also activate novel potential pathways to modulate dopaminergic neurotransmission.

The changes observed in GRK2, arrestin-3 and ERK1/2 expression, elicited by chronic PAOPA administration, can involve quite complex mechanisms, engaging multiple pathways. In our studies, the exact pathway linking D2 receptor activation by PAOPA to the increases in ERK1/2 is yet to be determined. One suggested mechanism could involve the increased recruitment of arrestin-3 to activated receptors, leading to the formation of signaling scaffolds. These scaffolds bring in closer proximity Raf isoforms, which phosphorylate MEK within the scaffold, which in turn phosphorylates and activates ERK1/2[43]. Increased ERK1/2 levels can have a range of cellular effects. One particular study demonstrated the involvement of the ERK1/2 pathway in the upregulation of GRKs, via activation of Sp-1 and Ap-2 transcription factors in neuronal cells[55]. Increased arrestin-3 levels could also promote formation of another key scaffold complex, involving dephosphorylation and activation of Akt using PP2A. This pathway leads to activation of glycogen synthase kinase-3 (GSK-3) which has shown to be

significantly involved in neuropsychiatric disorders, including schizophrenia[56]. The observations of this study have yielded a few possible mechanisms of action of PAOPA, and opened avenues to improve the understanding of this allosteric modulator's actions in altering dopaminergic neurotransmission.

The effects of PAOPA observed in this study can almost certainly be associated to be occurring via allosteric effects on the D2 dopaminergic system. Previous *in vitro* and *in vivo* studies have shown the specificity of PAOPA for the D2 receptor, with non-significant effect of agonist binding on the D1 and D3 receptors, as well as the α2-adrenergic receptor [24]. Radioligand binding studies have shown that PAOPA modulates agonist binding to D2 without competing with dopamine binding, suggesting an allosteric interaction [24,25]. PAOPA also has no statistically significant effect on specific binding of D2 antagonists such as [^{3}H]-spiperone [24]. Additionally, in animal models (e.g.: 6-hydropdopamine (6-OHDA); vacuous chewing movement models) PAOPA has shown to potentiate the activity of D2 agonists, such as apomorphine [57,58], and attenuate the effects of D2-specific antagonist haloperidol [58]. Finally, the increases in the expression of GRK2, arrestin-3 and ERK1/2 observed in this study were seen to occur within the striatum of PAOPA-treated rats. No such significant changes were observed to occur in the cerebellum of the same treated rats, which is generally used as a negative control to study D2 receptor-related effects, as it is considered to be devoid of these receptors[46–48]. Together, this implies that the observed changes in striatal protein expression can almost certainly be attributed to PAOPA's modulatory effect on the D2 dopaminergic system.

The effect of PAOPA on the internalization of the D2 receptor, as demonstrated in the triple transfected cellular model, adds further insight into the mechanistic actions of this allosteric drug. Agonist stimulation of the D2 receptor by quinpirole caused internalization of the D2 receptor, which was further enhanced by addition of PAOPA to the agonist treatment, as shown both, by live cell imaging, and [^{3}H]-sulpiride assays. As a positive allosteric modulator, PAOPA increases agonist binding to the D2 receptor, by converting low affinity (GTP bound) receptors to high affinity (GDP bound) receptors, and therefore, is expected to induce increased D2 internalization, as observed in our results[24]. Furthermore, D2 receptor internalization can help explain the biphasic dose-response curve characteristic of PAOPA, whereby, this drug increases agonist binding at lower concentrations, and decreases it at higher concentrations[24]. The decrease in agonist binding at higher concentrations of PAOPA could be related to down-regulation in the overall availability of the D2 receptor due to internalization, providing a possible explanation for the therapeutic effect of PAOPA in the hyperdopaminergic state observed consistently in schizophrenia[23].

This study focuses on the GRK-mediated internalization of the D2 receptor following treatment with PAOPA, which is internalization via the homologous desensitization pathway. However, it is worthy to note that additional pathways exist for receptor downregulation, including heterologous desensitization. This process involves the broad desensitization of receptors in a cell, following prolonged activation of other GPCRs within that cell, and is mediated by protein kinase C (PKC) for the D2 receptor [59,60]. Heterologous desensitization results in non-specific downregulation of GPCRs,

whereas GRK-mediated desensitization is specific for the D2 receptor, leading this study to primarily focus on the latter [40,61]. However, subsequent studies in the future will also study heterologous desensitization in response to PAOPA treatment.

PAOPA has previously shown preclinical efficacy in the prevention and attenuation of schizophrenia-like behavioural abnormalities in rodent models of the disease[26,27]. As a potential new antipsychotic drug, the alterations caused by PAOPA in signaling pathways can be compared to that of drugs currently available in the market. For example, chronic treatment with atypical antipsychotic drug, olanzapine, enhances ERK activation in the prefrontal cortex, similar to our observations with PAOPA treatment in the striatum. Additionally, recent studies have shown a range of effects of atypical antipsychotic clozapine in a multitude of brain regions, including increases in GRK5, arrestin-2 and long-term activation of ERK2 in the caudal caudate putamen region of the striatum[34]. Clozapine has also been shown to activate levels of ERK1/2 in the prefrontal cortex region, by interaction with the epidermal growth factor (EGF) receptor[33]. This could have implications in regulating synaptic plasticity and connectivity, which are important physiological features dysregulated in schizophrenia[62,63].

In conclusion, this study has identified changes in the expression of key regulators involved in the activity of the dopamine D2 receptor in the striatum as a result of chronic treatment with the allosteric modulator, PAOPA. As hyperdopaminergia has been linked to certain symptoms of schizophrenia, this study brings to light the importance of understanding the effects of therapeutics on GPCR regulatory and downstream proteins.

Changes in the expression of these regulatory molecules can have profound effects on receptor availability, and overall neurotransmission, thus making them vital therapeutic targets to attenuate pathological receptor phenotypes in various GPCR-related disorders. By identifying the affected signalling pathways, this study develops PAOPA into a prospective new antipsychotic drug which can lead to improved treatment of schizophrenia.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DB YT JB JJ PH RLJ RKM. Performed the experiments: DB YT JB JJ PH RLJ RKM. Analyzed the data: DB YT JB JJ PH RLJ RKM. Contributed reagents/materials/analysis tools: DB YT JB JJ PH RLJ RKM. Wrote the manuscript: DB YT JB JJ PH RLJ RKM.

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CHAPTER THREE

Cell-free protein synthesis and purification of human dopamine D2L receptor

AUTHOR'S PREFACE:

The objective of this study was to express and purify the dopamine D2 receptor using a novel cell-free technique. The dopamine D2 receptor was initially attempted to be expressed in conventional cellular system, including bacterial, and mammalian cells. These methods were not successful in producing the D2 receptor, due to the synthesis of insoluble aggregates resulting in very low yields of the protein. This can be attributed to the highly hydrophobic nature of this transmembrane GPCR, which leads to increased cellular toxicity when overexpressed in natural systems. Therefore, to overcome this challenge, this study helped develop a cell-free technique to express the dopamine D2 receptor. Attaining purified D2 receptor was a key crucial step to progress understanding of the mechanism of action of the parent compound, PLG, as this preparation was to be used in the next chapter to elucidate the nature of the D2 allosteric site used for interaction with PLG.

I was responsible for study design, data collection and analysis, as well as writing of the manuscript. Jessica Castellano and Nancy Thomas provided invaluable support with the study design and data collection for this project. Dr. Mishra assisted with study design and data analysis.

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Title: Cell-Free Protein Synthesis and Purification of Human Dopamine D2L Receptor

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3.1 ABSTRACT:

The human dopamine D2L receptor has significant implications in neurological and neuropsychiatric disorders such as Parkinson's disease and schizophrenia. Detailed structural knowledge of this receptor is limited due to its highly hydrophobic nature, which leads to protein aggregation and host toxicity when expressed in cellular systems. The newly emerging field of cell-free protein expression presents numerous advantages to overcome these challenges. This system utilizes protein synthesis machinery and exogenous DNA to synthesize functional proteins outside of intact cells. This study utilizes two different cell-free systems for the synthesis of human dopamine D2L receptor. These include the *Escherichia coli* (*E.coli*) lysate-based system and the wheat germ lysate-based system. The bacterial cell-free method used pET 100/ D-TOPO vector to synthesize hexa-histidine tagged D2 receptor using a dialysis bag system; the resulting protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity resin. The wheat germ system used pEU-glutathione-S-transferase (GST) vector to synthesize GST tagged D2L receptor using a bi-layer translation method; the resulting protein was purified using a GST affinity resin. The presence and binding capacity of the synthesized D2L receptor was confirmed by immunoblotting and radioligand competition assays respectively. Additionally, in-gel protein sequencing via Nano LC-MS/MS was used to confirm protein synthesis via the wheat germ system. The results showed both systems to synthesize microgram quantities of the receptor. Improved expression of this highly challenging protein can improve research and understanding of the human dopamine D2L receptor.

Abbreviations: adenosine triphosphate (ATP), complementary DNA (cDNA), cell- free (CF), cytosine triphosphate (CTP), dopamine D2 receptor short isoform (D2S), dopamine D2 receptor long isoform (D2L), enhanced chemiluminesence (ECL), *Escherichia coli (E.coli)*, feeding mixture (FM), glutathione-S-transferase (GST), G-protein coupled receptors (GPCRs), guanosine triphosphate (GTP), horseradish peroxidase (HRP), McMaster Institute for Molecular Biology and Biotechnology (MOBIX), Molecular weight (MWT), nickel-nitrilotriacetic acid (Ni-NTA), norpropylapomorphine (NPA), polyethylene glycol (PEG), phosphate-buffered saline (PBS), polymerase chain reaction (PCR), polyvinylidene fluoride (PVDF), reaction mixture (RM), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Tris buffered saline with Tween (TBS-T), uridine triphosphate (UTP)

3.2 INTRODUCTION

G-protein coupled receptors (GPCRs) are a large and diverse superfamily of proteins crucial to eukaryotic cell signalling and implicated in cell development, proliferation and recognition. Significance of GPCRs to proper cell functioning has led this class of receptor to be highly exploited as a drug target. Currently, approximately 36% of market therapeutics are known to act on some class of GPCR.[1] However, the therapeutic potential of GPCRs has not been fully exploited due to the lack of detailed structural and functional knowledge of these proteins. This is in large due to the difficulties in expressing and purifying these receptors. Overexpression of highly hydrophobic GPCRs in heterologous cell-based systems often lead to cell toxicity, protein aggregation and misfolding, thus negatively affecting cell viability and protein expression. Additionally, functional GPCR production is often hindered by shortage of membrane space, and inadequate cell machinery required for membrane insertion and post-translational modifications.[2-4] These numerous hurdles help explain why detailed knowledge on the structure and function of hydrophobic GPCRs lags far behind that of soluble proteins.

Studies over the past decade have developed the technique of cell-free (CF) protein synthesis to overcome the challenges and limitations of cellular expression of membrane proteins.[5-15] A CF system utilizes protein synthesis machinery and exogenous DNA to synthesize functional proteins outside of intact cells. Such a system can be highly advantageous for expression of difficult integral membrane proteins, such as GPCRs, as cellular toxicity is no longer an issue.[16,17] CF systems allow for continuous replenishment of depleting precursors, and removal of toxic build up, thereby allowing for high yields of

GPCRs when compared to conventional cell- based methods.[18,19] This method can be further modified to incorporate radiolabeled-isotopes and unnatural amino acids in proteins.[20,21] Moreover, it can also be optimized to suit the synthesizing requirements of each unique GPCR with the addition of specific combination of solubilising detergents, co-factors, inhibitors etc. Such modifications are not possible to accomplish in cellular protein expression systems. Upon optimization of CF synthesis conditions, high levels of GPCRs can be produced within a few hours of incubation, thus significantly accelerating the protein expression process.[2,22,23]

A GPCR that presents challenges in protein expression and purification is the highly hydrophobic dopamine D2 receptor. This receptor is of significant importance in human psychiatric and mental health disorders. The dopamine D2 receptor exists as two isoforms, namely the presynaptic short isoform (D2S) and the postsynaptic long isoform (D2L), which differ by 29 amino acids at the third intracellular loop.[24] The involvement of the D2 receptor has been firmly linked to the etiology of devastating, yet poorly treated disorders, such as schizophrenia, Parkinson's disease and Tourette's syndrome[24-26]. Advancements in the development of safer, more effective drugs for these disorders are limited by the inadequate structural and functional knowledge of this receptor. Evidently, improved expression of pure human D2 receptor is crucial to expedite the process of receptor understanding, and improved drug development.

In this study, two CF protein synthesis systems, *Escherichia coli* (*E.coli*) lysate- and wheat germ lysate-based, were used to synthesize the human D2 receptor, specifically the postsynaptic D2L isoform. The production and purification of the receptor protein was

investigated using SDS-PAGE with western blot analysis, and confirmed using radioligand competition assays. For the wheat germ lysate-based system, in- gel protein sequencing via Nano LC-MS/MS also confirmed D2L protein synthesis.

3.3 MATERIALS AND METHODS

3.3.1 Cell- free Protein Expression with *E.coli* Lysate:

Chemicals:

Adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), uridine triphosphate (UTP), creatine phosphate and creatine kinase were purchased from Roche Diagnostics Canada (Mississauga, ON). Amino acids, including methionine, T7 RNA polymerase and *E.coli* S30 extract were purchased from Invitrogen (Carlsbad, CA). All other chemical reagents were purchased from Sigma-Aldrich (Oakville, ON).

Construction of Expression Vectors:

The complementary DNA (cDNA) for D2L (**NM_000795**) was obtained from Missouri S&T cDNA Resource Centre (Department of Biological Sciences of Missouri University of Science and Technology, Missouri). The genetic coding region of D2L was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (Invitrogen, Carlsbad, CA) and primers synthesized by McMaster Institute for Molecular Biology and Biotechnology (MOBIX), which incorporated a CACC sequence prior to the ATG start codon of the D2L gene. The primer sequences are provided in **Table 3.1**. The resulting

Table 3.1: Primer sequences for *E.coli* and wheat germ cell-free systems

Primer Sequences
Primer for E.coli cell-free system: Fwd: CACCATGGATCCACTGAA Rev: AGACTCGAGTCAGCAGTGGA
Primer for wheat germ cell-free system: Fwd: GCCCGCTCGAGATGGATCCACTGAATCTGT Rev: GCCGGCGGCCGCTCAGCAGTGGAGGATCTTC

PCR products were subcloned by ligation into linear vector pET100/ D-TOPO with a hexa-histidine tag (Invitrogen, Carlsbad, CA) and GTGG complementary sticky overhangs, as shown in **Figure 3.1**. Positive constructs with D2L gene were confirmed by DNA sequencing by MOBIX.

Cell- free Protein Expression:

The general design of the CF synthesis system is shown in **Figure 3.2**, and includes a reaction mixture (RM) and a feeding mixture (FM) separated by a dialysis membrane (25,000 Da molecular weight (MWT) cutoff, Spectra/Por, Rancho Dominguez, CA). The RM consisted of high and low MWT compounds, including the D2L plasmid DNA, and was the site of protein synthesis, whereas the FM consisted only of low MWT precursors used to replenish depleting components of the RM. This design allowed for high yield protein synthesis due to continuous supply of fresh substrates to the RM, as well as the removal of toxic product build up from the RM by passing through the membrane along the concentration gradient. The composition of the RM and FM are provided in **Tables 3.2** and **3.3** respectively. The reaction containing the RM and the FM was gently incubated for 8 hours at 30°C in a beaker of distilled water, and stirred continuously to prevent settling of the heavier reagents.

Protein Purification:

Following synthesis, CF expressed protein, was solubilised by adding 27.5 μL of 20% N-lauryl sarcosine. A nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Invitrogen,

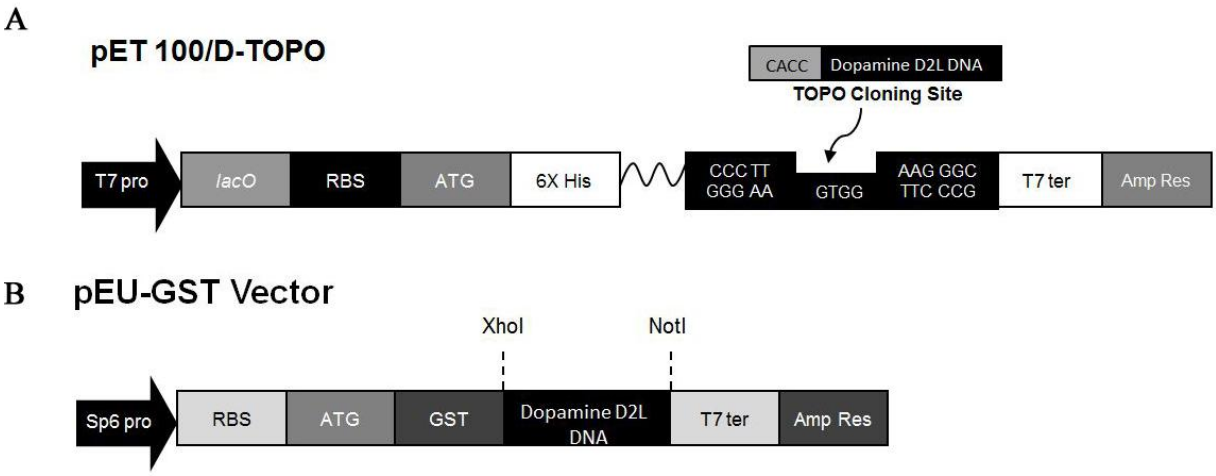


Figure 3.1: Vector diagram for constructs used in cell-free protein synthesis systems. **(A)** Hexa-histidine tagged pET100/D-TOPO vector used in *E.coli* lysate-based cell-free system (Invitrogen, Carlsbad, CA) **(B)** N-terminal GST-tagged pEU-GST vector used for wheat-germ cell-free system (CellFree Sciences Co., Ltd).

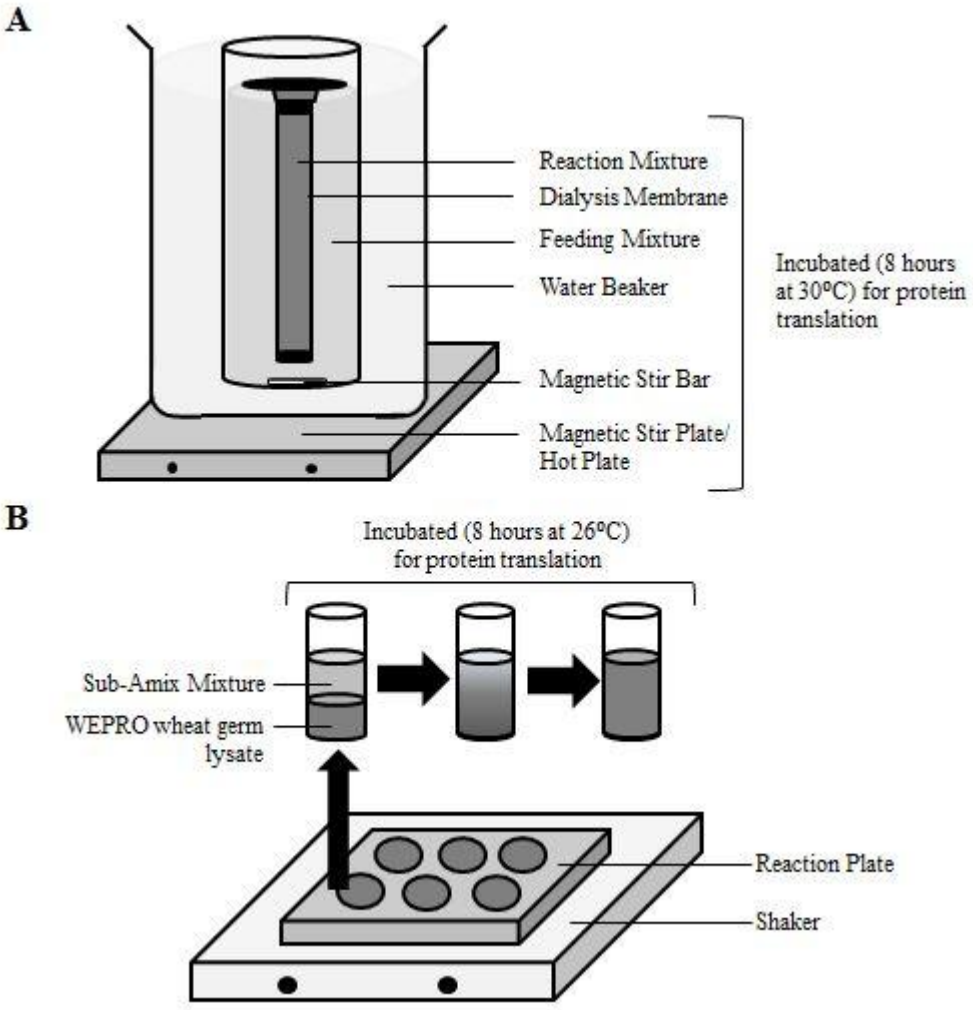


Figure 3.2: Cell-free synthesis setup diagram. **(A)** *E.coli* lysate-based cell- free system setup. **(B)** Wheat germ lysate-based cell-free system setup.

Table 3.2: Reagents for E.coli cell-free protein synthesis system (Reaction Mixture)

Chemicals	Volume	Final Concentration
1.6M Hepes-KOH (pH 7.5)	32.63 µl	58 mM
1M Dithiothreitol	2.07 µl	2.3 mM
100mM ATP	10.8 µl	1.2 mM
100mM CTP, GTP, UTP	8.1 µl/ each	0.9 mM/ each
1M creatine phosphate	72.9 µl	81 mM
20 mg/ ml creatine kinase	12.5 µl	250 ug/ml
40% polyethylene glycol 8000	90 µl	4%
5 mg/ml 3'-5' cyclic AMP	37.7 µl	0.64 mM
1mg/ml formyl 5,6,7,8 tetrahydrofolic acid	31.5 µl	35 ug/ml
10 mg/ml *E.Coli* tRNA	15.3 µl	170 ug/ml
3.33 M potassium glutamate	54 µl	200 mM
1 M ammonium acetate	24.93 µl	27.7 mM
1 M magnesium acetate	9.63 µl	10.7 mM
10% sodium azide	10 µl	0.05%
50 mM each amino acids (-Met)	27 µl	1.5 mM each
75 mM methionine	18 µl	1.5 mM
0.5 mg/ml D2 receptor DNA	46 µl	10 ug/ml
T7 RNA polymerase	20uL	66.7 ug/ml
E.coli S30 extract	300 µl	-

5% Brij 35	36 µl	0.2%
Final volume with nuclease free water	900 µL	

Table 3.3: Reagents for E.coli cell-free protein synthesis system (Feeding Mixture)

Chemicals	Volume	Final Concentration
1.6 M Hepes-KOH (pH 7.5)	362.5 µl	58 mM
1 M DTT	23 µl	2.3 mM
100 mM ATP	120 µl	1.2 mM
100 mM CTP, GTP, UTP	90 µl/ each	0.9 mM/ each
1 M creatine phosphate	810 µl	81 mM
40% polyethylene glycol 8000	1ml	4%
5 mg/ml 3'-5' cyclic AMP	420 µl	0.64 mM
1 mg/ml formyl 5,6,7,8 tetrahydrofolic acid	350 µl	35 ug/ml
10 mg/ml E.Coli tRNA	170 µl	170 ug/ml
3.33 M potassium glutamate	600 µl	200 mM
1 M ammonium acetate	277 µl	27.7 mM
1 M magnesium acetate	149 µl	14.9 mM
10% sodium azide	111 µl	0.05%
50 mM each amino acids (-Met)	300 µl	1.5 mM each
75 mM methionine	200 µl	1.5 mM
5% Brij 35	400 µl	0.2%
Final volume with nuclease free water	10 mL	

Protein Purification:

Following synthesis, CF expressed protein, was solubilised by adding 27.5 μL of 20% N-lauryl sarcosine. A nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Invitrogen, Carlsbad, CA) was used to purify the solubilised hexa-histidine tagged recombinant D2L expressed from pET100/D-TOPO vector. Following binding of the crude protein lysate to the column, washes were conducted twice with the following buffers respectively: denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8), denaturing wash buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.0) and another denaturing wash buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 5.3). Bound protein was then eluted with a denaturing elution buffer (8 M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4.0).

Protein Folding in Phospholipid Vesicles:

500 μL of a prepared phospholipid mix (1 phosphotidyl choline: 1 phosphotidyl serine: 1 phosphotidyl ethanol amine: 1 dipalmitoylphosphatidylcholine) (Sigma Aldrich, St. Louis, MO) was added to the purified D2L protein to promote proper folding. The phospholipid vesicles were synthesized by passing the mixture 10 times through a mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL). The presence of excessive detergents can disrupt folding of membrane proteins into their native conformations, and also St. Louis, MO) was added to the purified D2L protein to promote proper folding. The phospholipid vesicles were synthesized by passing the mixture 10 times through a mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL). The presence of excessive detergents

can disrupt folding of membrane proteins into their native conformations, and also interfere with downstream analysis.[10] Thus, excess detergent was removed from the solubilised D2L by dialyzing the CF synthesized protein in 1% Carbiosorb (Calbiochem, Mississauga, ON) dissolved in phosphate-buffered saline (PBS). This solution was continuously refreshed at 18, 32, 40 and 56 hours, and the resulting protein was resuspended in sodium dodecyl sulphate (SDS) buffer.

3.3.2 Cell- free Protein Expression with Wheat Germ Lysate

Construction of Expression Vectors:

The D2L protein synthesized using wheat germ lysate CF expression system was produced for Dr. Ram K. Mishra under license from CellFree Sciences Co., Ltd. (CFS) by Abnova (Taiwan) Corporation. Human D2L with a N-terminal glutathione-S-transferase (GST) tag was introduced into the pEU-GST vector (**Figure 3.1)**, using the XhoI and NotI restriction sites, using PCR primer sequences provided in **Table 3.1**.

Cell- free Protein Expression:

D2L-pEU mRNA was formed from the DNA by incubating components of the transcription solution (16 mM $Mg(CH_3COO)_2$, 5 mM NTP, 0.1 µg/ µL dopamine D2L DNA, 0.5 U/ µL RNase inhibitor, 0.5 U/ µL SP6 polymerase, 1X Transcription buffer) for 6 hours at 37°C in a thermal cycler. Subsequently, the transcribed solution was centrifuged at 4,000g for 20 minutes to remove white magnesium phosphate precipitate, and the supernatant was analyzed, by agarose gel electrophoresis, for mRNA quality, to

ensure lack of degradation. High quality D2L mRNA was used for the protein translation method.

D2L mRNA was used to synthesized D2L protein using a "bilayer" method.[23,27,28] A translation mixture, which included 120 OD/ mL of the WEPRO wheat-germ lysate (CellFree Sciences Co. Ltd., Yokohama, Japan), also included 250 µg dopamine D2L mRNA and 40 ng/ µL creatine kinase**.** 500 µL of this heavier translation mixture was layered below 5.5 mL of the lighter SUB-AMIX mixture (CellFree Sciences Co. Ltd., Yokohama, Japan) which contained ATP, GTP and complete set of 20 amino acids, to form a bilayer of translation mixture as shown in **Figure 3.2**. The bilayer design allows for the lighter waste byproducts to float to the upper substrate solution, and depleting precursors to move along the concentration gradient from the upper, to the lower translation solution, thus allowing for continuous protein synthesis. The bilayer translation was incubated in a 6-well plate for 8 hours at 30°C, and the resulting D2L protein was collected for purification.

Protein Purification:

Glutathione Sepharose 4B (GE Healthcare BioSciences, Baie d'Urfe, QC) was used to purify the GST tagged D2L wheat germ lysate CF synthesized protein. The sepharose was equilibrated with equilibration buffer (8.1 mM disodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate, 2.68 mM potassium chloride, 137 mM sodium chloride, pH 7.4), and loaded with crude lysate, and repeated 10 times with the flow through. The column was washed with 10 volumes of wash buffer (same as

equilibration buffer) and eluted with the elution buffer (50 mM Tris-HCl, 10 mM Glutathione, pH 8.0). Pure D2L was stored at -80°C for future protein expression analysis.

Immunoblotting

Coomassie staining and immunoblot of the prepared samples were performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described by our lab.[29] The anti-rabbit hexa-histidine primary antibody (Invitrogen, Carlsbad, CA) and anti-mouse D2L primary antibody (Abnova, Burlington, ON) were incubated at 1:5000 dilution, overnight at 4°C. Following primary antibody incubation, anti-rabbit exposed membranes were incubated with anti-rabbit IgG horseradish peroxidase (HRP) linked whole secondary antibody from donkey (Sigma Aldrich, St. Louis, MO) at a 1:5000 dilution, and anti-mouse exposed membranes were incubated with anti-mouse IgG HRP linked whole secondary antibody from sheep (Sigma Aldrich, St. Louis, MO) at a 1:5000 dilution for 1.5 hours at room temperature. Subsequently, all membranes were developed by incubation with enhanced chemiluminesence (ECL) reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and exposed to Kodak XAR film. Developed films were used for qualitative protein expression level analysis.

Radioligand Binding Assay:

Synthesized D2L protein was used for radioligand binding assays to investigate the binding capabilities of these receptors. The maximal amount of synthesized protein

was used for the assays, which for the bacterial system, was 19 μg for crude and 0.15 μg for pure fraction, and for the wheat germ system, was 1 μg pure. The D2L protein was incubated with 1 nM [^{3}H] norpropylapomorphine (NPA), or 1 nM spiperone, brought to a total volume of 250 μL with assay buffer (50 mM Tris- HCl pH 7.4, 5 mM MgCl2, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, Bacitracin 100 μg/ mL, Soybean trypsin 5 μg/ mL). Percent displacement was determined by calculating the ratio in radioactivity binding in the presence and absence of 1 μM dopamine, and expressed per μg of D2 protein. The tubes were incubated for 20 hours, at 4˚C with gentle rocking. Following incubation, 100 μL of 0.375% γ-globulin and 150 μL of 40% polyethylene glycol (PEG) were added to the assay tubes. Subsequently, the tubes were incubated at 4˚C for 15 minutes, followed by rapid filtration through a Brandel cell harvester (Brandel, Hertfordshire, UK) using 10% PEG filtration buffer. The radioactivity bound on filter paper disks were measured by placing them in plastic scintillation vials filled with scintillation fluid. Radioactivity counts were measured using a Beckman LS5000 liquid scintillation counter Model LS5KTA (Beckman Coulter, Mississauga, ON).

In- gel Protein Sequencing:

Wheat germ synthesized D2L protein was separated by SDS-PAGE and stained with Coomassie blue. Bands which superimposed to the D2 band, as seen on the immunoblot, was excised with a stainless steel surgical blade around the expected MWT and sent for sequencing by ProtTech Inc. (Norristown, PA). Briefly, the protocol involved

destaining, cleaning, and digesting proteins in- gel with sequencing grade modified trypsin (Promega, Madison, WI). The resulting peptide mixture was analyzed by a Finnigan ion trap mass spectrometer coupled to a high performance liquid chromatography system with a 75 µm inner diameter reverse phase C18 column. The mass spectrometric data acquired were used to search the most recent non-redundant protein database with ProtTech's proprietary ProtQuest software.

Data Analysis:

Radioligand competition assays were analyzed using GraphPad Prism 4.03 (GraphPad Software Inc. San Diego, CA). Statistical differences was calculated using Student's t-test to compare between control and experimental conditions. A change was considered significant when $p<0.05$. Data is presented as mean ± SEM from three independent experiments.

3.4 RESULTS

Synthesis of dopamine D2L receptor using E.coli cell- free system:

CF expression using an *E.coli* system showed successful D2L protein synthesis as analyzed by SDS-PAGE, and probed with anti-hexa-histidine antibody (**Figure 3.3A**).The immunoblots showed clear bands around the 47 kDa MWT, which is close to the 50.65 kDa predicted size, as calculated from the amino acid sequence. The purified CF synthesized lysate (second lane) contained the D2L protein, but to a lesser amount compared to the crude lysate (first lane) as a result of a loss of protein during the

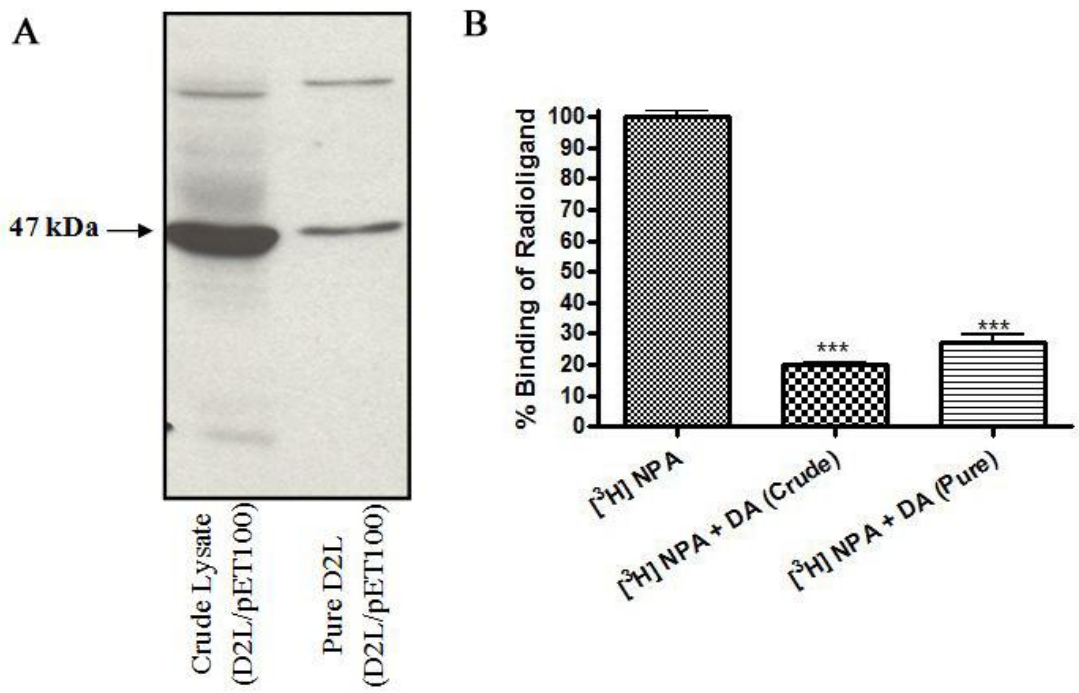


Figure 3.3: Confirmation of dopamine D2L receptor expression using *E.coli* lysate-based cell-free system. **(A)** Immunoblot using anti-hexa-histidine antibody to demonstrate expression of tagged dopamine D2L receptor in crude and purified lysate. **(B)** [^{3}H]-NPA/ dopamine (DA) radioligand competition assay of *E.coli* lysate cell-free synthesized D2L receptor using pET100/D-TOPO construct of crude and pure lysate.

purification process. The total amount D2L protein synthesized using the bacterial CF system, as measured by spectrophotometry, was ~17 µg/ mL reaction volume.

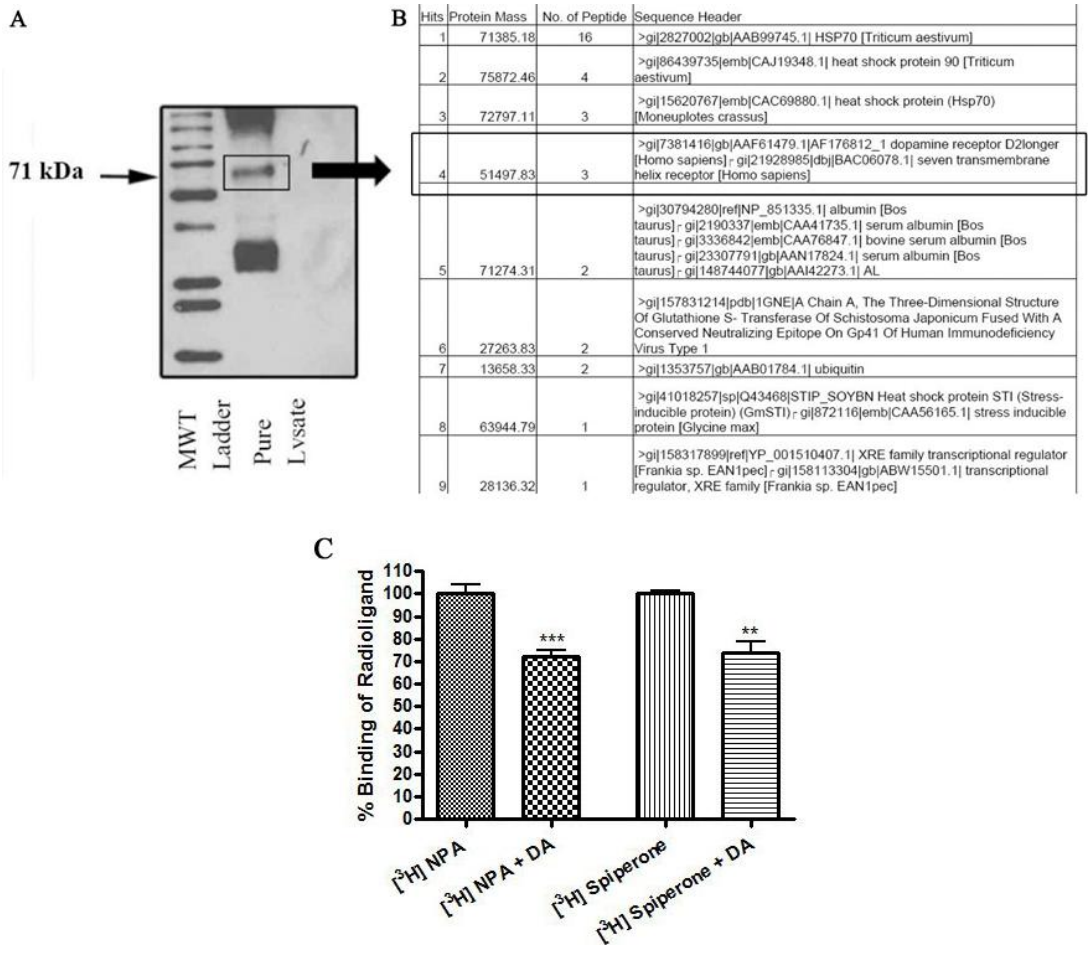
Ligand Binding Properties of E.coli Cell- free Synthesized Dopamine D2L Receptor:

To confirm the synthesis and purification of D2L receptor protein, in addition to immunoblotting, radioligand competition assays were also employed. These assays showed that both the crude and purified bacterial CF generated D2L protein, were capable of binding the receptor-specific agonist [^{3}H]-NPA, which was displaced by unlabelled dopamine. The % displacement, as shown in **Figure 3.3B**, was a highly significant 79.8% for the crude D2L and 76.9% for the purified D2L, demonstrating the ability of the receptor to clearly bind and displace ligands. This displacement can be used to estimate a yield of 0.83 µg/ mL (15 pmol D2L/ mg of total protein) for fully folded and functional D2L using the *E.coli* CF system.

Synthesis of Dopamine D2L Receptor using Wheat Germ Based Cell- free System:

Immunoblot of D2L synthesized using a wheat germ based CF system and purified using a 25 kDa GST tag showed a clear band at around 71 kDa, which is the similar to the expected 75 kDa size expected for this protein with a GST tag (**Figure 3.4A)**. The total amount of protein in the purified preparation as estimated by spectrophotometry was ~9.4 µg/ mL reaction volume.

The western blot for the purified CF D2L, also showed two other dense, non-specific bands around 30 and 150 kDa. Thus, in order to firmly confirm the



Hits	Protein Mass	No. of Peptide	Sequence Header
1	71385.18	16	>gi|2827002|gb|AAB99745.1| HSP70 [Triticum aestivum]
2	75872.46	4	>gi|86439735|emb|CAJ19348.1| heat shock protein 90 [Triticum aestivum]
3	72797.11	3	>gi|15620767|emb|CAC69880.1| heat shock protein (Hsp70) [Moneuplotes crassus]
4	51497.83	3	>gi|7381416|gb|AAF61479.1|AF176812_1 dopamine receptor D2longer [Homo sapiens] ┌ gi|21928985|dbj|BAC06078.1| seven transmembrane helix receptor [Homo sapiens]
5	71274.31	2	>gi|30794280|ref|NP_851335.1| albumin [Bos taurus] ┌ gi|2190337|emb|CAA41735.1| serum albumin [Bos taurus] ┌ gi|3336842|emb|CAA76847.1| bovine serum albumin [Bos taurus] ┌ gi|23307791|gb|AAN17824.1| serum albumin [Bos taurus] ┌ gi|148744077|gb|AAI42273.1| AL
6	27263.83	2	>gi|157831214|pdb|1GNE|A Chain A, The Three-Dimensional Structure Of Glutathione S- Transferase Of Schistosoma Japonicum Fused With A Conserved Neutralizing Epitope On Gp41 Of Human Immunodeficiency Virus Type 1
7	13658.33	2	>gi|1353757|gb|AAB01784.1| ubiquitin
8	63944.79	1	>gi|41018257|sp|Q43468|STIP_SOYBN Heat shock protein STI (Stress-inducible protein) (GmSTI) ┌ gi|872116|emb|CAA56165.1| stress inducible protein [Glycine max]
9	28136.32	1	>gi|158317899|ref|YP_001510407.1| XRE family transcriptional regulator [Frankia sp. EAN1pec] ┌ gi|158113304|gb|ABW15501.1| transcriptional regulator, XRE family [Frankia sp. EAN1pec]

Figure 3.4: Confirmation of dopamine D2 receptor expression using wheat germ lysate-based cell- free system. **(A)** Immunoblot using anti-D2 receptor antibody to confirm expression of synthesized and purified dopamine D2L receptor. **(B)** In- gel sequencing results of SDS-PAGE band corresponding to the D2L receptor, showing presence of this receptor **(C)** Radioligand competition assay of pure wheat germ lysate cell-free synthesized D2L receptor using dopamine (DA) competing with [^{3}H]-NPA or [^{3}H]-spiperone.

western blotting results, in-gel sequencing for this same preparation was also performed. The coomassie stain corresponding to the immunoblot in **Figure 3.4A** showed a faint band around the same size (data not shown). This band was excised and sequenced in- gel using mass spectrometry Nano LC-MS/MS by Prottech Inc. (Norristown, PA) as described under the "*Materials and Methods"* section. Sequencing detected the conclusive, direct evidence for the presence of peptides corresponding to the human D2L protein, as synthesized by the wheat-germ CF system (**Figure 3.4B)**.

Ligand Binding Properties of Wheat Germ Cell- free Synthesized Dopamine D2L Receptor:

Similar to the *E.coli* CF system, the wheat germ CF system synthesized D2L was studied for its ability to bind and displace ligands, using radioligand competition assays. The addition of dopamine caused a 27.8% displacement of the D2 specific agonist, [^{3}H]-NPA. To confirm this displacement, this assay was conducted using a D2 specific antagonist, [^{3}H] spiperone, which also showed a 26.0% displacement, with the addition of 1 μM dopamine. These results demonstrate the ability of the wheat germ synthesized protein to specifically bind and displace D2 receptor radioligands, suggesting proper receptor folding. Based on the results, the yield of fully functional and folded purified receptor can be calculated to be about 0.04 μg/ mL (0.4 pmol D2L/ mg of total protein).

3.5 DISCUSSION AND CONCLUSIONS

GPCRs have always provided a robust challenge for the field of protein expression, due to their hydrophobicity causing protein aggregation and host cell toxicity. To add to this challenge, each GPCR has a unique set of conditions for expression and purification, thus substantiating the need to investigate each GPCR individually.[30] Prior to the CF techniques used in this study, conventional methods were attempted in our laboratory to produce human D2L, including bacterial (*E.coli*) and mammalian (human embryonic kidney 293, and Chinese hamster ovarian) cellular systems. These techniques were unsuccessful, leading to synthesis of up to ~4 times lower quantities of insoluble protein, which could not be purified (data not shown). This demonstrated the limitations of cellular methods for expression of GPCR proteins, leading to our investigation into CF techniques for the expression and purification of human D2L.

Past studies have used *E.coli* CF systems to generate ~30 GPCRs including β2 adrenergic receptor,[10] muscarinic acetylcholine receptors,[8,10] neurotensin receptor,[10] melatonin receptor 1B,[12] and a variety of olfactory receptors,[5,7,8,15] and chemokine receptors.[8] In addition, *E. coli* 70S ribosomes have demonstrated to have a significantly faster rate of protein translation, which could result in the synthesis of high protein yields.[31] Due to these reasons, we chose the *E.coli* CF system for the synthesis of our GPCR of interest. The wheat germ system, on the other hand, was more recently developed, and investigations utilizing this system has mostly been targeted towards the production of soluble eukaryotic proteins, particularly enzymes and heat-shock proteins.[28] Eukaryotic protein translation systems have an advantage over prokaryotic systems, as

their 80S ribosomes produce proteins at a 10 times slower rate, and therefore are capable of synthesizing large eukaryotic proteins in properly folded, soluble forms.[31] Due to the eukaryotic nature of the wheat germ CF system, this method was also chosen to synthesize the human D2L.

The bacterial and wheat germ systems were both successful in synthesizing the D2L protein, which were purified using Ni-NTA and GST columns respectively. The bacterial CF reaction was continued for 8 hours, following the protocol by Ishihara *et al.*,[10] who were able to successfully synthesize neuroreceptors similar to the D2L within this timeframe. The wheat germ CF reaction was also continued for the same 8 hours, to allow for comparison of yields between the systems. Spectrophotometer measurement of the purified D2L protein showed that in 8 hours, the bacterial system was able to produce ~17 µg/ mL, which was higher than the wheat germ system yield of ~9.4 µg/ mL of pure D2L. This is as expected, since the bacterial ribosomal system has a higher rate of translation compared to the wheat germ system.[31]

There are key differences between *E.coli* and wheat germ translation mechanisms which affect the solubility and folding of the synthesized protein. Although, the higher rate of translation in bacterial systems can result in higher protein yields, large eukaryotic proteins expressed within these systems often form into aggregates, called inclusion bodies.[31] Therefore, proteins synthesized in *E.coli* CF systems are often solubilised in detergent during or after the reaction, and reconstituted post-translationally into proteoliposomes, to promote proper folding. [6,10,12] In CF reactions, the most compatible detergents are usually the polyoxyethylene derivatives, with Brij-35 being the most

commonly used detergent of choice, motivating our choice of this detergent for the synthesis of D2L.[5,12] Wheat germ systems, on the other hand, have a much slower 80S ribosomal unit, which synthesize protein at a rate of 3-5 peptide bonds per second. In this system, folding is co-translational, and occurs during protein synthesis, without requiring further post-translational solubilising or folding. In fact, the wheat germ ribosomal unit is significantly more efficient at folding protein into its native conformation compared to the bacterial wheat germ system,[32] eliminating the need for adding an extra refolding step to the CF synthesis protocol.[7]

Folding efficiency of our cell free systems were calculated by comparing the protein yield from spectrophotometry, to the yield from radioligand competition assays. Based on radioligand competition assays, the E.coli system generated 0.83 µg/ mL (15 pmol D2L/ mg of total protein) and the wheat germ synthesized 0.04 µg/ mL (0.3-0.5 pmol D2L/ mg of total protein), of properly folded protein. This is the actual amount of folded, functional protein, and shows the efficiency of refolding to be 5.0% for the bacterial system, and 0.4% for the wheat germ system. These refolding numbers are hard to compare between studies, as no other CF literature, to the best of our knowledge, present similar data comparing total yield and total yield of folded, functional protein. The yield of the bacterial system reconstituted in proteoliposomes appears to surpass that of the wheat germ system, for the synthesis of properly folded D2L receptor.

Finally, in addition to protein folding, another concern associated with CF expression is the possible lack of post-translational modifications and formation of higher order complexes, such as dimers.[27,33,34] In its native state, the D2L receptor is

glycosylated at the N-terminal and can homo- as well as hetero-dimerize.[35,36] Depending on the type of protein and expression system used, past studies have shown the ability of eukaryotic lysates to incorporate certain post-translational modifications such as glycosylations.[22] Based on the MWT, as observed on the immunoblot for the wheat germ system (**Figure 3.4A**), there was bands present at about twice the size of the D2L band at 71 kDa. This suggests possible formation of dimers, although it was not confirmed by in-gel sequencing. These modifications were not present in the bacterial CF system. However, the open nature advantage of these CF systems, allows for the addition of enzymatic and other forms of additives which could help with such modifications.

Past studies on D2 receptor expression and purification have been exceptionally limited. Studies conducted in the late eighties have reported extraction of D2 receptors from bovine striatal membranes.[37,38] Additional studies have focussed on the short isoform of the human D2 receptor, D2S. D2S constructs have been expressed in various yeast cell lines including *Pichia Pastoris*,[39] *Saccharomyces cerevisiae*[40] and *Schizosaccharomyces pombe*,[40] and two baculovirus insect cell lines including *Spodoptera frugiperda*[41] and *Trichoplusia ni*.[41] In all cases, protein expression was confirmed by SDS-PAGE with western blotting, combined with saturable radioligand binding assays, although no in- gel sequencing was performed. The amount of D2S receptor expression in relation to the total protein varied depending on the cell line used, and ranged from ~3-15 pmol/ mg of total protein. However, similar expression studies on the D2 receptor long isoform, D2L, have been significantly lacking. Our present study is one of the first to demonstrate the expression and purification of the human D2L protein.

The bacterial CF system utilized by our group was able to produce about 15 pmol/ mg, which is comparable, if not more, than the *in vivo* protein levels observed for D2S expression. Additionally, our CF system was much less time consuming and laborious, as the protein was synthesized in approximately 8 hours, with no need for cell line maintenance or transfection. The smaller reaction volumes used for studies can be scaled up to increase protein yield from µg to mg levels.[15,42-44]

The D2 is a GPCR with substantial implications in central nervous system disorders such as schizophrenia, Parkinson's disease, attention-deficit-hyperactivity disorder (ADHD), Tourette's syndrome and various addictions.[24] The antagonism of D2 receptors has been long accepted as the primary, most effective mechanism of action for antipsychotic drugs used in the treatment of schizophrenia, which is characterized by striatal hyperdopaminergic activity.[26,45,46] However, current methods of treatment lead to serious metabolic[47,48] and movement side effects, [49,50] thus warranting better understanding of this postsynaptic receptor. The three-dimensional structural knowledge of D2 receptor would significantly add to our understanding of ligand binding sites and allow for the design of highly discriminating, and therefore, safer therapeutics, targeting various central nervous system disorders. Such structural studies have, unfortunately, been largely unfeasible due to the inability of producing large enough quantities of the pure protein. Our study provides partial resolution of this situation by presenting novel methods to synthesize and purify the human D2L. The results of this study can be used as an avenue that leads towards better research, and improved drug design, for a receptor highly implicated in human mental health.

ACKNOWLEDGEMENTS

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CHAPTER FOUR

Expression of dopamine D2 receptor mini-proteins to determine putative binding site of allosteric modulator prolyl-leucyl glycinamide (PLG)

AUTHOR'S PREFACE:

As stated in Chapter three, a purified dopamine D2 receptor protein was attained, which was designed to be used as a tool to elucidate the exact nature of the PLG allosteric site. Briefly, the established protocol included binding the purified D2 receptor with PLG, followed by subsequent trypsin digestion and identification of the segment used for ligand binding, using mass spectrometry. However, these attempts at mass spectrometry were unsuccessful, as the detergent content to keep the D2 receptor solubilised during purification was too high to allow for clear separation of the peptides by mass spectrometry. Therefore, to overcome this challenge, a new approach was used to narrow down the amino acids composing the allosteric binding site. The objective of this chapter was to clone and express mini-proteins corresponding to parts of the full length D2 receptor, and conduct competitive ligand binding assays with each D2 mini-protein, to determine the allosteric binding site of the parent compound, PLG.

I was responsible for study design, data collection, analysis and writing of the manuscript. Mattea Tan provided remarkable support to the project with cloning of the genes for the mini-proteins. Similarly, Nancy Thomas assisted with study design as well as autoradiography data collection. Dr. Mishra provided support with study design and analysis. We are also grateful to Dr. Johnson for providing his expertise in this project, and discussing the feasibility of many of its aspects.

This manuscript is currently being prepared for submission, and we are presently in the process of determining an appropriate choice for a journal. We are expecting to have this ready for submission in the short few months to follow.

Title: Expression of dopamine D2 receptor mini-proteins to determine putative binding site of allosteric modulator prolyl-leucyl glycinamide (PLG)

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4.1 ABSTRACT

Allosteric modulators are an emerging concept in the field of drug discovery which present significant advantages over currently used orthosteric ligands. These ligands present a novel approach of precise, fine-tuned modulation of G-protein coupled receptors (GPCRs), which could provide an improved method of treating a vast range of pathophysiologies. A GPCR of particular interest is the dopamine D2 receptor, which has repeatedly shown implications in complex neuropathologies including Parkinson's disease and schizophrenia. Previous studies by our group have developed novel allosteric modulators targeting the D2 receptor based on the pharmacophore of the endogenous neuropeptide L-prolyl-L-leucyl-glycinamide (PLG). PLG, and its potent peptidomimetics, have shown robust preclinical efficacy in models of Parkinson's disease, tardive dyskinesia, depression and schizophrenia, making them excellent candidate for further research. The objective of this study was to elucidate the functional domain required for the allosteric interaction of PLG with the dopamine D2 receptor. PLG can be predicted as being 99% charged at a physiological pH of 7.4, and will not be able to cross the plasma membrane, leading to the hypothesis that the allosteric binding site most likely exists on extracellular loops or transmembrane regions exposed to the extracellular milieu. To investigate this, the full-length D2 receptor was expressed in human embryonic kidney (HEK293) cells as four smaller mini-proteins, and the specific binding capacity of each mini-protein was visualized using autoradiography with a radioiodinated PLG peptidomimetic. Results of this study demonstrate the most consistent specific binding to occur at D2 fragment 4, corresponding to amino acids 372-432 of the full length D2

receptor. Future studies will use homology-based computer modeling combined with site directed mutagenesis to confirm this region of the D2 receptor as an allosteric binding site of PLG.

Abbreviations: GPCR, G-protein coupled receptor; AM, allosteric modulator; PLG, L-prolyl-L-leucyl-glycinamide; 6-OHDA, 6-hydroxydopamine; K_D, dissociation constant; PCR, polymerase chain reaction; MOBIX, McMaster Institute for Molecular Biology and Biotechnology; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; HEK 293, human embryonic kidney cells 293; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescence; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid.

4.2 INTRODUCTION

Modulation of G-protein coupled receptors (GPCRs) using allosteric modulators (AM) is emerging as a highly effective method of altering receptor activity. These ligands bind to a site topographically distinct from that bound by the endogenous ligand, to subtly modify its receptor binding properties, or activate novel signaling pathways. As an AM, these ligands incur significant advantages over orthosteric ligands. AMs can be designed to be highly receptor-subtype specific, as allosteric sites exhibit much greater sequence divergence compared to orthosteric sites. Additionally, AMs can work with the natural physiological tone of a region, as they can be designed to be inactive by themselves, thus working primarily by enhancing or attenuating endogenous receptor activity in a fine-tuned, subtle manner (Christopoulos, 2002; Christopoulos & Kenakin, 2002; Conn et al., 2009; May et al., 2007).

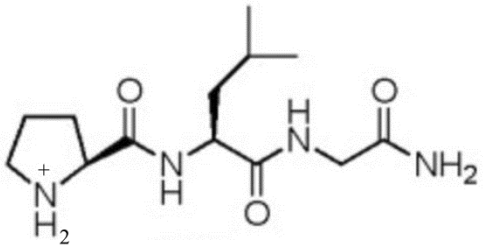
Previous studies from our lab have worked to develop novel AMs for the dopamine receptor. This receptor can be divided into a few subtypes (D1-D5). The D2 subtype, has been implicated in a range of neurological and neuropsychiatric disorders, including Parkinson's disease and schizophrenia (Seeman, 2010; Howes & Kapur, 2009). Past studies in our lab have developed AMs of the D2 receptor, including the tripeptide L-prolyl-L-leucyl-glycinamide (PLG). PLG has shown to be effective in the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease, suggesting its ability to attenuate the hypodopaminergic state of this disease (Ott et al., 1996; Mishra et al., 1997). PLG also antagonizes the antipsychotic (haloperidol) induced vacuous chewing movements in rats, a well accepted preclinical model of tardive dyskinesia (Castellano et

al., 2007). PLG has been used as a foundation to develop further peptidomimetics in the lab, with increased efficacy, or photoaffinity labeling properties. 3(R)- [(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide, PAOPA, has been shown to be the most potent analogue of PLG, and demonstrates antipsychotic properties in amphetamine and MK-801-sensitized preclinical animal models of schizophrenia (Beyaert et al., 2013; Dyck et al., 2011). Additionally, we have developed a photoaffinity peptidomimetic, analogue 157, which can be used as a scientific tool to improve our study of PLG (Mann et al., 2010). The structures of PLG and 157 are shown in **Figure 4.1.**

PLG and 157 have been shown to interact with the same allosteric site on the dopamine D2 receptor (Mann et al., 2010). PLG has been shown to modulate dopamine binding in a biphasic manner, enhancing dopamine binding at lower concentrations, and decreasing it at higher concentrations. Scatchard analysis of radioligand binding studies of agonist binding have shown PLG to significantly decrease the dissociation constant (K_D) while causing no significant changes in B_{max}, indicating this AM to have an effect by increasing the percentage of high affinity D2 receptors, but not the total number of receptors (Srivastava et al., 1988; Verma et al., 2005). Considering the therapeutic effect of PLG and its peptidomimetics, more studies are required to understand the mechanism of action of this AM.

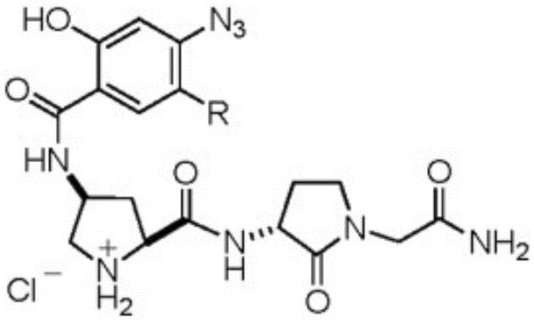
The objective of this study was to determine the putative allosteric site of interaction of PLG, on the dopamine D2 receptor. The pKa of PLG can be predicted, based on the known pKa values of other pyrrolidine-based compounds, to be around 9.4-10.0. Thus, at a physiological pH of 7.4, the Henderson-Hasselbach equation suggests

A



PLG

B



157

Figure 4.1: Chemical structure of **(A)** PLG and **(B)** 157

that approximately 99% of the tripeptide would be present in a protonated state as shown in **Figure 4.1**. Therefore, assuming there are no PLG transporters located on the cell membrane, this study hypothesizes the most likely binding site of the charged PLG molecule to be located on the extracellular or transmembrane regions of the dopamine D2 receptor, which are exposed to the extracellular matrix. This study therefore segments and expresses the dopamine D2 receptor into four regions, each representing different transmembrane and extracellular loops of the full receptor. Competitive ligand binding assays are conducted with these expressed mini-proteins, to determine the putative PLG interaction site.

4.3 MATERIALS AND METHODS

4.3.1 Cloning of dopamine D2 receptor mini-genes

The genetic coding region of each mini-protein was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (Invitrogen, Carlsbad, CA) and primers synthesized by McMaster Institute for Molecular Biology and Biotechnology (MOBIX). The primer sequences are provided in **Table 4.1**. The resulting PCR products were subcloned by ligation into pcDNA 3.1/ His C (Invitrogen, Carlsbad, CA) vector using EcoRI and XhoI restriction sites. Positive constructs were confirmed by DNA sequencing by MOBIX.

Table 4.1: Primer sequences for cloning of the vectors used to express the dopamine D2 receptor mini-proteins

MINI-PROTEIN FRAGMENT	PRIMER SEQUENCES
Fragment 1	*Fwd:* GAATTCATGGATCCACTGAATCTGTCCTG
	Rev: CTCGAGCTCGCGGGACACAGCCAT
Fragment 1+2	*Fwd:* GAATTCATGGATCCACTGAATCTGTCCTG
	Rev: CTCGAGTGTGTACCTGTCGATGCTGAT
Fragment 3	*Fwd:* GAATTCCGCCGGGTCACCGTC
	Rev: CTCGAGGTAGATCTTGATGTAGACCAGCAGG
Fragment 4	*Fwd*: GAATTCACTCAGATGCTCGCCATTG
	Rev: CTCGAGCTCAATGTTGAAGGTGGTGTAGAT

4.3.2 Expression of dopamine D2 receptor mini-genes

The dopamine D2 receptor mini-proteins were expressed as shown in **Figure 4.2**. This was done by transfecting the mini-genes in human embryonic kidney 293 (HEK293) cells with Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA). Cells were seeded and grown overnight in 100mm plates in Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Transfections were performed at 90% confluency, using 24 µg of recombinant vector DNA and 60 µL Lipofectamine 2000 diluted in 1.5 mL DMEM each. The DNA and Lipofectamine solution were combined and incubated for 20 mins at room temperature. 3 mL of this transfection solution was added to 15 mL DMEM per plate. Following 12 hours incubation (37°C, 5% CO_2), the transfection solution was removed, and replaced with complete media (DMEM, 10% FBS, 50 U/ mL pencillin, 50U/ mL streptomycin) to allow for the cells to recover, and continue to overexpress the transfected DNA. Cells were collected 48 hours following transfection, using citrate saline. Collected cells were pelleted at 2000xg for 5 mins, and resuspended in Tris-EDTA buffer with Complete Mini, EDTA-free protease inhibitor tablet (Hoffmann La-Roche, Mississauga, ON). Cells were lysed using the One shot model cell disrupter (Constant Systems, Kennesaw, Georgia, USA), thereby releasing the overexpressed dopamine D2 receptor mini-proteins, which were used for the rest of the study.

A

1 MDPLNLSWYD DDLERQNWSR PFNGSDGKAD RPHYNYYATL LTLLIAVIVF GNVLVCMAVS

61 REKALQTTTN YLIVSLAVAD LLVATLVMPW VVYLEVVGEW KFSRIHCDIF VTLDVMMCTA

121 SILNLCAISI DRYTAVAMPM LYNTRYSSKR RVTVMISIVW VLSFTISCPL LFGLNNADQN

181 ECIIANPAFV VYSSIVSFYV PFIVTLLVYI KIYIVLRRRR KRVNTKRSSR AFRAHLRAPL

241 KGNCTHPEDM KLCTVIMKSN GSFPVNRRRV EAARRAQELE MEMLSSTSPP ERTRYSPIPP

301 SHHQLTLPDP SHHGLHSTPD SPAKPEKNGH AKDHPKIAKI FEIQTMPNGK TRTSLKTMSR

361 RKLSQQKEKK ATQMLAIVLG VFIICWLPFF ITHILNIHCD CNIPPVLYSA FTWLGYVNSA

421 VNPIIYTTFN IEFRKAFLKI LHC

X = Fragment 1 X+X = Fragment 1+2 X = Fragment 3 X = Fragment 4

B

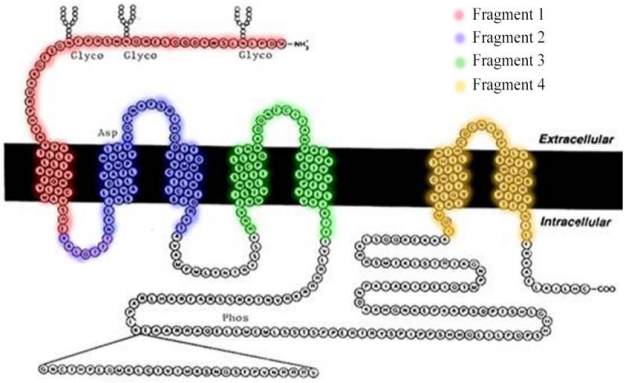


Figure 4.2: Mini-proteins designed for partial expression of the full-length dopamine D2 receptor. **(A)** Amino acid sequence of the dopamine D2 receptor (Accession #: AAB26274) with highlighted mini-protein fragments expressed in HEK293 cells. **(B)** Structure of the dopamine D2 receptor with the same mini-protein fragments highlighted as (A).

4.3.3 Western immunoblot analysis

The positive expression of the dopamine D2 receptor mini-proteins was confirmed using western immunoblot analysis. The concentration of the lysed protein was estimated using Bradford assay, and 40 µg of lysed protein was separated on a 16.5% Mini-Protean Tris-Tricine precast gel (Biorad, Mississauga, ON). Separated proteins were transferred to Immobilon-P^{SQ} 0.2 µM polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a wet transfer technique in a methanol transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol). Membranes bound to transferred proteins were blocked in 5% skim milk, and probed overnight at 4°C with anti-Xpress primary antibody (1:5000; Invitrogen, Carlsbad, CA), to recognize the eight amino acid Xpress tag (-Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys-) expressed by the recombinant proteins. Primary antibody bound membranes were exposed for 1.5 hours to anti-mouse IgG horseradish peroxidase linked whole secondary antibody from sheep (1:5000; Sigma Aldrich, St. Louis, MO), and visualized using enhanced chemiluminescence (ECL) substrates. Resulting chemiluminescence was developed on a CL-X Posure film (Thermo Scientific, Ottawa, ON).

4.3.4 Iodination of photoaffinity PLG peptidomimetic 157

Iodionation of the photoaffinity PLG peptidomimetic was conducted as previously described by our lab(Mann et al., 2010). Briefly, a C18 (18%) column was equilibrated with 5 mL methanol twice, and 5 mL distilled water twice. Two iodination beads (Pierce, Rockford, IL, USA) were washed twice for 5 minutes with 1 mL of phosphate buffered

saline (PBS). The washed iodination beads were incubated with 1 mCi of Na-[^{125}I] for 15 minutes in a reaction vial. One mg of the PLG peptidomimetic photoaffinity-labeling agent, 157, was added to the reaction vial and incubated for an added 15 minutes. Following incubation, the reaction was transferred to the equilibrated C18 column, and allowed to pass through. The column was washed five times using 1 mL distilled water, followed by a wash in 1 mL of 25% and 1 mL 50% methanol. The iodinated photoaffinity labeled agent, [^{125}I]-157, was collected by eluting the complex with 1 mL of 100% methanol. This eluted [^{125}I]-157 was collected and stored at 4°C and used for the experiments in this study.

4.3.5 Competitive ligand binding assay

50 µg of each dopamine D2 receptor mini-protein was individually incubated with 2 µM ^{125}I-157. Unlabelled 157 was used as the displacing agent for these assays. Reactions were incubated in a total of 250 µL reaction volume for 1 hour at 25°C in assay buffer (50 mMTris–HCl, 5 mMKCl, 4 mMMgCl2, 1 mM ethylenediaminetetraacetic acid (EDTA) and 120 mM NaCl (pH 7.6)) with or without the displacing agent. The binding complexes formed during the competitive ligand binding assay were covalently linked by exposing the samples to UV radiation for 5 mins. Samples were pelleted by centrifuging at 16,600xg for 30 mins to remove free radioactivity, and resuspended in 1X tricine sample buffer for autoradiography.

4.3.6 Autoradiography

Proteins were separated on a 16.5% Mini-Protean Tris-Tricine precast gel (Biorad, Hercules, CA). Radioactivity intensity was analyzed by exposing the gel to a phosphor-screen for 5 minutes, and scanning the screen using a Typhoon 9410 Molecular Imager (Molecular Dynamics, Ramsey, MN). The resulting image was analyzed for change in receptor bound radioactivity.

4.3.7 Data Analysis

The intensity of the radioactivity spots observed from the autoradiography was quantified using ImageJ 1.43M (National Institutes of Health). Data was presented as the percent change in spot intensity compared to control without the addition of the displacing agent, and expressed as percent change ± SEM.

4.4 RESULTS

4.4.1 Expression of dopamine D2 receptor mini-proteins

The cloned mini-genes representing segments of the D2 receptor at the transmembrane and extracellular regions, were transiently expressed in HEK293 cells, as shown in **Figure 4.2**. Fragment 2, the area between the second and third transmembrane region, was attempted to be expressed using a range of techniques, but with negative results (data not shown). This could possibly be due to the higher hydrophobicity of this fragment compared to the other fragments, which leads to increased cellular toxicity when overexpressed, resulting in lower protein yields. Therefore, the Fragment 2 segment

was expressed by being attached to Fragment 1, to decrease overall hydrophobicity. This resulted in Fragment 1 ranging from amino acids 1-62, fragment 1+2 ranging from amino acids 1-134, fragment 3 ranging from amino acids 150-213, and fragment 4 ranging from amino acids 372-432. The positive expression of these fragments in HEK293 cells was confirmed by immunoblotting, as shown in **Figure 4.3**. Fragment 1+2 and fragment 4 were the two mini-proteins that expressed within the system 48 hours following DNA transfection. The other two mini-proteins were expressed at both 24 and 48 hour following DNA transfection, with fragment 1 expressing more optimally at 24 hours, and fragment 3 expressing more optimally at 48 hours.

4.4.2 Autoradiography to elucidate putative PLG binding site

The positively expressed D2 receptor mini proteins were used for competitive ligand binding assays to determine which fragment was capable of specifically binding and displacing a radioiodinated peptidomimetic of PLG. Autoradiography was used to quantify bound radioactivity, and the results are shown in **Figure 4.4**. The results were repeated in three individual experiments, and show fragments 1, 1+2, and 3 to not have significant positive displacement with the addition of non-radioiodinated PLG peptidomimetic, 157. However, fragment 4, which corresponds to amino acids 372-432, showed consistent positive displacement of [^{125}I]-157 with non-radioiodinated 157. The results, therefore, suggest the amino acids composing the fragment 4 mini-protein as putatively having important sites required for PLG binding.

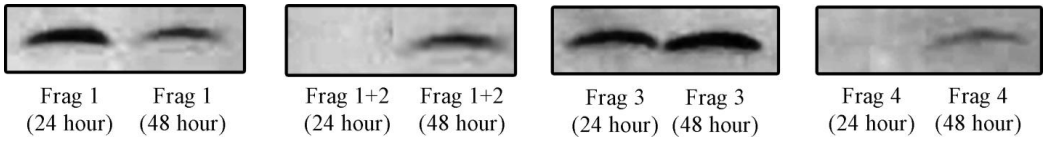


Figure 4.3: Expression of the dopamine D2 receptor mini-protein fragments. Immunoblot using anti-Xpress antibody (Invitrogen, Carlsbad, CA) to recognize the protein tag, confirming expression of the mini-proteins. The molecular weights of the expressed proteins are: fragment 1 (7.2 kDa), fragment 1+2 (15.25 kDa), fragment 3 (7.1 kDa) and fragment 4 (7.0 kDa).

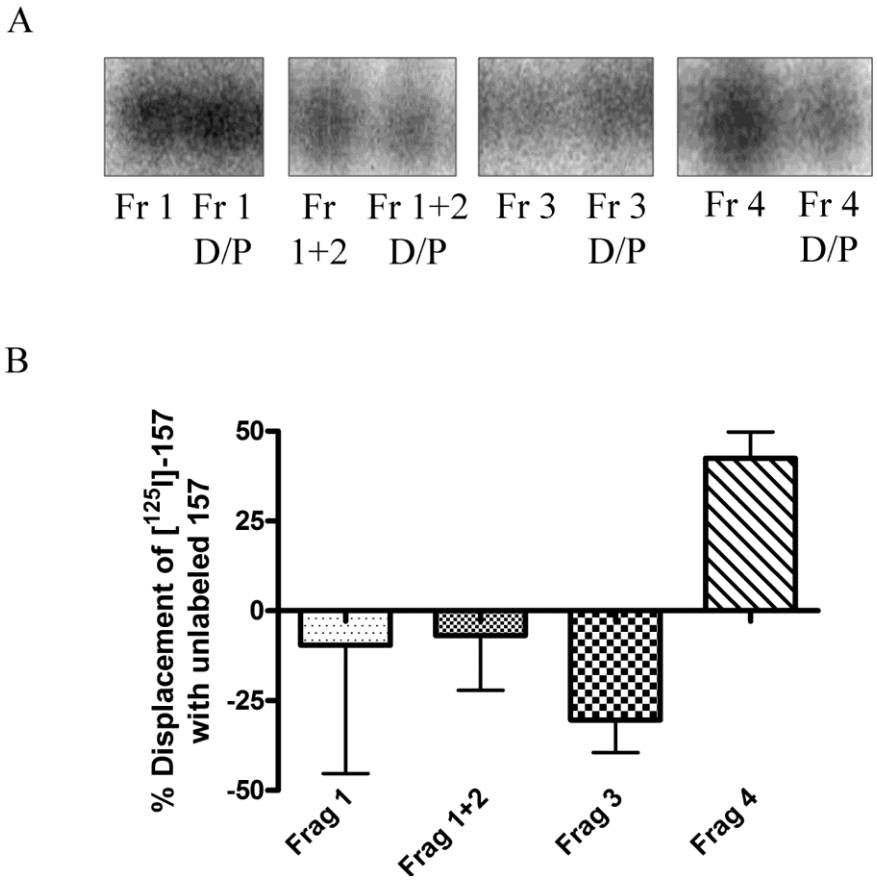


Figure 4.4: Autoradiography results of the competitive ligand binding assay of dopamine D2 receptor mini-proteins bound with PLG photoaffinity peptidomimetic, 157, and displaced with unlabeled 157 **(A)** Representative autoradiography spots of the fragment bound with [^{125}I]-157 (left panel in each box) and displaced (D/P) with unlabeled 157 (right panel in each box) **(B)** Displacement of [^{125}I]-157 with unlabeled 157 for each dopamine D2 receptor mini-protein, averaged over three individual experiments. Data are presented as a percentage of control ± SEM.

4.5 DISCUSSION

The preliminary data presented in this study shows the fragment 4 mini-protein, corresponding to amino acids 372-432 of the dopamine D2 receptor, to putatively bind the PLG peptidomimetic 157. Previously conducted competition assays from our lab have shown the photoaffinity peptidomimetic, 157, to have the same binding site as PLG (Mann et al., 2010). Therefore, fragment 4, seen as capable of specifically binding to 157, could be inferred as putatively also being the binding site of PLG. PLG could not be directly used within this study, as it cannot be radiolabeled as 157. This study presented a unique method to narrow down allosteric binding site of a GPCR, with the use of mini-proteins. The mini-proteins technique has been used by other studies to determine peptide-peptide interactions in a range of scenarios (Klammt et al., 2007; Agnati et al., 2006; Lee et al., 2007). This study, therefore, highlights an innovative solution which could help resolve allosteric interactions for a range of additional receptors.

There are several challenges associated with studying binding properties for hydrophobic, transmembrane proteins, such as the dopamine D2 receptor. A common emerging technique in elucidating binding sites combines computational modeling with site directed mutagenesis. Computer modeling efforts were initially based on the only resolved GPCR X-ray crystal structure of bacteriorhodopsin (Ballesteros et al., 2001; Costanzi et al., 2009). In the last five years, significant technical advances have been made in X-crystallography methods, allowing for the elucidation of additional 60 crystal structures of nine GPCR subtypes (rhodopsin, β2-adrenergic, β1-adrenergic, A2A–adrenergic, CXCR4, dopamine D3, histamine 1, M2 muscarinic and M3 muscarinic)

(reviewed in Jacobson & Costanzi, 2012). All these GPCRs belong to the Type A family, and have allowed for improved computer prediction of ligand binding sites for these receptors. For receptors, such as the D2 receptor, which do not have a solved crystal structure, prediction of ligand binding has proven to be more challenging. Studies have revealed that computer modeling can be more unpredictable in these cases, with predictions on the involvement of the extracellular binding loops proving to be more inaccurate, than predicting transmembrane interactions (Goldfeld et al., 2011; Costanzi, 2012). Therefore, despite the strong implications of the D2 receptor in the etiology of neuropsychiatric disorders, designing ligands targeting its allosteric site has always presented a strong challenge.

Improved computational capabilities and X-ray crystallography techniques will eventually allow for more efficient virtual screening of GPCR specific ligands. In fact, studies have been showing increased accuracy when modeling for unresolved receptor binding interactions, if these predictions are based on a crystallized structure with high sequence homology (Jacobson & Costanzi, 2012). Though the crystal structure of the D2 receptor has not been resolved, that of the D3 receptor has very recently been determined (Chien et al., 2010). The D3 has a fairly high homology to the D2 receptor of about 52%, which increases to 78% if only transmembrane regions are compared (Civelli et al., 1993; Sibley & Monsma, Jr., 1992; Levant, 1997). The D2 and D3 receptors share a range of similarities, including the same orthosteric ligand binding site, and both receptors exert an inhibitory effect on the downstream activity of adenylyl cyclase (Ahlgren-Beckendorf & Levant, 2004). Therefore, future studies to progress the understanding of the PLG

allosteric binding site will utilize the known dopamine D3 crystal structure to model possible points of functional interaction with the D2 receptor, using methods outlined in a recent study (Platania et al., 2012). This homology-based modeling will be combined with site directed mutagenesis at likely sites, including at fragment 4, followed by radioligand binding assays and functional assays to observe the effects of the mutations on PLG's allosteric activity. Finally, future studies will also directly sequence the binding of fragment 4 to peptidomimetic 157, using the mass spectrometry technique of Nano liquid chromatography mass spectrometry.

It is currently feasible for medicinal chemists to target amino acid residues near the pharmacophore of a ligand, to design an improved ligand with greater efficacy and affinity (Congreve et al., 2012; Deflorian et al., 2012). This makes identification of allosteric binding sites a crucial step in developing improved GPCR-targeting ligands. Despite its strong implications, determining such sites have proven to be very challenging, and studies are just currently emerging, which can help advance this field of drug discovery. The results of this study provide a contribution to the determination of the nature of a D2 allosteric site which can be modulated to have preclinical effects in attenuating behavioural abnormalities associated with schizophrenia. Elucidation of the PLG binding site could lead to the eventual design of novel antipsychotic drugs for the improved treatment of the millions suffering worldwide.

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CHAPTER FIVE

DISCUSSION

The collection of studies presented here advance our understanding of the mechanisms of action of novel allosteric modulators of the dopamine D2 receptor, including the parent compound PLG, and its peptidomimetic, PAOPA. These studies help develop original methodologies which can be used to study the diverse and challenging nature of allosteric modulators, whether they target the D2 receptor, or other physiologically crucial GPCRs. As mentioned above, PLG and PAOPA have both demonstrated efficacy in attenuating symptoms of Parkinson's disease, depression, tardive dyskinesia. Additionally, PAOPA, as a more potent peptidomimetic of PLG, has most interestingly shown robust preclinical efficacy in models of schizophrenia. To improve the understanding of PAOPA's mechanism of action, the downstream *in vivo* effects of this ligand on GPCR regulatory molecules, as well as its *in vitro* effects on D2 receptor internalization, was investigated in the study entitled "*Effects of Dopamine D2 Allosteric Modulator, PAOPA, on the Expression of GRK2, Arrestin-3, ERK1/2, and on Receptor Internalization".* To further progress our understanding of the nature of modulation elicited by PLG and PAOPA, the binding site of the parent compound, PLG, to the dopamine D2 receptor was investigated. A cell-free protocol was adopted to circumvent the *in vitro* challenges of expressing the D2 receptor using conventional cellular techniques, which resulted in publishing of the study "*Cell-free protein synthesis and purification of human dopamine D2L receptor"*. The purified D2 receptor from the cell-free system was initially employed to determine the nature of the PLG allosteric binding site. Technical limitations resulted in a shift in protocol, to the design and

expression of short D2 receptor mini-proteins, which helped narrow down the allosteric binding site, resulting in the study entitled "*Expression of dopamine D2 receptor mini-proteins to determine putative binding site of allosteric modulator prolyl-leucyl glycinamide (PLG)".* Overall, the key studies presented within this body of work highlight original approaches which can be employed to improve understanding of allosteric modulators, and have significantly progressed the elucidation of PLG and PAOPA's mechanism of action.

5.1 Effects of PAOPA on the Expression of GRK2, Arrestin-3, ERK1/2, and on Receptor Internalization

5.1.1 Global Significance

GPCRs, specifically D2 receptors, are involved in a vast range of human pathophysiologies including schizophrenia, Parkinson's disease, depression and ADHD (Missale et al., 1998; Beaulieu & Gainetdinov, 2011). Research of methods to regulate the activity of these GPCRs can provide novel approaches on improving the treatment of these complex, and poorly treated D2 related disorders. A prominent method of D2 regulation involves internalization of these receptors subsequent to prolonged agonist stimulation, a process which is tightly controlled by proteins such as GRK2 and arrestin-3 (Ito et al., 1999; Kim et al., 2001). Changes in these regulatory proteins can have significant phenotypic consequences (Premont & Gainetdinov, 2007). In fact, recent understanding suggests that targeting downstream signaling molecules, including ones

involved in GPCR regulation, can provide more subtle receptor modulation, allowing for the design of therapeutics with improved safety profiles (Beaulieu & Gainetdinov, 2011). Additionally, studies have also revealed the many advantages of using allosteric modulators, in contrast to orthosteric modulators, to target GPCRs (Conn et al., 2009; May et al., 2007). These novel developing concepts reveal the strong significance of the work presented here, since PAOPA, as an allosteric modulator which targets regulatory proteins in the D2 pathway, can provide the more subtle alternative to current orthosteric site drugs used to treat schizophrenia. This can lead to significant improvements in incidences of movement and metabolic adverse events, which are strongly associated with current APDs, eventually leading to improved patience compliance, symptom management, and overall quality of life.

5.1.2 FUTURE STUDIES

This study entitled *"Effects of Dopamine D2 Allosteric Modulator, PAOPA, on the Expression of GRK2, Arrestin-3, ERK1/2, and on Receptor Internalization"* has opened up vast avenues to future research which can be used to highlight the mechanisms of action of D2 allosteric modulators. Firstly, it is worthy to note, that although this study focused on PAOPA's effects on GPCR regulatory and downstream molecules, as well as on D2 internalization, similar results have also been obtained by our lab, for the parent allosteric modulator compound, PLG (unpublished). However, as PAOPA was the preclinically relevant and more potent compound, this particular ligand shall remain the focus for future studies.

As mentioned above, this study investigated the effects of PAOPA on GRK2 and arrestin-3, two prominently involved molecules within the D2 regulatory system. Future studies can progress to understand the effects of PAOPA on additional neuronal GRK and arrestin subtypes including GRK3-6 and arrestin-2. Previous studies have shown the majority of these regulatory molecules to not be significantly involved in D2 regulation, although future studies may present different results (Premont & Gainetdinov, 2007). An exception to this finding is GRK6, which has shown to have effects on D2 receptor internalization (Gainetdinov et al., 2003; Premont & Gainetdinov, 2007). In addition to GRKs, RGS molecules, specifically RGS-9-2 subtype, have also shown importance in regulating the activity of the D2 receptor (Rahman et al., 2003). Therefore, future studies will investigate the role of PAOPA on all GRKs, arrestins, and RGS molecules, but with particular focus on the GRK6 and RGS9-2 and subtypes.

In addition to examining the effects of PAOPA on additional GRKs and arrestins, future studies can also investigate the effects of this D2 allosteric modulator on additional brain regions, besides the studied striatum and cerebellum. The internalization related proteins were primarily measured in the striatum, a D2 rich region highly implicated as having hyperdopaminergic activity in schizophrenia (Howes & Kapur, 2009). Since PAOPA has shown to be highly interactive at the D2 site, as well as preclinically efficacious in schizophrenia, our generated hypothesis was to test for D2 internalization related effects within the striatum, as a method to decrease the hyperdopaminergia observed within this region. In addition to the striatum, protein levels were also measured in the cerebellum, which expresses negligible quantities of the D2 receptor (Suhara et al.,

1999), as a method to associate the observed striatal protein changes to the D2 system. Future studies will advance these findings by measuring the effects of PAOPA in other neuronal regions which have repeatedly been implicated in the etiology of schizophrenia, particularly cortical regions and the nucleus accumbens, which have shown dopaminergic dysregulation in this disorder (Howes & Kapur, 2009).

Subsequent to these large scale studies which can better map out the global neuronal effects of PAOPA, future studies should also include more detailed investigation on the exact pathways utilized by PAOPA to increase levels of GRK2, arrestin-3 and ERK1/2. One possible hypothesis which should be the focus of future investigations is the formation of arrestin-mediated scaffolds. These scaffolds mediated by arrestin-3, bring in closer proximity, and activates, a range of downstream molecules, including ERK 1/2 (DeWire et al., 2007). Arrestin-3 scaffolds can also activate glycogen synthase kinase 3β (GSK3β) molecule, which has shown significant associations with D2-related behaviours, and will also be studied in future investigations (Beaulieu et al., 2009; Freyberg et al., 2010). Additionally, further studies could investigate the effects of PAOPA on D2 oligomerization states, as theses states are emerging as being implicated in the etiology of schizophrenia (Perreault et al., 2011). Finally, it will be important for future studies to delineate the difference in effect of PAOPA on D2S, the short isoform, compared to D2L, the long isoform.

The results of this study provide ample evidence that create a framework implicating PAOPA's effects to be primarily on the dopaminergic system. This is based on *in vitro* evidence from radioligand binding assays which have shown PAOPA to have

the strongest pharmacological effect on the D2 receptor (Verma et al., 2005; Yu et al., 1988). The observed effects of PAOPA were in the striatum, which is primarily composed of the D2 receptor. Additionally, PAOPA has shown to be effective *in vivo* in drug-induced models which clearly have dopaminergic effects, including a vacuous chewing model induced by haloperidol, a D2 specific antagonist (Castellano et al., 2007), and a schizophrenia model induced by amphetamine, a dopamine releasing drug (Beyaert et al., 2013). From these evidence, it can be convincingly inferred that PAOPA's effects on the downstream signaling molecules GRK2, arrestin-3 and ERK1/2, are more than likely occurring by interaction with the dopaminergic, specifically, the D2 site. However, to get direct evidence of this inference, the effect of knock-down/out studies and the effect of blocking pathways using antagonists, could be conducted. It is worthy to note that the above stated study, although demonstrating the effects of PAOPA on GPCR regulatory molecules, and D2 internalization, has yet to clarify the extent to which these changes in protein levels and receptor availability are responsible for the therapeutic effects of PAOPA in preclinical models of schizophrenia. Antagonist and knockdown/out studies will allow for clarification of this unknown. D2 receptor, as well as arrestin-3 knockout mice, have previously been established by other studies and could be used to delineate the downstream effects of PAOPA (Klinker et al., 2013; Skinbjerg et al., 2009a). In this case, PAOPA would be administered to the knockout mice, and changes in the GPCR regulatory and downstream proteins will be observed, and are expected to not change. Additionally, if it is possible, models of schizophrenia can be established in these knockout rodent models, and PAOPA's ability to prevent and reverse these abnormalities

can be measured. Although this will help delineate the extent to which the D2 receptor and arrestin-3 play a role in the therapeutic effect of PAOPA, there could be technical limitations in establishing such models of schizophrenia in gene deletion animals. Currently, a full knockout of GRK2 is non-viable, although future studies may find techniques to circumvent this limitation, in which case, these can be used for future studies. Overall, the studies suggested herein will help directly establish the extent to which the GPCR regulatory proteins are involved in the therapeutic effects of PAOPA.

The final proposed future work stemming from this study would be to expand beyond PAOPA's role within the dopaminergic realm, and investigate its possible effects on additional neurotransmitter systems, via direct or indirect routes. A recent receptor binding screening conducted by National Institutes of Mental Health's Psychoactive Drug Screening Program revealed PAOPA to potentially act as an orthosteric inhibitor of the 5-HT7 serotonin receptor. Future studies will work to confirm this preliminary screening result, and will also use methods such as microdialysis combined with high performance liquid chromatography (HPLC), to determine PAOPA's effects on neurotransmitters levels of dopamine, serotonin, GABA, and glutamate, all of which have been systems implicated in the etiology of schizophrenia (Stone et al., 2007).

Overall, the studies conducted so far are a promising start to elucidating the novel mechanism of PAOPA's action and act as a guide to open up avenues for future studies which can lead towards improved understanding of allosteric modulators, and their therapeutic use for treating GPCR-related pathologies.

5.2 "CELL-FREE PROTEIN SYNTHESIS AND PURIFICATION OF HUMAN DOPAMINE D2L RECEPTOR"

5.2.1 GLOBAL SIGNIFICANCE:

Detailed studies on the structure and function of GPCRs have been limited in comparison to the work done on cytoplasmic proteins, largely owing to the significant challenges encountered when expressing and purifying these highly hydrophobic transmembrane receptors. Overexpressed hydrophobic proteins form misfolded aggregates in cellular systems and often lead to host cell toxicity, resulting in poor protein yields (Katzen et al., 2009; Wagner et al., 2006; Grisshammer, 2006). Limitations in available protocols for the expression and purification of GPCRs has resulted in a lack of fundamental structural knowledge, as is reflected by the fact that high resolution X-ray crystallography data exists for only a very select handful of receptors (Jacobson & Costanzi, 2012). The emerging concept of cell-free protein synthesis presents an innovative method to overcome the issues of GPCR overexpression, as it combines cellular lysate and exogenous deoxyribonucleic acid (DNA) outside of intact cells to generate the protein (Endo & Sawasaki, 2006; He, 2008; Katzen et al., 2005; Klammt et al., 2007; Schneider et al., 2010). The development of this methodology has had major positive implications in the field of GPCR protein expression, although, such advancements have largely been missing in application towards expressing the D2 receptor. Considering the highly robust implications of the D2 receptor in the etiology of neuropsychiatric disorders, studies developing the expression and purification of this

receptor appear to be of high importance. Our study tackles this lack of information by presenting two different cell-free techniques to express the D2 receptor, and purify it. This study highlights the challenges faced in obtaining purified D2 protein, and overcomes these using novel techniques, bringing to realization, that in order to advance studies on allosteric interactions, proper expression and purification of GPCRs are a highly crucial prerequisite.

5.2.2 FUTURE STUDIES:

The study entitled *"Cell-free protein synthesis and purification of human dopamine D2L receptor"* was initially undertaken to develop a protocol to obtain reasonable amounts of the D2 receptor, to further our studies on the nature of the PLG allosteric interaction site. Although, this study was able to accomplish D2 expression and purification, attempts at identification of the D2/PLG peptide fragment using Nano liquid chromatography-mass spectrometry (Nano LC-MS) proved to be unfeasible. Unfortunately, the high concentration of detergents required to keep the receptor soluble during purification steps prevented accurate mass-based separation of the D2 digests. The strength of this study, however, is based on its ability to overcome the challenges of expressing a GPCR, such as the D2 receptor, in order to improve research on this receptor, using more compatible techniques. Future studies can utilize cell-free protein synthesis technique to generate larger amounts of the pure D2 receptor, which can be used to study allosteric interactions using more compatible methods, such as radioligand binding assays. Additionally, the cell-free protein synthesis techniques generated in this

study, can be transferrable to the expression of other similar GPCRs, which future studies can use to expand our knowledge on the mechanisms of action utilized by other allosteric modulators.

5.3 Expression of dopamine D2 receptor mini-proteins to determine putative binding site of allosteric modulator prolyl-leucyl glycinamide (PLG)

5.3.1 Global Significance

Improved understanding on the nature of allosteric sites can lead to improved drug design targeting these sites. In fact, studies have shown medicinal chemists to have developed stepwise protocols which can utilize binding site information to modify receptor targeting, and improve the overall efficacy and affinity of existing drugs (Congreve et al., 2012; Deflorian et al., 2012). This demonstrates the significance of our current study, which was conducted to aid in narrowing down the allosteric binding site used by the potentially therapeutic D2 targeting parent compound, PLG. The preliminary results localizing the PLG functional allosteric interaction towards the C-terminus of the D2 receptor helps open up avenues towards future studies which can focus on this region to further pinpoint specific amino acids required for this binding. Additionally, it guides us towards elucidating the nature of the allosteric binding site of PLG peptidomimetics, allowing further improvements on these preclinically therapeutic ligands.

5.3.2 FUTURE STUDIES

The technique of homology-based computer modeling has been making remarkable progress in the past 5 years, owing to the significant advances made in determining high resolution X-ray crystal structures of GPCRs (Jacobson & Costanzi, 2012). Initial methods of such modeling were plagued by high variability in predictions, as they were technically limited in using the only known GPCR crystal structure of rhodopsin. However, the confidence in computational modeling has been significantly increasing with increased number of resolved GPCR crystal structure, and studies have shown improved predictability when there is a high homology between the GPCR used as a model, and the one for which binding predictions are being conducted (Jacobson & Costanzi, 2012). Therefore, future studies from this project will capitalize on the very recently resolved X-crystal structure of the dopamine D3 receptor, which shows a significant 52% homology with the D2 receptor (Civelli et al., 1993), to model possible PLG binding sites, using protocols as developed by another study (Platania et al., 2012). The predictions of these models are expected to coincide with the results from our study, which suggests amino acids 372-432 of the D2 receptor to contain the functional allosteric domains for binding of PLG. Future research will solidify the predictions of our study, as well as that of the homology-based modeling, using site directed mutagenesis according to many previously stated protocols. The combination of our current study, with the future research protocols, will help solidify the exact amino acids required for the allosteric binding of PLG to the dopamine D2 receptor.

CLOSING REMARKS

Allosteric modulation is a very interesting, and yet challenging, research concept in the field of drug discovery. The precise and diverse mechanisms of their action impose on to them a complexity which interplays well with the complexities observed in natural physiological systems. Receptor signaling within these physiological systems can be modulated in a range of methods to overcome various disease states, and allosteric modulators are highlighting a promising new method of attaining this goal. This thesis presents the importance of targeting post-receptor modalities, such as those involved in receptor trafficking and downstream events, to induce subtle changes in receptor activity. In addition, studies presented here also bring to realization, that in order to advance our knowledge on allosteric interactions, proper expression and purification of GPCRs are a highly crucial prerequisite. Finally, original research methods were also introduced in these studies, to overcome the lack of X-ray crystal structures for the D2 receptor, and present novel ways to narrow down the nature of the PLG allosteric binding site. Allosteric modulators can present unique methods of altering receptor activity to improve the treatment of GPCR-related disorder. The overall impact of this study is thus, to contribute to this promising field by improving our understanding of involved signaling cascades and the nature of functional interaction domains. This research can open up avenues to progress research on allosteric modulators of other physiologically crucial GPCRs, eventually leading to significant positive improvements in the treatment and management of a range of pathologies impacting human health worldwide.

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