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G PROTEINS AND PARKINSON'S DISEASE: THE ROLE OF SIGNAL TRANSDUCING G PROTEINS IN MEDIATING DOPAMINE RECEPTOR SUPERSENSITIVITY IN PARKINSON'S DISEASE

By

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G PROTEINS AND PARKINSON'S DISEASE

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ABSTRACT

There is growing evidence that factors other than cell-surface receptors are involved in regulating the sensitivity of cells to external signals. In particular, G proteins have been implicated in the increased sensitivity of numerous receptor systems under a variety of conditions (Mishra et al., 1997). The goal of this research project was to determine the role of G proteins in mediating dopamine receptor supersensitivity in Parkinson's disease.

Preliminary studies of G protein levels in human post-mortem brain tissue proved inconclusive, due to the limited availability and variability of tissue samples. Subsequent studies in the 6-hydroxydopamine (6-OHDA) rat lesion model of Parkinson's disease revealed that stimulatory G protein levels are persistently elevated following denervation (Marcotte et al., 1994). These G proteins are presumably coupled to dopamine D_1 receptors, which show clear evidence of supersensitivity despite apparently normal receptor levels. This result supports the hypothesis that G proteins are involved in the maintenance of dopamine receptor supersensitivity (Marcotte and Mishra, 1997). Stimulatory G protein levels were differentially regulated in the MPTP mouse model, with decreased stimulatory G proteins acutely following MPTP treatment, and increased stimulatory G proteins after long-term recovery (Marcotte et al., 1998a). Although the significance of these findings is unclear, they provide additional support for the

hypothesis that G proteins are modulated in response to dopaminergic denervation.

Attempts to measure functional changes in stimulatory G protein activity in the rat striatum proved unsuccessful, consistent with the available literature. Specifically, neither the GTPase nor a specific GTP binding assay was able to consistently detect stimulatory G protein activity following dopamine D_1 receptor stimulation. To provide direct evidence for the role of Golf in mediating dopamine receptor supersensitivity, Golf antisense oligonucleotides were administered to 6-OHDA lesioned rats. Intrastriatal infusion of Golf antisense, but not control sense oligonucleotides, specifically reduced apomorphine-induced rotational behaviour and Golf levels. The effects of Golf antisense infusion were at least partially reversible, supporting a specific antisense mechanism of action. However, one of the control oligonucleotides, Golf missense, consistently reduced rotational behaviour and G protein levels in a non-specific fashion. This effect was dose- and sequence-dependent, and may be due to non-specific binding to other nucleotides or proteins (Marcotte and Mishra, 1998).

Taken together, these studies support the hypothesis that stimulatory G proteins are involved in mediating dopamine D_1 receptor supersensitivity. Further characterization of the effects of in vivo antisense oligonucleotides may provide more definitive conclusions regarding the role of G proteins in mediating this phenomenon.

ACKNOWLEDGMENTS

The truth is a hard master, and costly to serve, but it simplifies all problems
Brother Cadfael's Penance, by Ellis Peters

Like most things in life, this thesis and the years of research that it entailed would not have been possible without the support and encouragement of a large number of people. The work was carried out under the supervision of Dr. Ram K. Mishra, to whom I am indebted for his continual support and confidence. I would also like to thank the members of my supervisory committee, Drs. Len P. Niles and Eva S. Werstiuk, for their support and guidance throughout the process.

The quality of my research, and my life in general, has been greatly enhanced by association with the members of various laboratories at McMaster University. Although too numerous to mention, I would particularly like to thank Boris, Yvonne, and Cia for their friendship throughout the years. This thesis would also not have been possible without the love and support of my family, especially my parents, Paul and Diane Marcotte.

I am also indebted to the various institutions and agencies that provided financial support during my graduate career, including the Ontario Graduate Studentship (OGS), the J.R. Longstaffe Scholarship, the Mutual Group Scholarship, and McMaster University. This research was also supported by grants awarded to my supervisor by the Parkinson's Foundation of Canada and the National Institutes of Health (NIH), USA.

Finally, I would like to end with another quote from *Brother Cadfael's Penance* that I think reflects life in general, and academia in particular.

> In our various degrees, we are all sinners. To acknowledge and accept that load is good. Perhaps even to acknowledge and accept it and not entertain either shame or regret may also be required of us. If we find we must still say: Yes, I would do the same again, we are making a judgement others may condemn. But how do we know God will condemn it? ... If the sin is one which, with all our will to do right, we cannot regret, can it truly be a sin?
>
> In the end there is nothing to be done but to state clearly what has been done, without shame or regret, and say: Here I am, and this is what I am. Now deal with me as you see fit. That is your right. Mine is to stand by the act, and pay the price.
>
> You do what you must do, and pay for it. So in the end all things are simple.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3-MT	3-Methoxytyramine
6-OHDA	6-Hydroxydopamine
AC	Adenylyl cyclase
ANOVA	Analysis of variance
App(NH)p	5'-Adenylyl-imidodiphosphate
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CAC	Canadian Council for Animal Care
CAF	Central animal facility, McMaster University
cAMP	Adenosine 3',5'-cyclic-monophosphate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
C-terminal	Carboxyl terminal
DA	Dopamine
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis-(β-amino ethyl ether)N,N'-tetra-acetic acid
EPS	Extrapyramidal motor syndrome
G Protein	Guanine-nucleotide binding protein
GABA	γ-aminobutyric acid
GDP	Guanosine 5'-diphosphate
Gi	G inhibitory
Golf	G olfactory
Gpp(NH)p	5'-guanylyl imidophosphate
Gs	G stimulatory
GTP	Guanosine 5'-triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HPLC	High performance (pressure) liquid chromatography
HRP	Horse Radish Peroxidase
HVA	Homovanillic acid
IC_{50}	Concentration inhibiting 50% of receptor binding
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.s.	Intrastriatal
kDa	KiloDalton

L-DOPA	L-3,4-Dihydroxyphenylalanine
MAO	Monoamine oxidase
MCID	Microcomputer imaging device
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NPA	N-propylnorapomorphine
N-terminal	Amino terminal
PBS	Phosphate-buffered saline
PMSF	Phenylmethyl-sulfonyl fluoride
ROD	Relative optical density
RT-PCR	Reverse transcriptase polymerase chain reaction
s.c.	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.l.	Sublingual
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TH	Tyrosine Hydroxylase
U.V.	Ultraviolet

CHAPTER I

INTRODUCTION

The complexity of living systems requires a finely-regulated communication system between individual cells of every organism. To maintain homeostasis within a multicellular organism, a wide variety of cells types are required with the ability to recognize and process an even more diverse range of extracellular signals. These signal molecules include not only physical and sensory stimuli, but the hormones and transmitters released by other cells. As a general rule, these signals do not enter the target cell, but rather interact with specific receptors on the cell surface. In some cases, these receptors may be responsible for directly mediating intracellular responses to extracellular signals (e.g. ligand-gated ion channels, protein tyrosine kinase receptors, etc.). In the majority of instances, however, these receptors participate in a multi-level signaling system that governs the response of the cell to external signals.

Classically, the first step following ligand binding to these receptors is the activation of heterotrimeric guanine nucleotide binding proteins (G proteins) (Lefkowitz et al., 1993). These G proteins serve to transduce and amplify extracellular signals through modulation of the activity of effectors such as ion channels, transporters, and enzymes. These effectors in turn control the production of intracellular second

messengers (e.g. cAMP, inositol phosphates, diacylglycerol, etc.). Thus, many of the vital processes that occur within cells, including cell division, protein secretion, and presumably learning and memory, may be directly influenced by extracellular signals mediated through G proteins.

Given that the majority of peptide hormones and monoamine neurotransmitters signal through G protein-coupled receptors, the function and regulation of these receptors have received considerable attention in recent years. Typically, alterations in neurotransmitter or hormone status can result in either reduced or increased receptor activity, referred to as receptor desensitization and receptor supersensitivity, respectively. Although the role of G proteins and other signal transduction components in regulating receptor desensitization has long been appreciated (for recent reviews, see Hadcock and Malbon, 1993, and Freedman and Lefkowitz, 1996), the study of receptor supersensitivity has generally focused more narrowly on changes in receptor levels and affinity. The focus of this thesis is on the role of G proteins in mediating dopamine receptor supersensitivity.

In broad terms, receptor supersensitivity refers to the enhanced responsiveness of a given receptor pathway for its specific ligand or activating stimulus. Receptor supersensitivity can be brought about in a number of ways, most commonly by receptor antagonist treatment or through denervation (Srivastava and Mishra, 1994). In both cases, the dopamine receptor system has received a great deal of attention, and may be regarded as a prototype for the study of receptor supersensitivity. As will be discussed later in this Introduction, there are manifest differences in how dopamine receptor

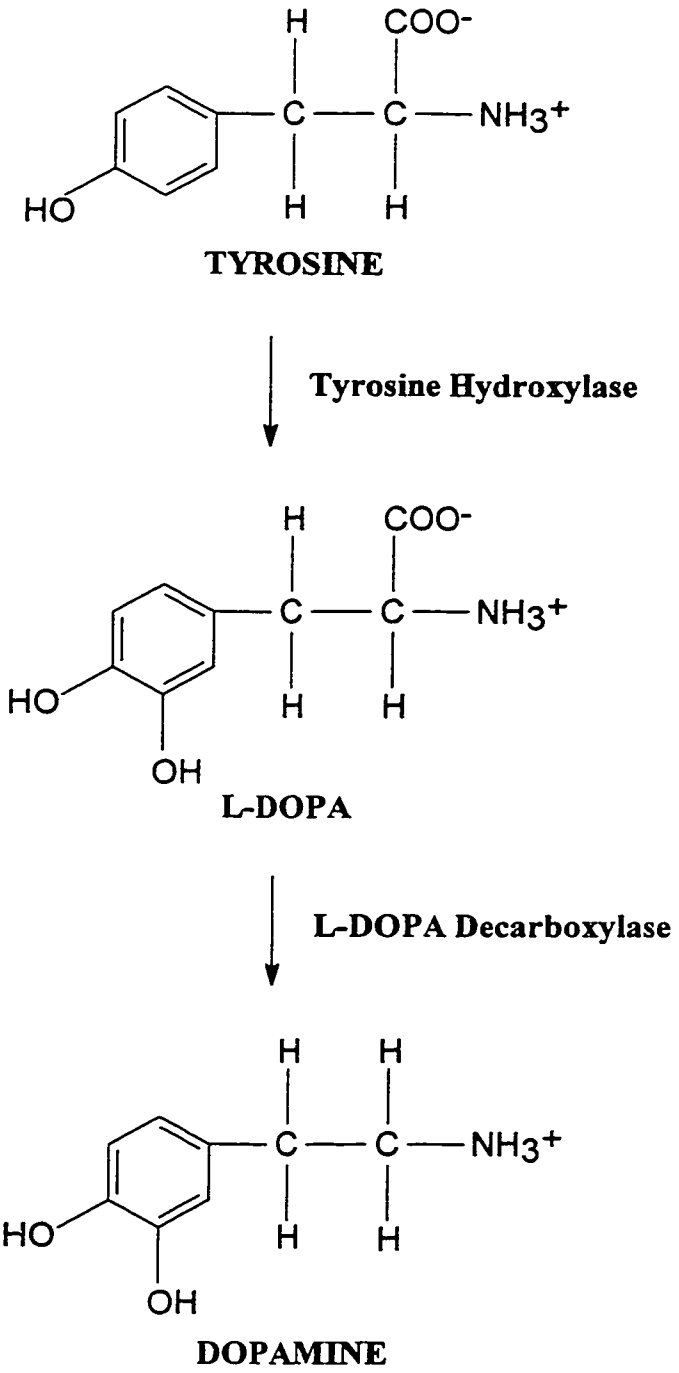
supersensitivity is maintained following denervation and chronic receptor blockade. The purpose of the research project described in this thesis was to examine the potential role of G proteins in mediating denervation-induced dopamine receptor supersensitivity in Parkinson's disease.

I.1. DOPAMINE NEUROTRANSMISSION

Although the critical role of dopaminergic neurotransmission in the CNS is well known, dopamine was initially considered to be merely an intermediate in the synthesis of epinephrine and norepinephrine (see Figure 1) (Cooper et al., 1997). The discovery of large quantities of dopamine in the basal ganglia led to the examination of dopamine as a potential neurotransmitter in its own right (Grace et al., 1998). Dopamine neurotransmission gained considerable attention following the discovery that dopamine was depleted in the striatum of Parkinson's disease patients. Moreover, it was soon realized that the metabolic precursor to dopamine, L-dihydroxyphenylalanine (L-DOPA), could reverse the clinical symptoms of Parkinson's disease (Hagan et al., 1997). Subsequently, dopamine has been demonstrated to play a pivotal role in a large number of neuropsychiatric and movement-related processes (Hyman and Nestler, 1993, Cooper et al., 1997).

Anatomically, the vast majority of dopamine-containing neurons exist in small, discrete nuclei found in the midbrain and telencephalon (Cooper et al., 1997, Grace et al., 1998). These give rise to three major dopaminergic projection systems in the brain; the

Figure 1. Dopamine synthesis from tyrosine



nigrostriatal system, which projects from the substantia nigra to the striatum (caudate-putamen); the mesolimbic system, which projects from the ventral tegmental area to various limbic structures; and the tuberoinfundibular system, which projects from the hypothalamus to the anterior pituitary (Hyman and Nestler, 1993, Grace et al., 1998). This research project focused on the nigrostriatal system, as it is the most amenable to experimental manipulation and dissection. Moreover, this system is directly involved in the pathophysiology of Parkinson's disease, and is believed to play a critical role in the coordination of motor movements through the basal ganglia (Mello and Villares, 1997).

I.1.1. Dopamine Synthesis and Regulation

Like all catecholamines, dopamine is synthesized from the amino acid precursor L-tyrosine (Figure 1). The rate-limiting step in catecholamine synthesis is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (Cooper et al., 1997, Elsworth and Roth, 1997). L-DOPA is subsequently converted to dopamine by the enzyme aromatic acid decarboxylase, also known as L-DOPA decarboxylase. Since L-DOPA is quickly converted enzymatically into dopamine, increased dopamine synthesis can readily be achieved by the exogenous administration of L-DOPA. This is evidenced by the use of L-DOPA as the primary treatment for Parkinson's disease (Chase, 1998b).

The enzyme tyrosine hydroxylase is under strict physiological regulatory control. End-product inhibition by dopamine, presynaptic inhibition by dopamine autoreceptors,

Figure 2. Schematic diagram of the dopaminergic synapse

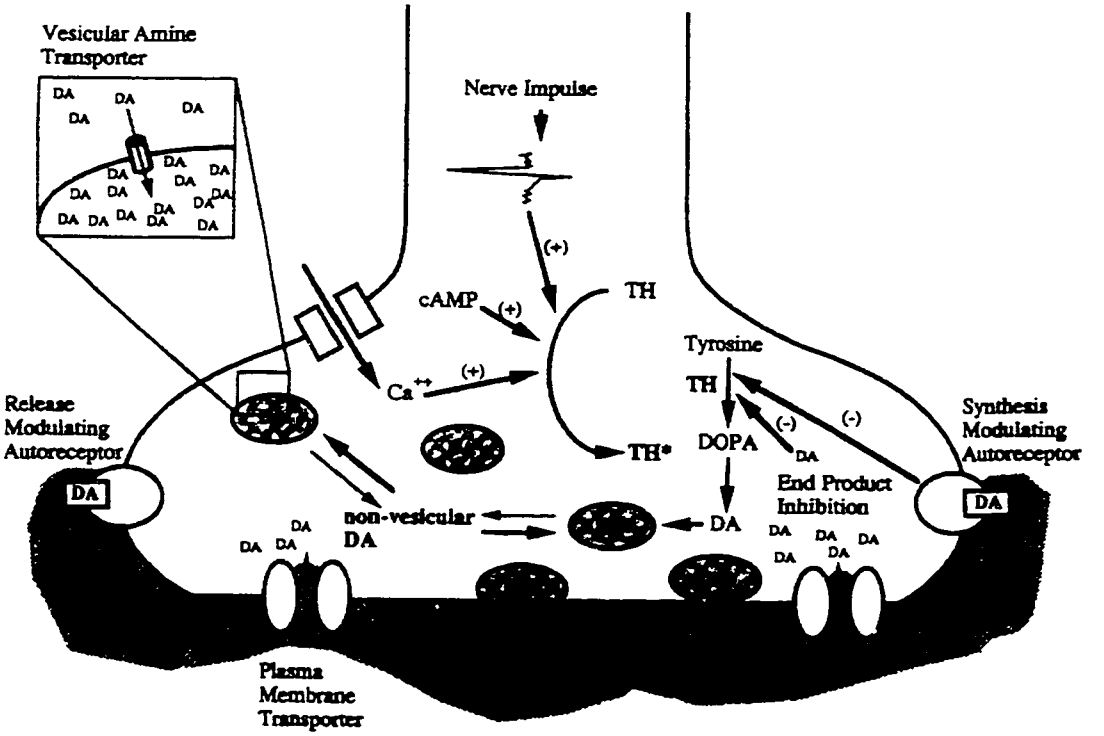


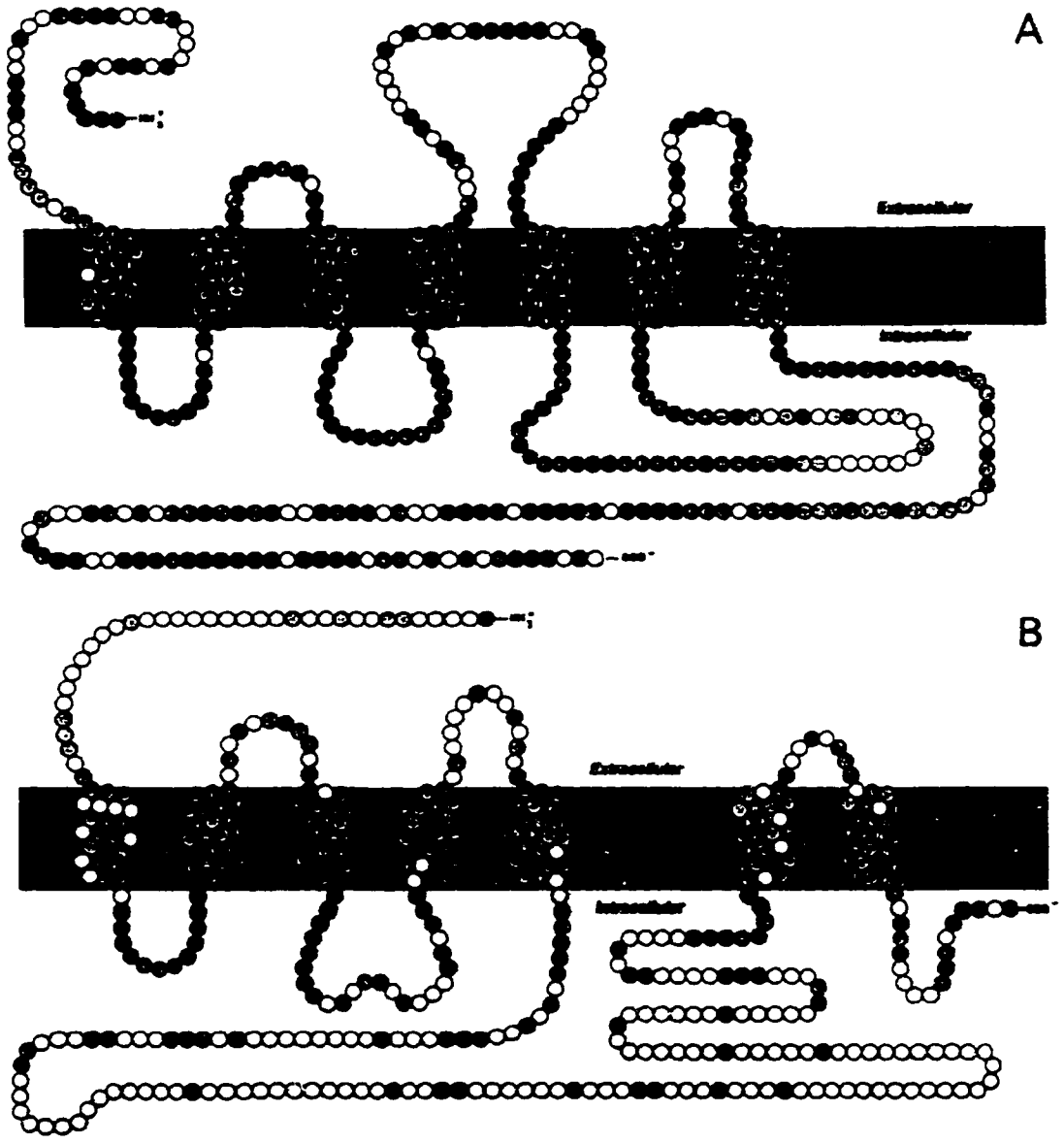
Illustration of the cycle of dopamine synthesis, storage, release, and uptake in the nerve terminal. Voltage-sensitive Ca^{2+} channels regulate dopamine synthesis and storage vesicle fusion and release in response to nerve impulses. Release is also modulated by presynaptic dopamine autoreceptors. The dopamine transporter mediates reuptake of released dopamine. The vesicular amine transporter regulates cytoplasmic storage of dopamine. DA, dopamine, TH, tyrosine hydroxylase. Taken from Cooper et al, 1996.

and the rate of impulse flow in the nigrostriatal pathway all serve to regulate its function (Cooper et al., 1997, Grace et al., 1998). Under normal synaptic transmission, dopamine is released into the extracellular space following arrival of electrical impulses into the terminal area. Following release, dopamine can bind to post-synaptic dopamine receptors which relay this trans-synaptic extracellular signal (see Figure 2). Dopamine signaling is rapidly terminated by the re-uptake of dopamine into the presynaptic terminal and surrounding glia through the dopamine transporter. Dopamine can then be re-packaged for synaptic release or broken down by its regulatory enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) (Elsworth and Roth, 1997). The release of dopamine from pre-synaptic terminals is also under the regulatory control of dopamine autoreceptors.

I.1.2. Dopamine Receptors

Dopamine receptors are members of a large seven-transmembrane domain receptor superfamily of G protein-coupled receptors (see Figure 3) (O'Dowd, 1993, Missale et al., 1998). Dopamine receptors fall into two main categories, initially classified as D_1 and D_2 based on their differing pharmacological profiles and opposing effects on adenylyl cyclase (Kebabian and Calne, 1979). With the advent of modern molecular cloning techniques, this characterization has broadened to include several subtypes, but the general D_1 / D_2 subcategories still hold (Lachowicz and Sibley, 1997, Baldessarini and Tarazi, 1996, Hartman and Civelli, 1997, Palermo-Neto, 1997). The D_2

Figure 3. Dopamine receptor amino acid structure



Theoretical transmembrane organization of the (A) D_1 and (B) D_2 families of dopamine receptors. Darkness of the circles represents the degree of amino acid homology among the different receptor subtypes. (A) Sequence of D1A as compared to D1B and D5, (B) D2L as compared to D3 and D4. Adapted from Gingrich and Caron, 1993.

receptor subfamily can be divided into a long and short form of the D_2 receptor, D_{2L} and D_{2S}, that differ in the length of the third cytoplasmic loop, as well as D_3 and D_4 receptors (Picetti et al., 1997, Jaber et al., 1996). The D_1 subfamily consists of just two members to date, D_1 and D_5 receptors (see Table I) (Sokoloff and Schwartz, 1995, Kulkarni and Ninan, 1996). Although further subdivisions may also exist (e.g. D_4 receptors in the human population Van Tol et al., 1992), the general outline presented above and in Table I is sufficient for the purposes of this examination. It should be noted, however, that while the general regional distributions of these various subtypes have been mapped out, their potentially different roles in signal transduction have not been unequivocally established due to a relative lack of specific pharmacological agents. Moreover, the exact localization of these receptors may affect their signaling pathways. Thus, the signaling pathways summarized in Table I for each of the receptor subtypes may not accurately reflect the physiological response to the dopamine receptor stimulation under all conditions or in all areas.

Dopamine receptor supersensitivity can most easily be detected in the caudate-putamen region of the basal ganglia, also referred to as the striatum. For the purpose of this thesis, only the prototypical D_1 and D_2 receptor subtypes will be considered as these appear to be the predominant forms in the striatum (Table I) (Civelli et al., 1993). However, as will be discussed shortly, various G protein subtypes may be involved in mediating dopamine receptor function in this area.

Table I. Properties of dopamine receptor subtypes

	D_1	D_2	D_3	D_4	D_5
Genetics					
Chromosome	5q35.1	11q22-23	3q13.3	11p15.5	4p
Pharmacology					
Selective agonists	SKF38393 dihydrexedine	(+)PHNO bromocriptine	PD128907 7-OH-DPAT	--	--
Prototypic antagonists	SCH23390	spiperone	spiperone	spiperone	SCH23390
Selective antagonists	--	haloperidol raclopride	nafadotride	L745870 U101387	--
Signal Transduction					
G protein	Gs	Gi/Go	Gi/Go	Gi/Go	Gs
Adenylyl cyclase	activation	inhibition	inhibition (?)	inhibition (?)	activation
Phospholipase	activation	activation	n.e.	n.e.	activation (?)
Ca^{2+} channel	activation	inhibition	inhibition	inhibition	n.d.
Arachidonic acid	n.d.	activation	inhibition (?)	activation	n.d.
Localization	caudate-putamen nucleus accumbens olfactory tubercle amygdala	caudate-putamen nucleus accumbens substantia nigra pituitary gland	nucleus accumbens hypothalamus olfactory tubercle substantia nigra	frontal cortex hippocampus cerebellum midbrain	hippocampus parafasicular hypothalamus

n.d., not determined; n.e., no effect; ?, contradictory data, -, not available. Adapted from Seeman and Van Tol, 1994, Sokoloff and Schwartz, 1995, Baldessarini and Tarazi,1996, Alexander and Peters, 1997, Lachowicz and Sibley, 1997, Hartman and Civelli,1997, Missale et al.,1998.

I.1.3. Dopamine Receptor Supersensitivity

There are several potential means by which dopamine receptor supersensitivity can be elicited. As previously mentioned, dopamine neurotransmission plays a critical role in a large number of neuropsychiatric illnesses, including schizophrenia (Willner, 1997, Hietala and Syvalahti, 1996). The standard pharmacological treatment of schizophrenia involves the use of dopamine receptor antagonists known as antipsychotics or neuroleptics (Leiberman and Koreen, 1993). By antagonizing dopamine receptor function, these agents would be expected to increase the sensitivity of dopamine receptor pathways. In fact, many of the side effects and long-term adverse effects of neuroleptics are thought to result from dopamine receptor supersensitivity (Hyman and Nestler, 1993, Sunahara et al., 1993).

A similar situation is believed to occur in Parkinson's disease, where the degeneration of substantia nigra neurons results in the depletion of striatal dopamine. Reduction of normal dopaminergic signaling would thus be expected to increase dopamine receptor sensitivity in the striatum in the long-term (Korczyn, 1995). As in the case of schizophrenia (Busatto and Kerwin, 1997), many of the long-term adverse consequences of dopamine replacement therapy in Parkinson's disease are believed to result from dopamine receptor supersensitivity. These include abnormal or dyskinetic movements, collectively referred to as extrapyramidal motor syndrome (EPS), wearing-off phenomena, and on-off phenomena that are frequently manifested in later stages of Parkinson's disease (Nutt, 1990, Jeste and Caligiuri, 1993). Increasing clinical evidence

suggests that the intermittent stimulation of dopamine receptors, resulting from current treatment regimens, contributes significantly to the development of these complications (Chase, 1998a). Despite the superficial similarity to neuroleptic-induced tardive dyskinesia, the specific pattern of dyskinetic activity in end-stage parkinsonian patients differs markedly from that observed in schizophrenics (Tanner, 1986).

In addition to the primary deficiencies in dopaminergic neurotransmission, a large number of other neurotransmitter and neuropeptide systems are also known to be altered in Parkinson's disease. Most notably, alterations in GABA, acetylcholine, glutamate, and norepinephrine neurotransmission have all been reported in Parkinson's disease and its related animal models (Hallet, 1993, Albin et al., 1995, Mello and Villares, 1997, Bergman et al., 1998). Similarly, alterations in a variety of striatal neuroactive peptides, such as substance P, met-enkephalin, dynorphin, neurotensin, and somatostatin have also been observed (Graybiel, 1990, Gerfen, 1992b). The potential interactions of these various transmission systems, and their relevance to dopamine receptor supersensitivity, will discussed in more detail in Sections I.4 and II.2.3.

I.2. THE ROLE OF G PROTEINS

I.2.1. G Protein Structure and Function

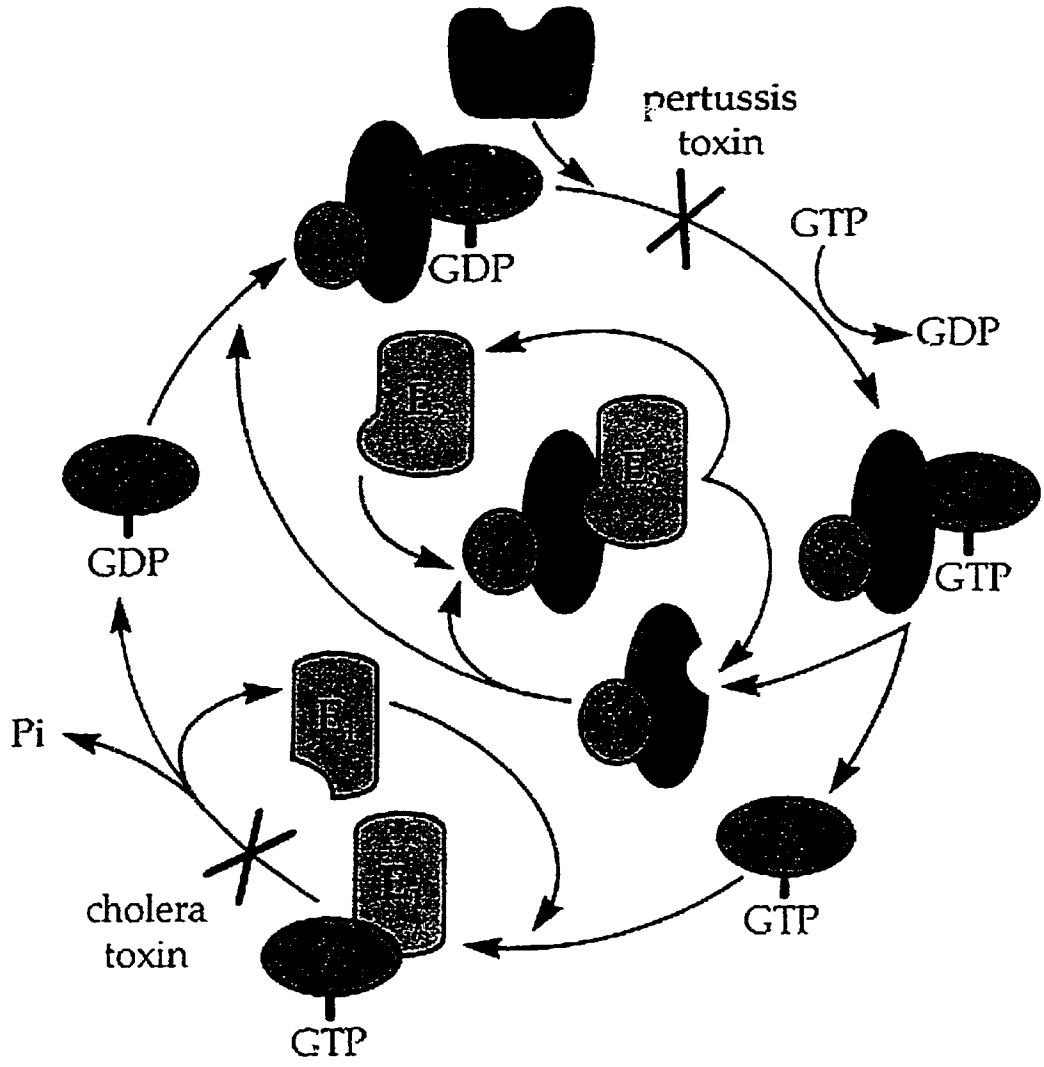
G proteins are members of a large family of guanine nucleotide-binding proteins that include the Ras-like proteins as well as the ribosomal protein synthesis elongation

and initiation factors (e.g. EF-Tu) (Spiegel et al., 1992). G proteins are heterotrimers consisting of α, β, and γ subunits, and are generally classified by their α subunits. The α subunits of all G proteins, as well as Ras and EF-Tu, possess a GTP binding/GTPase domain that allow these proteins to act as "molecular switches" in regulating cellular function (Spiegel et al., 1992, Gudermann et al., 1996). An overview of G protein signaling is presented in Figure 4. G protein subunits can undergo one or more conformational changes in response to receptor activation, facilitating the exchange of GTP for GDP (Savarese and Fraser, 1992, Lefkowitz et al., 1993). The binding of GTP to the α subunit promotes dissociation of the α subunit from the βγ complex. GTP-liganded α subunits, and possibly βγ complexes, are then capable of modulating different effector systems within the cell. This G protein response is terminated by hydrolysis of the bound GTP by the intrinsic GTPase activity of the α subunit, leading to re-association of the subunits, and allowing the cycle to repeat.

At least 23 distinct α subunits have been identified, including several splice variants of the more common G protein subtypes (Gudermann et al., 1997). Generally, α subunits are divided into four major categories, as depicted in Figure 5. These include the Gs family, which stimulate adenylyl cyclase, the Gi family, which inhibit adenylyl cyclase, and the Gq and G_{12} families, which stimulate phospholipases (Birnbaumer, 1993). All four signaling systems may play a role in mediating dopamine receptor signal transduction.

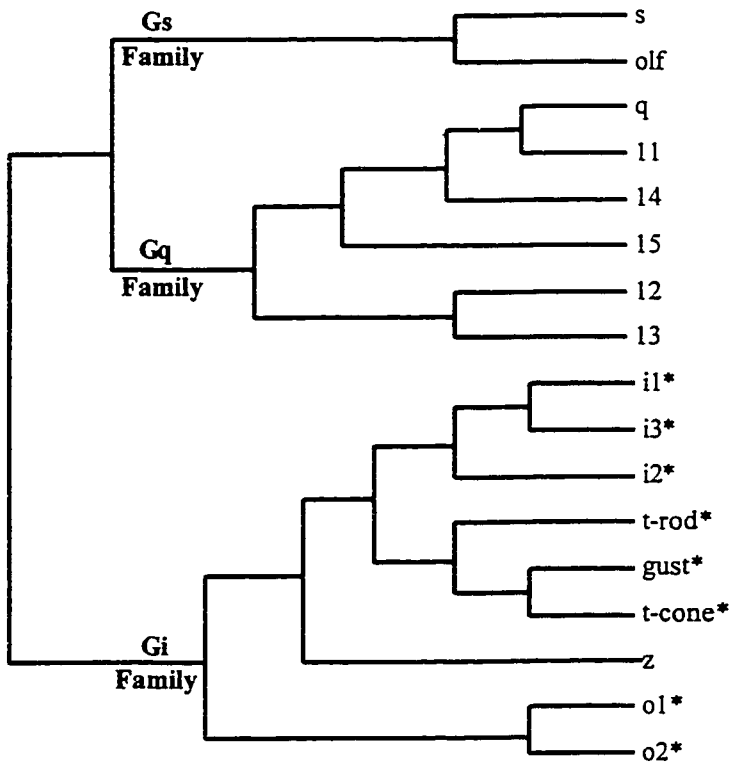
There are two main subtypes of stimulatory G proteins, Gs and Golf, both of which are present in the striatum, where they are believed to couple to dopamine D_1

Figure 4. The G protein cycle



Standard representation of the receptor-G protein cycle. Activation of a receptor by its ligand results in the substitution of the G protein α-subunit-bound GDP for GTP, with subsequent dissociation of the receptor / G protein subunits. Inactivation of the α-subunit occurs through the hydrolysis of GTP to GDP, allowing the re-initiation of the cycle. Greek letters denote G protein subunits, E_1 refers to the first effector, E_2 a second effector, and Pi is inorganic phosphate. Taken from Milligan, 1993.

Figure 5. G protein classification



Phylogenetic family tree of sixteen G protein α subunits. * denotes pertussis sensitive subunits. Adapted from Birnbaumer, 1993.

receptors (Hervé et al., 1993). Golf, so named because it was initially discovered in the olfactory tubercle (Jones and Reed, 1989), is believed to be the predominant stimulatory G protein in the striatum (Hervé et al., 1993). The Gi family includes multiple subtypes including Gi_1, Gi_2, and Gi_3, which are involved in the inhibition of adenylyl cyclase, and Go_1 and Go_2, which stimulate K^+ channels and inhibit Ca^{2+} channels (Birnbaumer, 1993). Both subtypes are believed to be coupled to dopamine D_2 receptors in the striatum. The Gq and G_{12} sub-families consist of several subtypes involved in the stimulation of phospholipases C and D (Gudermann et al., 1996). Although Gq and G_{12} are not generally believed to play a significant role in dopamine signal transduction, this view has recently been challenged (Wang et al., 1995b).

I.2.2. G Protein - Dopamine Receptor Interactions

As previously mentioned, one of the likely consequences of dopamine deficiency, whether produced by denervation or receptor blockade, is the increased sensitivity of post-synaptic dopamine receptors. The most obvious mechanism by which this may occur is receptor upregulation due to increased receptor synthesis or reduced degradation. Alternatively, other signal transduction elements, such as G proteins, may play a role in mediating changes in receptor sensitivity. However, given that hundreds of G protein-coupled receptors interact with a limited number of G proteins, the specificity of this coupling needs to be addressed.

In the simplest possible case, an individual receptor would couple to a single G

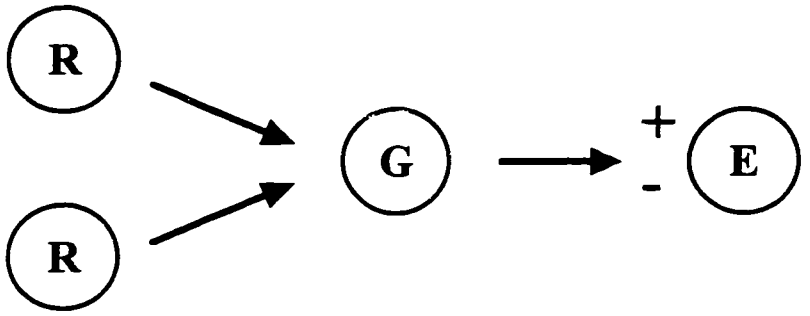
protein, activate it, and initiate a signal transduction cascade (Figure 6A). However, in most systems, there are multiple receptor types available to interact with a variety of G protein subtypes. Thus, multiple receptors could "converge" on a single G protein (Figure 6B). Direct evidence for this model has recently been provided by the use of targeted Gi gene disruption in transgenic mice (Rudolph et al., 1996). Following Gi knockout in these animals, parallel decreases in adenylyl cyclase inhibition were observed for three separate receptor agonists (Rudolph et al., 1996). Alternatively, a given receptor could potentially activate more than one G protein, leading to "divergent" signaling within cells (Figure 6C). This model was first demonstrated for the β-adrenergic system in the retina, where Gt (transducin), Gs, and even Gi activation have been shown to occur following β-adrenergic receptor stimulation (Asano et al., 1984).

Despite the general reliance on an α subunit nomenclature, G protein βγ subunits have been shown to have direct stimulatory and inhibitory effects on a variety of second messenger systems (Clapham and Neer, 1993). In particular, βγ subunits have both stimulatory and inhibitory effects on various adenylyl cyclase isoforms in a type-specific manner (Iyengar, 1993, Tang and Gilman, 1991). Concurrent stimulation of both Gs and Gi G proteins could result in the net suppression of Gs α subunit activity by the thermodynamic inhibition of βγ subunits released by activated Gi (Figure 7). This stems from the fact that Gi levels are higher than Gs in virtually all known cell types (Birnbaumer, 1993). This indirect mechanism allows one G protein to conditionally suppress the spontaneous activation of another. However, it is also possible that Gsα and Giα may directly compete with each other in the regulation of effector function.

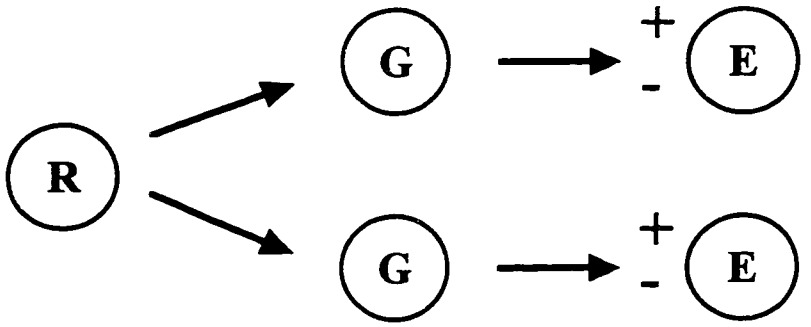
Figure 6. Signaling mechanisms for receptor / G protein interactions

A. Linear Signaling (R => G => E)

B. Convergent Signaling by Multiple Receptors

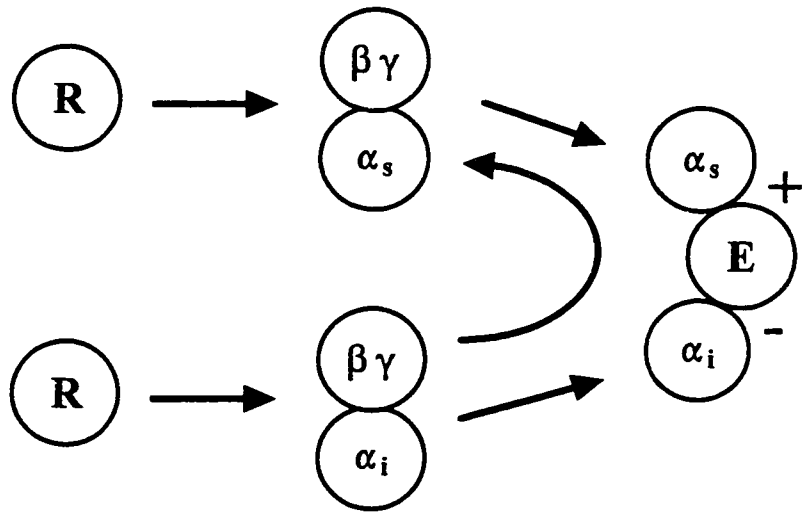


C. Divergent Signaling by One Receptor



Signaling mechanisms based on standard proposed receptor / G protein interactions. R, Receptor; G, G protein; E, Effector. From Hildebrandt, 1997.

Figure 7. Subunit dissociation and G protein activation



Signaling mechanisms based on subunit dissociation as a mechanism of G protein activation. The specific example demonstrates Gs and Gi G proteins regulating the effector adenylyl cyclase. R, Receptor; E, Effector. From Hildebrandt, 1997.

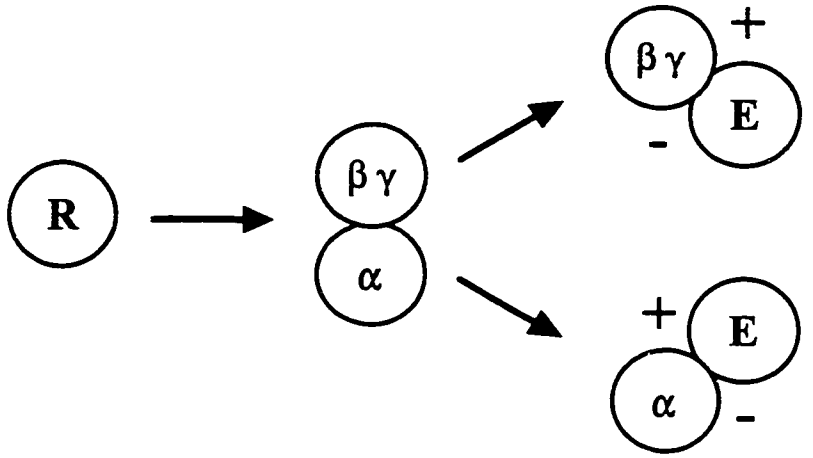
βγ Subunits may also directly modulate the function of various effector systems (Figure 8A). There is now an extensive number of effectors known to be regulated by βγ subunits (Clapham and Neer, 1993). However, just as βγ subunits could inhibit α subunit-mediated activation, excess α subunits liberated by G protein activation could suppress βγ subunit-mediated effects (Figure 8B). Moreover, it has been suggested that a single G protein may preferentially activate different signal transduction pathways depending on the level of receptor expression (Zhu et al., 1994). Clearly, the linear conceptualization of signal transduction pathways presented in Figure 6A is inadequate in light of recent experimental results. Nevertheless, the interaction of a given G protein and receptor in a specific cell type may still be governed by a high degree of selectivity (Gudermann et al., 1997).

In terms of modulating cellular responses to extracellular signals, alterations at the receptor level offer the potential advantage of maintaining signal specificity. Alterations at the G protein level would generally be expected to result in a corresponding loss of selectivity for a given receptor system. However, the integration of diverse receptor-mediated transduction systems through a common G protein (as presented in Figure 6B) offers the potential advantage of offsetting the loss of one neurotransmitter by increased signaling through a common G protein. G proteins may thus serve as potential sources of redundancy and integration among diverse neurotransmitter systems.

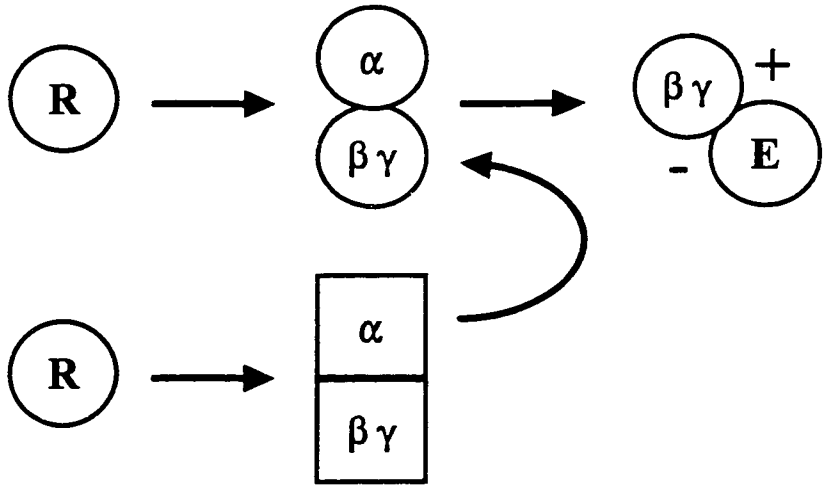
In terms of a single receptor-mediated signaling pathway, increased levels of either G proteins or their corresponding receptors would be expected to increase the sensitivity to specific receptor agonists. Specifically, increased receptor number is

Figure 8. Signaling mechanisms involving G protein subunits

A. Dual Signalling Pathways Activated by a Single G Protein



B. Suppression of βγEffects by Excess α subunits Liberated by Activation of Other G Proteins



Signaling mechanisms based on the effects of both activated α subunits and free βγ dimers. R, Receptor; E, Effector.From Hildebrandt, 1997.

expected to increase the potency of receptor agonists, as measured by the lower concentration required to elicit a given physiological response. This is generally depicted as a leftward shift of the concentration-effect (dose-response) curve, as shown in Figure 9A. Increased G protein activity would also be expected to increase the potency of receptor agonists. However, G protein upregulation also offers the potential advantage of increasing the maximum physiological response to receptor stimulation, as shown in Figure 9B (Ross, 1992). In addition, alterations in G protein activity could also affect other aspects of the concentration-effect relationship (i.e. modulate the 'steepness' of the curve), thus allowing for greater adaptive control over receptor-mediated signal transduction. The validity of this theoretical model, and its applicability to dopamine receptor supersensitivity will be discussed in detail in Section II.2.3.

I.2.3. Evidence for G Protein Dysfunction in Human Disease States

In support of the pivotal role of G proteins in regulating intracellular events, alterations in G protein levels and function have been reported for a number of disorders, as described in Table II. There is evidence for a primary G protein abnormality in several of these disorders, including acromegaly (Spada et al., 1992), Albright's hereditary osteodystrophy (Weinstein et al., 1990), McCune-Albright syndrome (Weinstein et al., 1991), pseudoparahypothyroidism type Ia with or without precocious puberty (Carter et al., 1987, Iiri et al., 1994), congenital stationary night blindness (Dryja et al., 1996), and

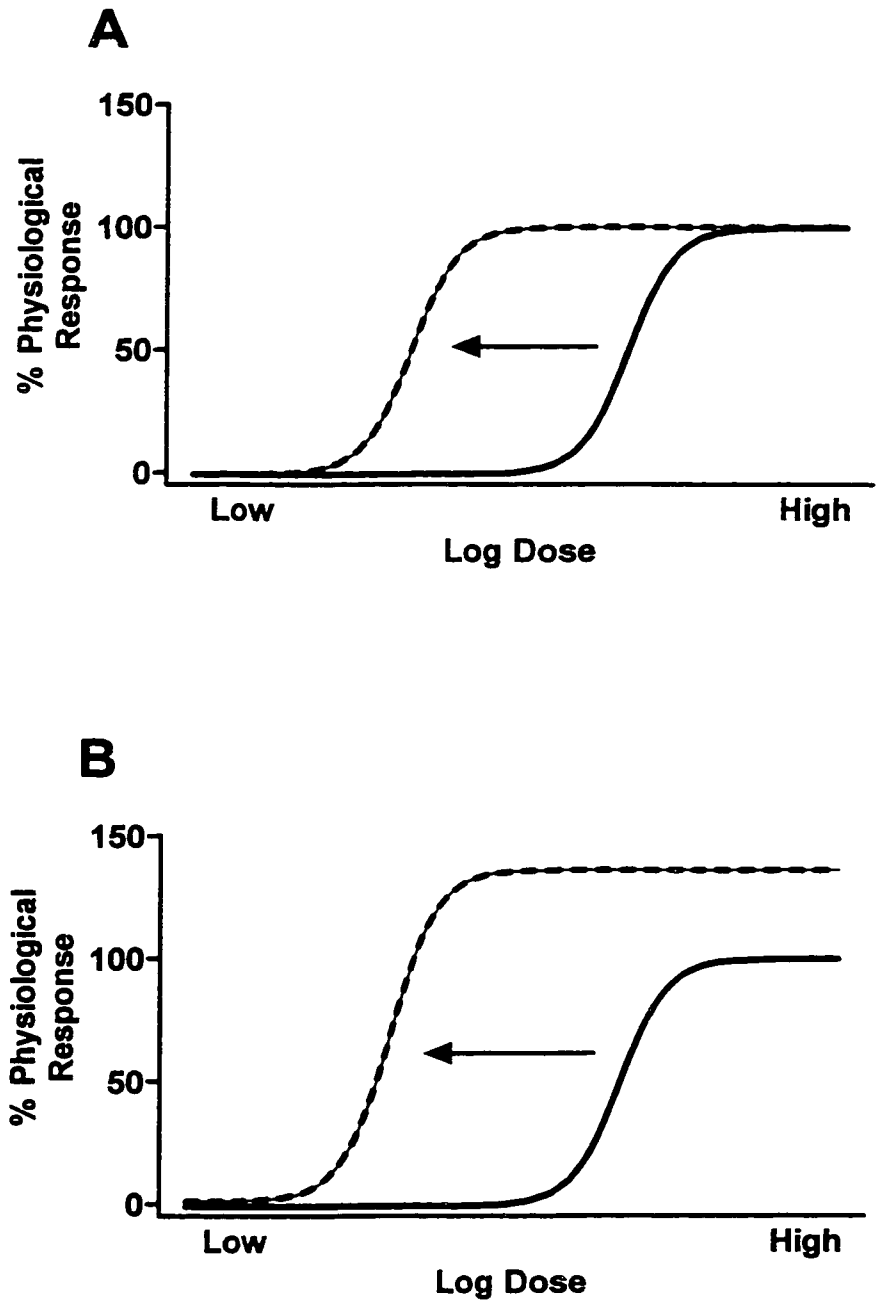
Figure 9A. Effects of receptor upregulation

The typical response to increasing drug concentrations is a greater physiological response, up to a certain maximum (100% physiological response in the figure). The resulting dose response curve (solid line) is thus sigmoidal shaped. Receptor upregulation would be expected to increase the sensitivity of the receptor system, thus resulting in a given physiological response at lower concentrations (i.e. higher potency). This is demonstrated by the leftward shift of the concentration-effect (dose-response) curve, depicted as a dotted line. Note that the maximum physiological response remains unaltered, however.

Figure 9B. Effects of G protein upregulation

Using the same receptor system presented in Figure 9A, G protein upregulation may also produce a similar increase in drug potency as measured by the leftward shift of the dose response curve (dotted line). However, G proteins may also increase the maximum physiological response to receptor stimulation (e.g. 140 % in the figure). Other parameters of the concentration-effect (dose-response) relationship, such as the range of the concentrations over which the response is manifested (i.e. the "steepness" of the curve) may also be altered by G protein manipulation.

Figure 9. Theoretical dose response curves



Adapted from Ross, 1992

Table II

Diseases involving G Protein up- and down-regulation

Disease	G Protein	Regulation	Reference
Neurological Disorders:			
Bipolar Affective Disorder	Gsα	up	Young et al., 1993, Manji et al., 1995
Depression	Gsα	up	Manji, 1992
Huntington's Disease	Gsα	down	De Keyser et al., 1989
Olivopontocerebellar Atrophy	Gsα	up	Kish et al., 1993
	Giα	down	
Schizophrenia	Giα, Goα	down	Okada et al., 1990,
	Giα, Goα	down	Nishino et al., 1993
Others:			
Acromegaly	Gsα	up	Spada et al., 1992
Albright Hereditary Osteodystrophy	Gsα	down	Weinstein et al., 1990
Alcoholism	Gsα	down	Diamond et al., 1987
Cardiomyopathy	Giα	up	Bohm et al., 1990
Congenital Stationary Night Blindness	Gt (transducin)	down	Dryja et al., 1996
Congestive Heart Failure	Gsα	down	Horn et al., 1995
Fibrous Dysplasia of bone	Gsα	down	Candeliere et al., 1997
Left ventricle failure	Gsα	down	Longabaugh et al., 1988
McCune-Albright syndrome	Gsα	up	Weinstein et al., 1991
Mitral Valve Prolapse Dysautonomia	Gsα	up	Davies et al., 1987,
	Gsα	up	Davies et al., 1991
Ovarian and adrenocortical tumours	$Gi_2\alpha$	up	Lyons et al., 1990

Disease	G Protein	Regulation	Reference
Pseudohypoparathyroidism (PHP) type Ia	Gsα	down	Iiri et al., 1994, Carter et al..,1987
PHP Ia with precocious puberty	Gsα	down/up	Iiri et al., 1994
Septic shock	Giα	up	Bohm et al., 1995

endocrine tumors (Lyons et al., 1990). In the remainder of the cases presented in Table II, G protein dysfunction is presumably secondary to the principle disease pathophysiology. However, this does not negate the potential significance of G proteins in these disorders; in fact, the results presented in this table clearly demonstrate that G proteins are susceptible to modulation by a variety of disease processes.

Alterations in G protein levels have also been demonstrated in several experimentally-induced models of human disease, as shown in Table III. Also included in this table are examples of alterations in G protein activity following denervation or chronic drug treatment. Of particular relevance to this thesis, are stimulus deprivation of the pineal (Babila and Klein, 1992) and chronic morphine treatment (Van Vliet et al., 1993, Eriksson et al., 1992b, Eriksson et al., 1992a). These experimental manipulations provide a precedent for the examination of G protein function in denervation-induced dopamine receptor supersensitivity. Of particular relevance, the behavioural supersensitivity that occurs following chronic morphine treatment has been shown not to include upregulation of opiate receptors (Reddy et al., 1993).

More recently, advances in transgenic molecular techniques have allowed the development of specific gene "knockout" animal models for a variety of expressed proteins and enzymes, including G proteins. Targeted disruption of the $Gi_2\alpha$ subunit gene in transgenic mice has been shown to result in a lethal form of ulcerative colitis with adenocarcinomas of the colon, similar to the human disease (Rudolph et al., 1995). Local increases in CD4+T cell levels and proinflammatory cytokines have also been observed in these animals (Hornquist et al., 1997). However, these animals lack any obvious

Table III

G protein levels in animal models of disease

Disease	G Protein	Regulation	Reference
Experimentally-Induced Disorders:			
Alcoholism	Giα	up	Wand et al., 1993
Cerebral Infarct	Goα	up	Murayama et al., 1989
Chagas' Disease	$Gi_1\alpha$, $Gi_2\alpha$	up	Huang et al., 1997
	Gsα	down	
Diabetes	Giα	down	Gawler et al., 1987
Encephalitis	Goα	up	Murayama et al.., 1989
Hyperthyroidism	$Gi_1\alpha$, $Gi_2\alpha$	down	Orford et al., 1992
Meningitis	Goα	up	Murayama et al., 1989
Spontaneous Hypertension	$Gi_2\alpha$, $Gi_3\alpha$	up	Thibault and Anand-Srivastava, 1992
Stimulus deprivation of the pineal	Gsα, Gβ	up	Babila and Klein, 1992
Chronic Drug Administration:			
Antidepressants	Gqα, $G_{12}\alpha$	up	Colin et al., 1991, Lesch and Manji, 1992
	$Gi_1\alpha$, $Gi_2\alpha$, Gsα	down	
Cocaine	Giα, Goα	down	Nestler et al., 1990
Glucocorticoids	Gsα	up	Saito et al., 1989
	Giα	down	
Morphine	Gsα	up	Van Vliet et al., 1993, Eriksson et al.,
	Giα	down	Eriksson et al., 1992a

neuropathology, despite the critical role of these G proteins in neuronal function. This surprising result may reflect compensatory changes in other G protein subunits, most notably $Gi_1\alpha$ and $Gi_3\alpha$. Upregulation of both inhibitory G protein subunits has been observed in these animals (Rudolph et al., 1996). In contrast, the recent transgenic knockout of $Gq\alpha$ has been shown to render platelets unresponsive to platelet activators, with little evidence of compensatory changes in $Gi\alpha$ or $\beta\gamma$ subunits (Offermanns et al., 1997). The effects of these knockouts on neuronal development are unknown.

I.3. SIGNAL TRANSDUCTION IN THE BASAL GANGLIA

The basal ganglia are a collection of subcortical nuclei that are involved in the control of movement (Cote and Crutcher, 1991, Mello and Villares, 1997). They are not directly connected to the corticospinal or extrapyramidal motor pathways, but receive innervation from neurons in the cerebral cortex and project back to the motor cortex through the thalamus. The precise role of the various basal ganglia structures in motor control is not entirely clear, but they are believed to play a general regulatory role in the initiation and propagation of voluntary movements.

I.3.1. Structure and Function of the Basal Ganglia

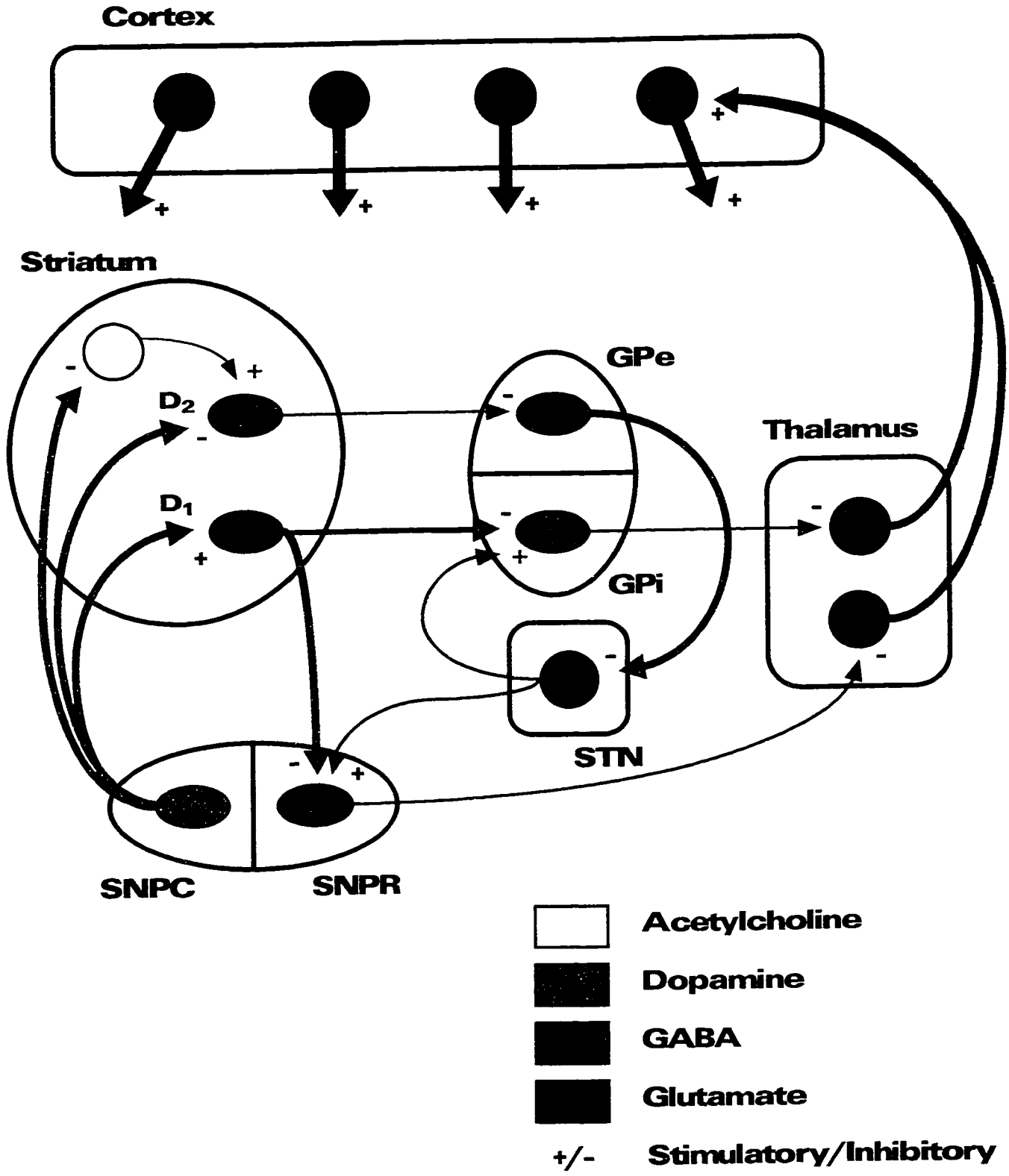
As illustrated in Figure 10, the basal ganglia consist of the substantia nigra (pars compacta and pars reticulata segments), striatum (caudate and putamen), globus pallidus

(external and internal segments), subthalamic nucleus, and the nuclei of the thalamus. Despite their apparent segregation from the corticospinal and extrapyramidal motor pathways, attempts to separate out the specific contributions of the basal ganglia to motor control have proven difficult due to the extensive interconnections of this network (Cote and Crutcher, 1991).

The precise cellular localization of dopamine receptors and their respective G proteins needs to be addressed in any meaningful discussion of dopamine receptor supersensitivity. If D_1 and D_2 receptors are co-localized on the same neuron, increased signaling through Giα would be expected to inhibit Gsα-stimulated adenylyl cyclase. This could occur through the direct competition of Giα for adenylyl cyclase, or through thermodynamic inhibition of Gsα as a result of the greater levels of inhibitory $\beta\gamma$ subunits released by activated Giα, as previously described in Section I.2.2 (Figure 7). There is some evidence for the co-localization of D_1 and D_2 receptors on striatal neurons, based mainly on electrophysiological and immunohistochemical findings (Surmeier et al., 1993). However, the majority of available evidence, gathered from a large number of pharmacological and biochemical studies, suggests that the majority of D_1 and D_2 receptors are located on discrete populations of striatal neurons (Gerfen, 1992b, Graybiel, 1995, Albin et al., 1995, Korczyn, 1995). Nevertheless, these so-called output neurons may be extensively collateralized, providing branches to multiple basal ganglia structures (Parent and Cicchetti, 1998). The standard model of striatal function within the basal ganglia, consisting of separate and distinct D_1 and D_2 receptor output pathways, is presented in Figure 10.

Figure 10. Basal ganglia neurochemistry and neuroanatomy

This figure depicts the main neurochemical connections between basal ganglia nuclei. Thickness of the arrows indicate the relative strength of each of the connections between groups of neurons, with +/- indicating whether the effect is stimulatory (excitatory) or inhibitory. All nuclei depicted in this figure, with the exception of the globus pallidus, receive glutamatergic afferents (input) from the cortex (omitted for the sake of clarity). Similarly, thalamic glutamatergic efferents (output) to the striatum are also omitted. STN refers to the subthalamic nucleus, GPe and GPi refer to the external and internal segments of the globus pallidus, and SNPC and SNPR refer to the substantia nigra pars compacta and pars reticulata, respectively. The predominant striatal efferent pathways consist of GABAergic neurons under the control of excitatory glutamatergic cortical neurons, modulated by substantia nigra dopaminergic neurons. Dopamine stimulation at D_1 receptors is believed to activate a subpopulation of striatal GABAergic neurons that project to the internal segment of the globus pallidus and the substantia nigra pars reticulata, which in turn project to the thalamus ("direct" pathway). D_2 receptor stimulation results in the activation of GABAergic neurons that project predominantly to the external globus pallidus ("indirect" pathway). These neurons in turn are connected to the thalamus through the subthalamic nucleus and internal globus pallidus. Note that the ultimate effect of dopamine release in the striatum is a relatively low level of inhibition of excitatory thalamic neurons. Adapted from Graybiel, 1990, Carlsson and Carlsson, 1990, Gerfen, 1992, Albin et al, 1995, Calabresi et al, 1996, and Mello and Villares, 1997.


Cortex
Striatum
GPe
Thalamus
D2
D1
GPi
STN
SNPC
SNPR
Acetylcholine
Dopamine
GABA
Glutamate
+/- Stimulatory/Inhibitory

Interestingly, co-activation of D_1 and D_2 receptors is often required to elicit a given behavioural response. This permissive interaction between dopamine receptors has been referred to as D_1/D_2 synergism (Robertson, 1992a). The physiological basis of this phenomenon is unclear. One possible explanation for this effect is suggested by the nature of the interconnections of the various basal ganglia nuclei. Specifically, although D_1 and D_2 receptors are likely segregated on different populations of neurons at the level of the striatum, signals from these distinct output paths may ultimately converge further downstream, for example at the level of the thalamus. Alternatively, the acute collateralization of striatal output pathways may allow for more local interactions between closely associated structures, such as the globus pallidus and substantia nigra pars reticulata.

The various nuclei that make up the basal ganglia comprise a diverse range of neurotransmitter and neuropeptide systems (Graybiel, 1990, Albin et al., 1989, Graybiel, 1995, Gerfen, 1992a, Albin et al., 1995). For example, in addition to the pivotal roles of dopamine and glutamate in the striatum, this region also contains the largest concentration of acetylcholine in the CNS (Gilman and Newman, 1992). Accordingly, the nature of the interactions between these neurotransmitter systems is extremely complex. Based on the standard model presented in Figure 10, dopamine receptors are primarily located on medium-size spiny neurons that use the inhibitory neurotransmitter γ-aminobutyric acid (GABA). These GABAergic neurons comprise the predominant efferent or output pathways from the striatum. Under resting conditions, these output neurons are under the direct control of excitatory glutamatergic cortical neurons (Mello

and Villares, 1997). Dopamine, released from the striatal terminals of substantia nigra neurons, modulates the activity of these neurons through D_1 and D_2 dopamine receptors.

According to this model, dopamine stimulation at D_1 receptors activates a subpopulation of GABAergic striatal output neurons that predominantly project to, and inhibit, GABAergic neurons in the internal segment of the globus pallidus and the substantia nigra pars reticulata. Neurons in these regions in turn project topographically to the thalamus, where they inhibit predominantly glutamatergic neurons. The thalamus provides excitatory feedback to the frontal cortex and striatum. This pathway is often referred to as the "direct" pathway, as it consists of only one intermediate group of neurons between the striatum and the thalamus. The second striatal output pathway, consisting of GABAergic neurons under the control of D_2 receptors, project predominantly to the external globus pallidus. This pathway is termed the "indirect" pathway as it is connected to the thalamus only indirectly through the subthalamic nucleus and internal globus pallidus (Mello and Villares, 1997).

In addition to the presence of specific dopamine receptor subtypes, the striatal output pathways can also be characterized by the neuropeptides they synthesize and release. Neurons of the direct output pathway are rich in the neuropeptides substance P and dynorphin, while the indirect output neurons contain met-enkephalin and possibly neurotensin (Gerfen et al., 1991). There are also a large number medium aspiny neurons in the striatum which are cholinergic in nature and contain the neuropeptides somatostatin and neuropeptide Y. These neurons are believed to serve primarily as interneurons involved in modulating dopamine/glutamate interactions in this area (Graybiel, 1990,

Kawaguchi et al., 1995). As previously mentioned, however, this model should be taken as only a crude approximation of the rich interconnections of neurotransmitter and neuropeptide systems in the basal ganglia. Each component of this model is actually part of a highly complex and organized network where the pattern of activation and inhibition form an extremely precise system (Parent and Cicchetti, 1998).

I.3.2. Integration of Signal Transduction Systems

Even if we limit our examination of the basal ganglia to the striatum, it is readily apparent that a large number of neurotransmitters and their receptors need to be taken into consideration. The confluence of neurotransmitter systems in this area leads to the interaction and integration of diverse signaling pathways at the level of G proteins, effectors, second messengers, and immediate-early genes. Specifically, dopamine is known to have direct interactions with many neurotransmitter systems in the striatum, including glutamate (Di Chiara et al., 1994), adenosine (Ferre et al., 1993, Ferre et al., 1996), acetylcholine (Imperato et al., 1994, De Klippel et al., 1993, Odagaki and Fuxe, 1995), opioids (Tirone et al., 1985, Groppetti et al., 1990, Dourmap and Costentin, 1994), and GABA (Hossain and Weiner, 1995).

Given the complex nature of the various signal transduction systems present in the striatum, alterations in G proteins could potentially have dramatic effects on the signaling of multiple neurotransmitter systems. As previously mentioned, G proteins provide a great degree of flexibility in the regulatory control of signal transduction. Specifically,

upregulation of G protein levels or function could produce a compensatory effect for the loss of one transmitter system by increasing the sensitivity to other concurrent inputs. Alternatively, this integration could occur at later stages of processing, such as at the level of effectors or second messengers. These possibilities will be discussed in more detail in various sections of this thesis.

I.4. PARKINSON'S DISEASE

I.4.1. Clinical Features and Neuropathology

Parkinson's disease is a progressive neurodegenerative disorder that generally manifests itself in late adulthood or early old age (Marsden, 1994a). In fact, Parkinson's disease is actually a collection of disorders that share a common set of characteristics and pathophysiology, sometimes referred to as Parkinson's syndrome. These characteristics include the related motor syndromes of akinesia, bradykinesia, muscle rigidity, and tremor at rest. Anatomically, all forms of parkinsonism show marked degeneration of the melanin-containing dopaminergic neurons of the substantia nigra pars compacta, with the subsequent depletion of striatal dopamine (Korczyn, 1995, Marsden, 1994a). Histologically, Parkinson's disease is also characterized by the presence of Lewy body inclusions in the nigra. The significance of these cytoplasmic inclusions to disease pathology is unclear.

The reduction in dopaminergic innervation of the striatum is believed to underlie

the motor impairment present in this disorder. The discovery that L-DOPA, the metabolic precursor to dopamine (Figure 1), can reverse the clinical signs of Parkinson's disease in early stages of the disease has stimulated intensive research into dopamine neurotransmission. Despite this effort, the cause(s) of dopaminergic cell death in idiopathic Parkinson's disease remain unknown. Increased oxidative stress and free radical production have frequently been suggested as potential candidates or contributing factors (Stern, 1997). It is likely, however, that oxidative processes are secondary to some other unidentified primary cause, such as genetic or environmental factors (Jenner, 1998). Nevertheless, there are several variants of Parkinson's disease where a causative agent has been identified, such as post-encephalitis parkinsonism (PEP), MPTP- or other drug-induced parkinsonism, and parkinsonism due to carbon monoxide poisoning (Rajput, 1992).

I.4.2. Basal Ganglia Neurochemistry

In terms of the standard model of basal ganglia function presented previously (Figure 10), the loss of dopamine in the striatum would be expected to have severe consequences for basal ganglia function. Dopamine depletion would result in the altered activity of almost all the basal ganglia nuclei, as depicted in Figure 11. Specifically, the loss of dopaminergic nerve terminals in the striatum would be expected to decrease the activity of the D_1 receptor-mediated direct pathway and increase the activity of the D_2 receptor-mediated indirect pathway. This is due to the differential response of these two

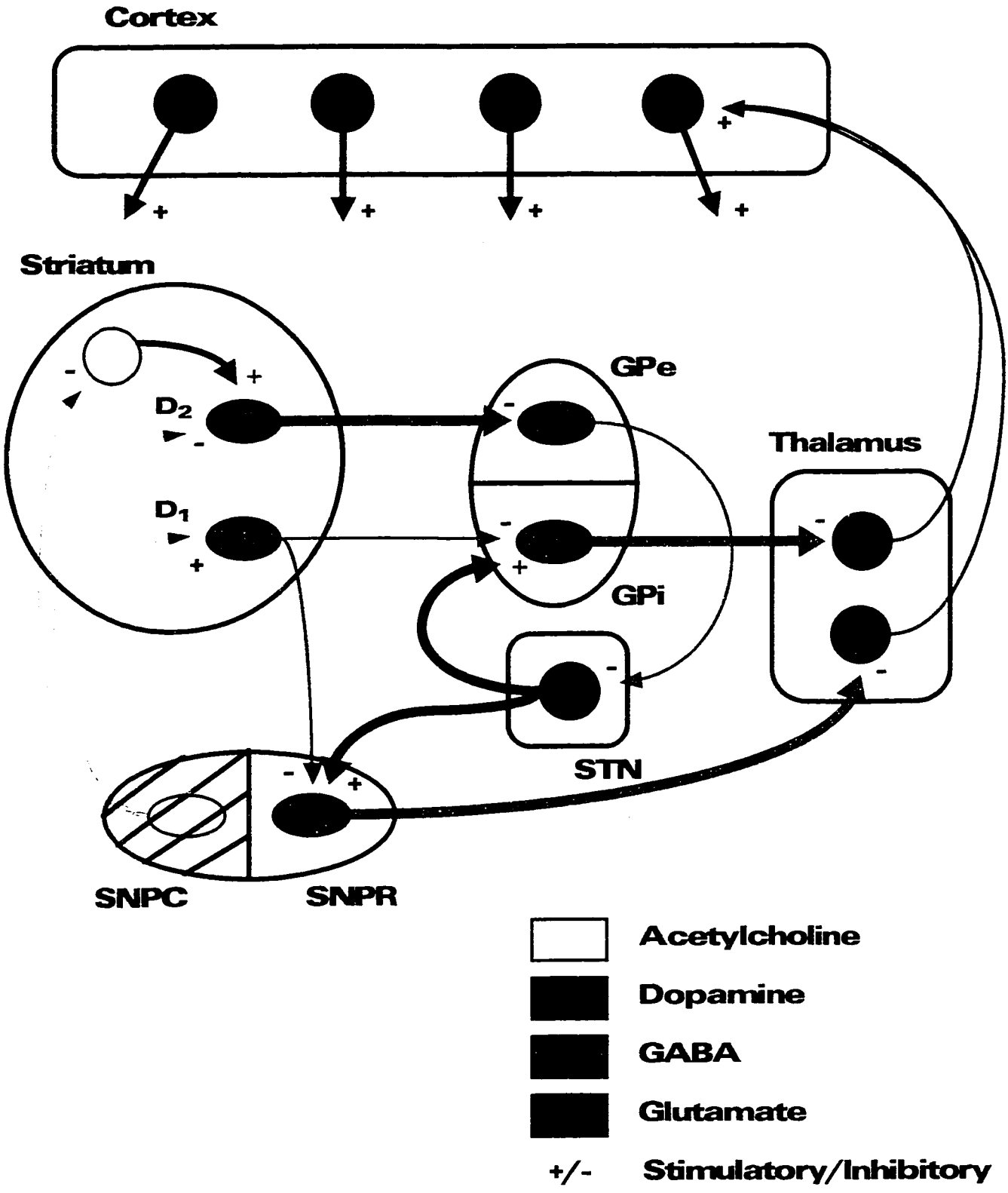
receptor subtypes to dopamine stimulation. The resulting imbalance in striatal output pathways would produce corresponding changes throughout the basal ganglia circuitry. The end result of these alterations would be an increased inhibition of excitatory thalamic neurons. The corresponding decrease in "thalamic drive" has been proposed to account for the clinical manifestations of akinesia and bradykinesia in Parkinson's disease (Marsden, 1994a).

Applications of this model have proven successful in providing a basis for surgical interventions in Parkinson's disease (Widner and Rehncrona, 1993, Krauss and Jankovic, 1996). In particular, the recent resurgence of posteroventral medial pallidotomy for severe Parkinson's disease (Lang et al., 1997), demonstrates the success of this sort of modeling approach. As appealing as the model presented in Figure 11 may be, however, it is ultimately an over-simplification of the long-term consequences of dopamine denervation on basal ganglia function. The model suffers from several important limitations, especially in its inability to accommodate more recent detailed anatomical and functional studies of the basal ganglia (Parent and Cicchetti, 1998). Unfortunately, formulating a coherent model of basal ganglia function remains problematic, and strict adherence to current arrow-type circuitry models has been severely criticized (Landau, 1993, Landau, 1990).

The lack of clear understanding of the underlying cellular mechanisms of Parkinson's disease has hampered the design of more effective therapeutic agents. Although dopamine receptor changes in Parkinson's disease have been well documented (Gerlach and Riederer, 1993, Seeman and Niznik, 1990), little is known about alterations

Figure 11. Basal ganglia in Parkinson's Disease

This figure depicts the neurochemical alterations in basal ganglia function in Parkinson's disease. As in Figure 10, the thickness of the arrows indicate relative strength of each of the connections between groups of neurons, with +/- indicating whether the effect is stimulatory (excitatory) or inhibitory. STN refers to the subthalamic nucleus, GPe and GPi refer to the external and internal segments of the globus pallidus, and SNPC and SNPR refer to the substantia nigra pars compacta and pars reticulata, respectively. The loss of SNPC dopaminergic neurons in Parkinson's disease, and the corresponding depletion of striatal dopamine levels, results in a reversal of the relative strengths of the efferent (output) pathways of the striatum. Specifically, activity of the "indirect" D_2 receptor-mediated pathway is enhanced, while the "direct" D_1 receptor pathway is reduced. These alterations are reflected throughout the circuits, culminating in a greatly increased inhibition of thalamic neurons. This in turns reduces the excitatory output of both thalamic and cortical neurons, and may underlie the clinical manifestations of akinesia and bradykinesia in Parkinson's disease. This model is adapted from Hallet, 1993, Marsden and Obeso, 1994, Graybiel, 1996, and Bergman et al., 1998.

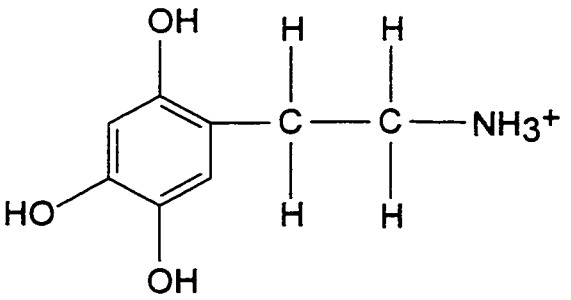

Cortex
Striatum
GPe
GPi
Thalamus
STN
SNPC
SNPR
D2
D1
Acetylcholine
Dopamine
GABA
Glutamate
+/- Stimulatory/Inhibitory

in other postsynaptic signal transduction components. As previously mentioned, integration of multiple receptor systems at the G protein level may produce beneficial effects to compensate for the loss of dopamine in the striatum. Interestingly, Parkinson's disease patients are asymptomatic for the disorder until a critical threshold of approximately 80% cell loss occurs. Although this apparent redundancy most likely occurs through presynaptic mechanisms that maintain relatively constant dopamine levels in the striatum, increased responsiveness of G proteins could play a role.

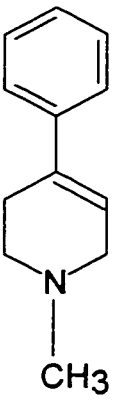
I.4.3. Animal Models of Parkinson's Disease

Like many neuropsychiatric and neurodegenerative disorders (Henn and McKinnery, 1987), Parkinson's disease has been extensively modeled in animals. Although the underlying cause of Parkinson's disease remains unknown, animal models can readily be developed that display many of the characteristics of the disease (i.e. good face validity). Similarly, animal models can be developed to test specific causative theories (construct validity), or to test novel therapeutic agents or procedures (predictive validity). Two of the animal models widely used in Parkinson's disease research are the 6-hydroxydopamine (6-OHDA) lesioned rat model and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model. The potential role of G proteins in mediating dopamine receptor supersensitivity was examined in both models for this thesis. The chemical structures of these two neurotoxins are presented in Figure 12.

Figure 12. Structure of the dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-tetrahydropyridine (MPTP)



6-HYDROXYDOPAMINE



MPTP

I.4.3.1. 6-Hydroxydopamine (6-OHDA) Lesioned Rats

Unilateral infusions of 6-hydroxydopamine (6-OHDA) into the substantia nigra pars compacta have long been used as a model of hemi-parkinsonism in the rat (Robertson, 1992b, Herrera-Marschitz and Ungerstedt, 1984). 6-OHDA is a selective neurotoxin for catecholaminergic neurons that is taken up through the dopamine transporter (see Figure 2). Although the precise mechanism of action of 6-OHDA remains controversial, the majority of available evidence supports the role of auto-oxidation and the production of reactive oxygen species in mediating cell death (Oertel and Kupsch, 1993, Kumar et al., 1995, Ma et al., 1995). Alternatively, the covalent binding of various quinone oxidation products of 6-OHDA to cellular proteins may also be involved in cell toxicity (Graham et al., 1978). Lesioning of the substantia nigra with 6-OHDA results in the corresponding depletion of the dopamine levels in the ipsilateral striatum. The resulting supersensitivity of dopamine receptors in the depleted striatum can be demonstrated behaviourally by administration of a dopamine receptor agonist such as apomorphine. In lesioned animals, apomorphine produces contralateral rotational behaviour (i.e. rotation away from the side of the lesion) due to the hemispheric imbalance in dopamine receptor sensitivity. The pharmacological characteristics of this rotational behaviour suggest two distinct forms, corresponding to the D_1 and D_2 output pathways (Herrera-Marschitz and Ungerstedt, 1984). For a detailed review of this lesion model, see Schwarting and Huston, 1996.

Although this unilateral lesion model shows relatively poor face validity, it has

several advantages over bilateral lesioning paradigms. The unilaterally lesioned animals are less severely impaired, thus requiring less care and intervention. The extent of lesioning can quickly and easily be determined by apomorphine-induced rotations (Schwarting and Huston, 1997). Moreover, the contralateral hemisphere can serve as an internal control for comparison in biochemical or molecular analyses. However, the potential compensatory changes in the contralateral hemisphere must be still be taken into account (Robinson, 1991).

Dopamine receptor supersensitivity observed in this model does not appear to be adequately explained by dopamine receptor upregulation. Increased dopamine D_2 receptor levels in the striatum have been reported in these animals, but the magnitude of this effect is generally small, in the order of 20-30% (Srivastava and Mishra, 1994). There are conflicting reports for striatal dopamine D_1 receptors in this model. Some studies have shown a transient or persistent increase in D_1 receptors, while most have found either no change or a decrease in D_1 receptor levels (see Table IV). The reason for this discrepancy in D_1 receptor studies is unclear. It may reflect differing lesioning methodologies, choice of dopamine ligands, or temporal changes in receptor expression. Interestingly, this pattern is not observed in chronic receptor blockade studies, where selective D_1 and D_2 antagonists have consistently been reported to increase respective dopamine receptor levels in the striatum (McGonigle et al., 1989). In the case of 6-OHDA lesioned rats, however, dopamine receptor supersensitivity does not appear to be maintained by an increase at the receptor level alone, at least not for the D_1 receptor system. This latter result is of particular interest since the ability of dopamine or D_1

Table IV

Dopamine receptor levels following 6-OHDA lesioning

Dopamine Receptor	Protein/mRNA Expression	Effect	Reference
D_1	Protein Levels	Increased	Buonamici et al., 1986, Porceddu et al., 1987
		Decreased	Ariano, 1988, LaHoste and Marshall, 1989, Marshall et al.,1989, Gerfen et al., 1990, Joyce, 1991a, Joyce, 1991b, LaHoste and Marshall, 1991, Blunt et al., 1992, Qin et al., 1994
		No Change	Altar and Marien, 1987, Breese et al., 1987, Savasta et al., 1988, Graham et al., 1990b, Cadet et al., 1991, Thomas et al., 1992, Chritin et al., 1993
	mRNA Levels	Increased	--
		Decreased	Gerfen et al., 1990, Qin, Chen and Weiss, 1994
		No Change	Chritin, Feuerstein and Savasta, 1993

Dopamine Receptor	Protein/mRNA Expression	Effect	Reference
D_2	Protein Levels	Increased	Thal et al., 1979, Heikkila et al., 1981, Stauton et al., 1981, Feuerstein et al., 1981, Neve et al., 1984, Savasta et al., 1987, Savasta et al., 1988, Marshall et al., 1989, Graham et al., 1990b, Joyce, 1991a, Blunt et al., 1992, Jenner and Marsden, 1992, Cadet and Zhu, 1992, Qin et al., 1994
		Decreased	--
		No Change	Breese et al., 1987, Thomas et al., 1992, Chritin et al., 1993
	mRNA Levels	Increased	Coirini et al., 1990, Gerfen et al., 1990, Angulo et al., 1991, Lisovoski et al., 1992, Qin et al., 1994
		Decreased	--
		No Change	Chritin et al., 1993

–, Not reported

receptor agonists to stimulate cAMP production is enhanced in the lesioned striatum (Barone et al., 1994, Gnanalingham et al., 1995).

I.4.3.2. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Treated Mice

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to produce a severe parkinsonian-like state in humans and several animal species (Gerlach and Riederer, 1996, Oertel and Kupsch, 1993). MPTP is highly selective for the dopaminergic neurons of the substantia nigra, and closely mimics the pattern of neurotransmitter depletion seen in Parkinson's disease. The mechanism of action of MPTP is not entirely understood, but is believed to involved oxidative stress through the biotransformation of MPTP into the cytotoxic metabolite 1-methyl-4-phenylpyridinium ion (MPP+) by monoamine oxidase B (Gerlach et al., 1991). In addition to free radicals produced by the reduction and oxidation of MPP+, impairment of mitochondrial respiration has also been proposed as a potential mechanism of action of this agent. Specifically, MPP+ can be taken up and concentrated within the mitochondria, where it blocks complex I of the electron transport chain (Tipton and Singer, 1993). This in turn results in the rapid depletion of cellular energy supplies in the form of ATP, and the increased production of free radicals. More recently, nitric oxide and the nitration of tyrosine have also been implicated in dopaminergic cell death following MPTP treatment (Przedborski and Jackson-Lewis, 1998).

Although much interest has focused on MPTP and MPTP-like compounds as

potential sources of idiopathic Parkinson's disease, not all of the structural and motor abnormalities of Parkinson's disease are reproduced by MPTP (Gerlach et al., 1991). Moreover, MPTP animal models tend to show behavioural recovery with time, in contrast to the progressive degeneration seen in humans (Gerlach et al., 1991, Burns, 1991). Although a variety of explanations for functional recovery have been put forward, including the regrowth or collateral sprouting of nerve terminals (Hallman et al., 1985), the underlying biochemical basis of this phenomenon is unclear (Gerlach and Riederer, 1996).

One popular animal model of MPTP lesioning involves the systemic administration of MPTP to C57 black mice (BL/6) (Sundstrom et al., 1990). Dopamine receptor supersensitivity can be difficult to establish in this animal model, however, due to the bilateral lesioning of the substantia nigra. Nevertheless, there are several reports of increased dopamine sensitivity in these animals (Lau and Fung, 1986, Lange, 1990, Fredriksson et al., 1994). Corresponding increases in D_1 or D_2 receptors are not generally found (Ogawa et al., 1987, Camps et al., 1989, Lange, 1990, Bhargava and Perlow, 1988), although small and transient increases in D_2 receptors have been reported (Lau and Fung, 1986, Peroutka et al., 1985). The similarity of the dopamine receptor response in MPTP and 6-OHDA lesion models suggests that comparable changes in G protein levels may also occur in these models.

I.5. ANTISENSE OLIGONUCLEOTIDES

One of the most popular approaches to studying the function and regulation of cellular constituents is the use of transgenic animals with targeted gene disruption. These so-called "gene knockout" animals allow the examination of the effects of specific gene deletions. Although conceptually elegant and technically effective, this approach is limited by various factors, including cost, difficulty, irreversibility, potential lethality of the mutations, and compensatory effects during development. An alternative approach to delineating the role of specific proteins is selective blockade using locally administered antisense oligonucleotides. These agents have the potential to temporarily "knockdown" gene expression while avoiding many of the difficulties inherent in transgenic models. However, antisense approaches have their own associated limitations, such as degree of effectiveness, stability of oligonucleotides, reliability, and potential toxicity. In order to help delineate the role of G proteins in mediating dopamine receptor supersensitivity, in vivo antisense oligonucleotides were administered to 6-hydroxydopamine (6-OHDA) lesioned rats.

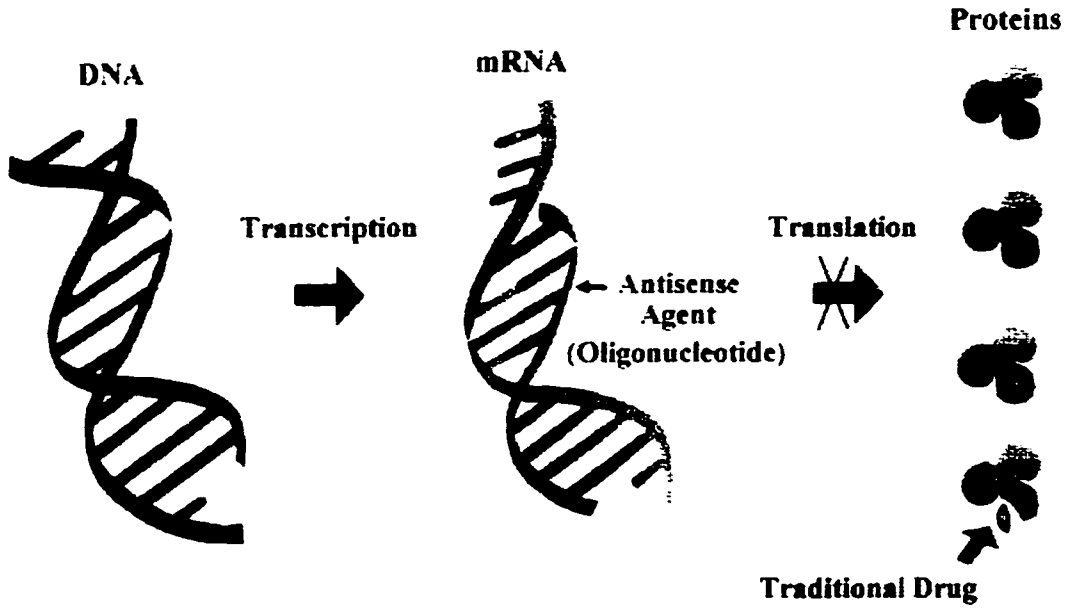
I.5.1. Mechanism of Action of Antisense Agents

Oligonucleotides can be designed to block protein synthesis in a number of ways. Oligonucleotides may be used to inhibit transcription by DNA strand invasion, triple-strand binding, or binding to transcription factors (Helene, 1991). Far more common,

however, is the use of oligonucleotides to inhibit translation via an antisense mechanism. Antisense oligonucleotides are short sequences of DNA that are directed against a complementary sequence of a target mRNA. The exogenously applied oligonucleotide hybridizes to the target sequence through the pairing of complementary base sequences (i.e. A with U, G with C) (Baertschi, 1994). The corresponding DNA:RNA hybrid, or duplex, may prevent protein translation in several ways, many of which are not fully understood at present. The most obvious mechanism is translational arrest, whereby the ribosomal peptide assembly cannot proceed with protein synthesis, as presented in Figure 13. Accordingly, a favorite mRNA target for oligonucleotide design is the translation initiation site (i.e. AUG). Another potential mechanism of action for antisense agents is the activation of RNase H, a ubiquitous enzyme that cleaves duplex RNA (Baertschi, 1994). Other possible mechanisms of action include oligonucleotide-induced cleavage of RNA or the destabilization of RNA metabolism (Crooke, 1994).

Although novel, oligonucleotide administration shares a similar conceptual framework to classical pharmacology. For example, oligonucleotides fulfill the pharmacological requirements of high affinity and specificity. These characteristics are achieved through hydrogen bonding of stacked bases and the selectivity of Watson-Crick base pairing, respectively (Crooke and Bennett, 1996). Oligonucleotide administration faces several potential difficulties, however, especially in regard to cellular uptake, nuclease stability, pharmacokinetics and toxicity, and potential non-specific effects. Of these factors, cellular uptake does not appear to be a major stumbling block. There is ample evidence that cells readily take up oligonucleotides through both adsorptive

Figure 13. Mechanism of action of antisense oligonucleotides

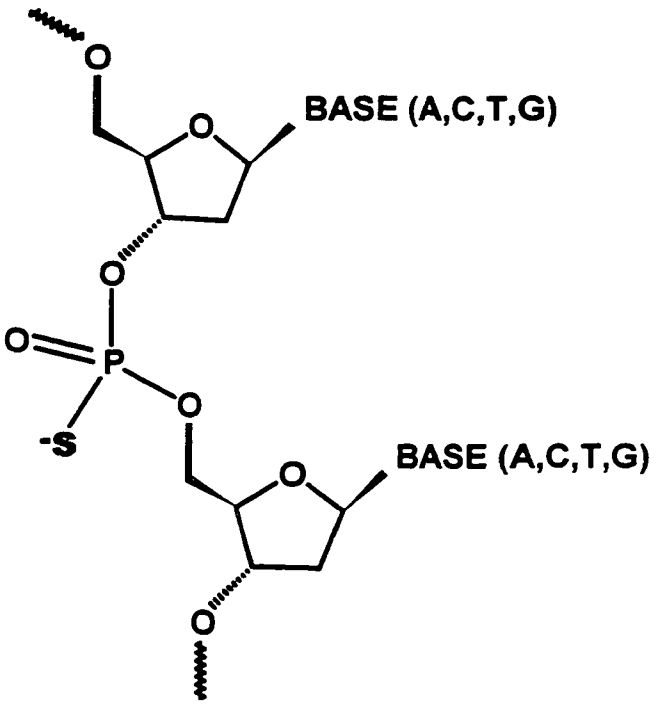


Antisense oligonucleotides are short sequences of DNA that are complementary to a target sequence of mRNA. Upon administration, antisense oligonucleotides selectively bind to the target mRNA. The resulting DNA:RNA hybrid can block protein translation in several potential ways (see text). Antisense oligonucleotides share many conceptual similarities to classical pharmacological agents, including high specificity and affinity. Traditional drugs, however, tend to act directly at the level of proteins. (Source: ISIS Pharmaceuticals)

endocytosis (pinocytosis) and receptor-mediated transport systems (Crooke and Bennett, 1996). Stability of oligonucleotides is a far greater concern, however, and has prompted the synthesis of nuclease-resistant oligonucleotide analogues. The most popular of these modifications is the replacement of a non-bridging oxygen atom with a charged sulfur atom in the sugar-phosphate backbone of the synthetic oligonucleotide (see Figure 14). These phosphorothioate analogues possess many of the same characteristics of unmodified phosphodiester oligonucleotides, but with the benefit of increased nuclease stability. Phosphorothioate oligonucleotides are water soluble, potent at nanomolar concentrations, capable of activating RNase H, and efficiently taken up by cells (Baertschi, 1994). However, they are also less sequence-specific at higher concentrations and may be more toxic than regular phosphodiester oligonucleotides. Moreover, although the pharmacokinetics of these agents have been well established in vitro, there is now ample evidence that in vivo pharmacokinetics may not be comparable (Crooke et al., 1995).

Perhaps the greatest obstacle to the successful application of oligonucleotide agents is their potential non-specific mechanisms of action. Non-specific binding to other nucleic acids and proteins are believed to represent the major sources of non-antisense effects. This binding can be both sequence-independent, sequence-specific or structure-specific. Moreover, although there are multiple studies that appear to demonstrate a specific antisense mechanism of action for these agents, there are also many cases where differentiation of antisense and non-antisense effects is difficult (Crooke and Bennett, 1996). Moreover, many antisense studies have proved to be difficult to reproduce,

Figure 14. Structure of phosphorothioate oligonucleotides



Schematic diagram of the sugar-phosphate backbone of DNA. Oligo-nucleotide bases (A,C,T,G) are linked to sugar residues that are joined by phosphate groups in a 5' to 3' orientation. Substitution of a charged oxygen species with a charged sulfur atom in the phosphate backbone increases nuclease resistance and stability of oligonucleotides.

especially with the addition of more stringent controls for non-specific effects (Robinson et al., 1997). The potential nature of these non-specific effects will be discussed more fully in Section IV along with the results of the G protein antisense studies performed here.

I.5.2. In Vivo Administration of Antisense Oligonucleotides

Antisense agents provide the opportunity for dissecting molecular processes in living systems as well as under in vitro conditions. Of particular relevance to this thesis, antisense oligonucleotides have been used successfully in inhibiting dopamine D_2 receptor expression in 6-OHDA lesioned mice (Weiss et al., 1993, Qin et al., 1995, Zhang and Creese, 1993, Silvia et al., 1994) (for a more detailed review of in vivo applications of antisense oligonucleotides, please see Crooke and Bennett, 1996). These studies have provided the impetus for the examination of the effects of G protein antisense oligonucleotides on dopamine receptor supersensitivity performed here.

Given the pivotal role of G proteins in regulating cellular events, it is not surprising that the effectiveness of antisense oligonucleotides in reducing G protein levels has been demonstrated both in vitro and in vivo, as summarized in Table V. As can be seen in this table, most of the in vitro studies were designed to illustrate the role of G proteins in mediating receptor-generated signals. Although relatively few in vivo studies have been performed using G protein antisense oligonucleotides, a similar trend can also be observed (Table V). Also of interest, all of the in vivo G protein studies performed to

Table V

Effects of G protein antisense oligonucleotides

G Protein	Model	Effect of Antisense Treatment	Reference
In Vitro			
$Go\alpha_1$, $Go\alpha_2$	IIC9 cell line	Growth in absense of mitogen	Weber et al., 1997
$Gi\alpha_3$, $G\beta_1$, $G\gamma_3$	Rat portal vein myoctes	Blockade of angiotensin AT_{1A} receptor signals	Macrez-Lepretre et al., 1997a
$Gq\alpha$, $G_{11}\alpha$, $G\beta_1$, $G\beta_3$, $G\gamma_2$, $G\gamma_3$	Rat portal vein myoctes	Inhibition of coupling of α_1-adrenoreceptors to Ca^{2+} channels	Macrez-Lepretre et al., 1997b
$Gq\alpha$, $G_{11}\alpha$, $Go\alpha_1$, $G_{14}\alpha$	RBL-2H3-hm1cell line	Differential effects on muscarinic m1 signaling	Dippel et al., 1996
$Gs\alpha$	HL-60 cell line	Regulation of cell differentiation	Meissner et al., 1996
$Go\alpha$	HL-60 cell line	Decreased Ca^{2+} response	Goetzl et al., 1994
$Gi\alpha_2$	HL-60 cell line	Decreased cAMP response	Goetzl et al., 1994
$Gs\alpha$	Hypothalamic neurons	Modulation of Ca^{2+} channel currents	Costa et al., 1995
$Gq\alpha/G_{11}\alpha$	Hypothalamic neurons	Inhibition of carbachol-stimulated K^+ currents	Buckley et al., 1995
$Go\alpha_1$, $G\beta_2$, $G\beta_3$, $G\gamma_3$,$G\gamma_4$	RINm5F cell line	Reduced galanin-induced inhibition of Ca^{2+} channels	Kalkbrenner et al., 1995
$Gi\alpha_3$	RINm5F cell line	Reduced galanin-induced inhibition of AC	de Mazancourt et al., 1994
$Gi\alpha_2$	ND8-47 cell line	Inhibition of opioid-induced increased Ca^{2+}	Tang et al., 1995
$Gi\alpha_1$	Nb 2 lymphoma cell line	Decreased soluble PLC activity	Akompong et al., 1994
$Gs\alpha$	Xenopus oocytes	Inhibition of TRH signaling	de la Pena et al., 1995
$Gq\alpha/G_{11}\alpha$	Xenopus oocytes	Blockade of muscarinic m3 signaling	Stehno-Bittel et al., 1995
$Go\alpha$	Xenopus oocytes	Decreased 5-HT-induced Cl^- currents	Quick et al., 1994
$Gq\alpha$	Xenopus oocytes	Decreased TRH-induced Cl^- currents	Quick et al., 1994
$Go\alpha$, $G_{11}\alpha$	Xenopus oocytes	Inhibition of $5\text{-}HT_{2C}$ receptor response	Chen et al., 1994
$Gi\alpha_2$	GH3 cell line	Decreased Ca2+ channel stimulation	Hescheler and Schultz, 1994

G Protein	Model	Effect of Antisense Treatment	Reference
$Gq\alpha/G_{11}\alpha$	GH3 cell line	Decreased PI stimulation	Hescheler and Schultz, 1994
$Gi\alpha_2$, $Gi\alpha_3$	GH3 cell line	Decreased TRH-stimulated Ca^{2+} channel	Gollasch et al., 1993
$Gi\alpha_2$	F9 stem cell line	Increased phospholipase C activity	Watkins et al., 1994
$Gi\alpha_2$	17/2.8 cell line	Increased phospholipase C activity	Watkins et al., 1994
$Go\alpha$	Cultured DRG neurons	Inhibition of coupling of $GABA_B$ receptors to Ca^{2+} channels	Campbell et al., 1993
In Vivo			
$Gi\alpha_1$, $Gi\alpha_3$, $Go\alpha$, $Gs\alpha$,$Gx/z\alpha$	ICV administration	Differential blockade of opioid analgesia	Standifer et al., 1996
$Gs\alpha$, $Gi\alpha_1$, $Gi\alpha_2$	ICV administration	Differential regulation of α2-adrenoreceptor antinociception	Raffa et al., 1996a
$Gi\alpha_2$	ICV administration	Differentiation of morphine antinociception	Raffa et al., 1996b, Raffa et al., 1994
$Go\alpha_A$, $Go\alpha_B$	ICV administration	Decreased nighttime food intake	Plata-Salaman et al., 1995
$Gi\alpha_1$, $Gi\alpha_2$	Periaqueductal gray	Blockade of opioid analgesia	Rossi et al., 1995

date have taken place in the central nervous system, further supporting the viability of the studies proposed here. However, most of the previously reported in vivo studies have involved intracerebroventricular (ICV) administration, which is by definition non-localized and therefore potentially less specific. To overcome this potential limitation, G protein antisense oligonucleotides were directly infused into the rat striatum using intracranial cannulas in these studies.

I.5.2. Clinical Applications of Antisense Technology

Despite the uncertainty surrounding the mechanism of action of antisense agents in vivo, several clinical trials involving antisense oligonucleotides are currently in progress, as listed in Table VI. In addition, there are also a large number of antisense agents under development as potential therapeutics. Many of these agents are currently being tested in animal models of human disease. Although antisense agents offer a great deal of potential in treating human disorders, there remains much basic research to be done. The first step in determining the efficacy and safety of antisense oligonucleotides is their administration in vivo. Moreover, the design of improved antisense agents will necessitate their testing in animal models. The expense and difficulty of clinical trials also mandate that the mechanism of action of these agents be clearly delineated before human experimentation proceeds. To date, this requirement has only been partially satisfied, further emphasizing the need for basic research into these agents.

Table VI

Current clinical trials involving antisense oligonucleotides

Disease	Target	Status	Company
CMV Retinitis in AIDS patients	HCMV	Phase III	Isis/CIBA
Crohn's Disease	ICAM-1	Pivotal Trial	Isis/Boehringer
Rheumatoid Arthritis	ICAM-1	Phase II	Isis/Boehringer
Psoriasis	ICAM-1	Phase II	Isis/Boehringer
Ulcerative Colitis	ICAM-1	Phase II	Isis/Boehringer
Renal Transplant Rejection	ICAM-1	Phase II	Isis/Boehringer
Cancer	PKC-α	Phase II	Novartis
	c-*raf* kinase	Phase I	Novartis
	c-*raf*	Phase I	Isis
	Ha-*ras*	Phase I	Isis
	c-*myb*	Phase I / II	?
AIDS	HIV	Phase I	Isis
	HIV	Phase II	Hybridon

Source: Crooke and Bennet, 1996, Hybridon, and ISIS Pharmaceuticals.

I.6. AIMS OF THIS STUDY

As described in this Introduction, the role of G proteins in modulating cellular responses to external signals has been increasingly recognized in recent years. The previous emphasis on receptor levels and expression as potential mediators of post-synaptic cellular response is understandable given their primary role in signal transduction. However, in cases such as dopamine receptor supersensitivity where receptor upregulation seems an insufficient explanation, examination of G protein levels and function is a reasonable course of action. There is increasing evidence for the role of G proteins and other signaling elements such as second messengers (Wanderoy et al., 1997) in mediating striatal dopamine receptor supersensitivity.

G protein levels were examined in this research project using antibodies raised against specific regions of various G proteins. Given the difficulties associated with post-mortem tissue analysis (see Section II.1), the majority of these studies were performed in animal models of Parkinson's disease (Sections II.2, II.3). As with any animal model of a human disorder, however, it is important to consider not only the applicability of these models, but their inherent limitations as well. As such, comparison of multiple models is frequently required to come to any meaningful conclusion regarding the nature of the phenomenon under study.

In addition to examination of G protein levels, direct functional measures of activity are required to establish the role of these proteins unequivocally. In this regard, several G protein functional assays were attempted for this research project. These

include measures of GTPase activity (Section III.1) and [α-^{32}P]-GTP binding (Section III.2) in the striatum. The functional roles of these proteins can also be determined by the effects of specific reductions in G protein expression on behavioural and biochemical measures of supersensitivity. Accordingly, antisense oligonucleotides targeted against specific G protein subunits were administered to 6-OHDA lesioned rats (Section IV). Despite the enthusiasm surrounding the potential clinical use of antisense oligonucleotides, the need for further basic research is paramount. Ultimately, antisense oligonucleotides may prove to be of greatest use as molecular tools in analyzing cellular processes. It is in this capacity that G protein antisense oligonucleotides were employed in this study.

CHAPTER II

ANALYSIS OF G PROTEIN LEVELS

The most effective way to characterize G protein subtypes in cell or tissue preparations is by Western blot analysis using selective antibodies which can differentiate protein subunits (Goldsmith et al., 1987). For the G protein analyses performed here, several G protein primary antibodies were used. The specificity and origin of these antibodies are described in Section II.0.1.4. The procedure for Western blot analysis is described below.

II.0.1. Methods and Materials

All reagents for Western blot analysis were purchased from various suppliers through Scientific Stores, McMaster University. For electrophoresis studies, all reagents were of electrophoresis grade. Specific suppliers are identified throughout the text where appropriate.

II.0.1.1. Tissue Homogenization and Protein Estimation

All tissue samples were homogenized in ice-cold Tris-EDTA buffer (50 mM Tris, 1 mM EDTA, pH 8.0 at room temperature). Frozen post-mortem human brain tissue samples (-70 °C) were homogenized using a polytron apparatus with a teflon homogenizer at 4 °C. Frozen animal tissue samples (-20 °C) were homogenized by hand in a 2.5 ml glass homogenizer, with 30-35 strokes per tissue sample. The volume of buffer varied according to the wet tissue weight, in order to yield approximately equivalent total protein concentrations. The total amount of protein loaded into gel lanes for separation by SDS-PAGE was 35 μg protein.

Protein content of crude homogenates was estimated by either the Lowry method (Lowry et al., 1951) or Bradford method (Bradford, 1976). For both methods, bovine serum albumin (BSA) was used as the protein standard, and all samples and standards were dissolved in Tris-EDTA buffer (50 mM Tris, 1 mM EDTA, pH 8.0). For the Lowry assay, the 2% sodium bicarbonate (Na_2CO_3), 0.01% cupric sulfate ($CuSO_4$), and 0.02% sodium potassium tartate in 0.1 N sodium hydroxide (NaOH) was added to each tube, followed by a folin-phenol reagent (Sigma Chemical Co, St. Louis, MO). For the Bradford assay, the Biorad protein reagent (Biorad, Mississauga, ON) was added to each sample and protein standard. Absorbance readings were taken at wavelengths of 700 nm (for Lowry) or 595nm (for Bradford) using a Beckman DU-20 spectrophotometer. Homogenate concentrations (mg / ml total protein content) were estimated by linear regression analysis of the standard curve constructed from absorbance readings. Although these two protein estimation methods differ in the protein content they estimate, each is internally consistent with its own standard curve. As such, all

comparisons between samples were confined to those estimated from the same sample curve.

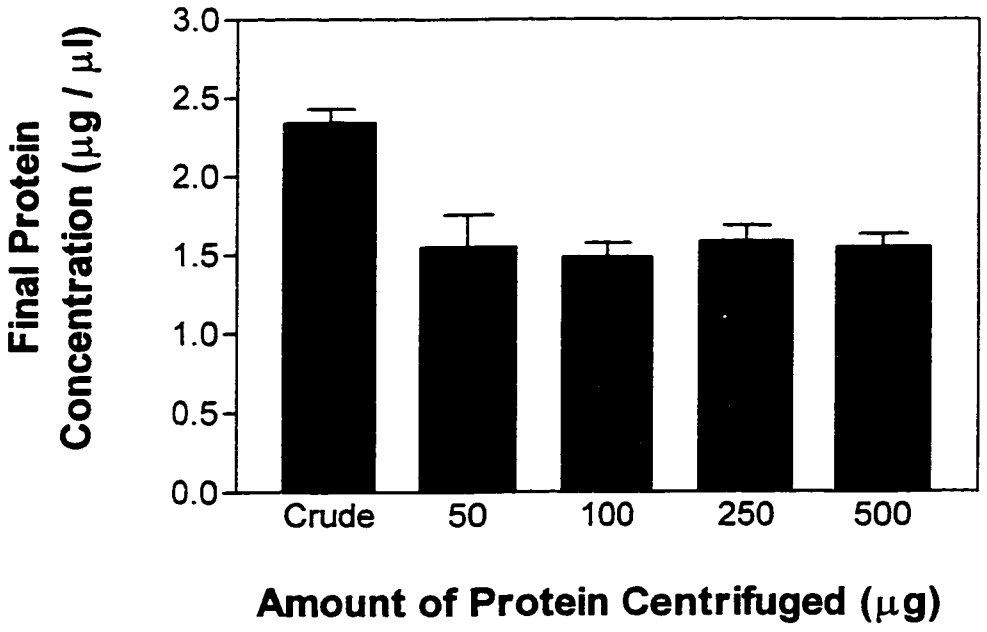
II.0.1.2. Sample Preparation

For SDS-PAGE gel electrophoresis, tissue sample homogenates from human post-mortem tissue were swamped with 3 volumes of gel loading sample buffer (0.625 M Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% β-mercaptoethanol, and 0.01% bromophenol blue, pH 6.8) and boiled for 4 min to denature the proteins. Prepared crude human tissue samples were then run immediately on SDS-PAGE gels.

For animal brain homogenates, samples were first centrifuged at 12,000 rpm (11,750 x g) for 15 - 20 min in an Eppendorf table-top centrifuge at 4°C. This step was required due to the dilute nature of these tissue samples following hand homogenization, and the limited lane volume available for gel electrophoresis. The resulting pellets were resuspended in gel loading sample buffer (0.625 M Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% β-mercaptoethanol, and 0.01% bromophenol blue, pH 6.8) and boiled for 4 min to denature the proteins. Prepared samples were run immediately on SDS-PAGE gels, or stored temporarily for up to 1 week at 4°C.

The extra centrifugation step required for animal tissue samples resulted in an expected loss of total protein content. To verify the linearity of protein loss in this semi-purified preparation, protein content was re-estimated following centrifugation in a pilot study (Figure 15). As demonstrated in this figure, protein loss was uniform

Figure 15. Loss of protein content due to centrifugation



Rat cortical tissue was homogenized by hand in Tris-EDTA buffer, and protein estimated by the Lowry method. Varying amounts of protein were spun down in an Eppendorf table-top centrifuge for 15 mins at 11,500 rpm (10,800 x g). Pelleted samples were resuspended in Tris-EDTA buffer and protein concentration was re-estimated using the same method. A sample of crude extract is shown for comparison (lane 1). As can be seen from the figure, a consistent loss of ~ 35% total protein content was observed for each of the samples.

(approximately 35% loss) across a wide range of concentrations. This result suggests that this method of sample preparation produces a consistent effect on total protein content.

II.0.1.3. SDS-PAGE Gel Electrophoresis

Polyacrylamide gels (10 - 12%) were cast according the method of Laemmli (Laemmli, 1970). SDS-PAGE was performed using either the Biorad Protean or Mini-Protean II gel electrophoresis systems (Biorad, Mississauga, ON), or the Helix Mini-gel electrophoresis system (Helix, Mississauga, ON). Acrylamide solutions (30% acrylamide (T), 2.67% N',N'-bis-methylene-acrylamide (C)) was prepared in advance and stored in the dark at 4°C for up to 60 day prior to use.

The separating gel solution (10 - 12% acrylamide solution, 0.375M Tris, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS), and 0.5% TEMED) was cast between two glass plates (Biorad, Mississauga, ON) or a polyurethane cassette (Helix, Mississauga, ON) separated by 1.5 mm for electrophoresis runs. The stacking gel (4% acrylamide solution, 0.125 M Tris, pH 6.8, 0.1% SDS, 0.05% APS, and 1.0% TEMED) was cast above the separating gel and a comb was inserted to create the lane indentations. Tissue samples (35 μg protein / lane) were loaded into the gel lanes using a pipetman with gel-loading tips. Electrophoresis gels were run in the presence of an electrophoresis running buffer (0.025 M Tris, pH 8.3, 0.20 M glycine, 1% SDS). An electrical potential of 150 - 175 V was applied to the gel apparatus for approximately 1 hour to achieve optimal separation of protein bands.

II.0.1.4. G Protein Antisera

As previously mentioned, Western blot analysis using selective G protein antibodies is the most effective way to characterize G protein levels (Goldsmith et al., 1987). For the G protein level analyses performed here, antibodies were developed by the selective immunization of rabbits with synthetic peptides corresponding to specific regions of the G protein subunit of interest. In particular, anti-peptide antibodies were raised against the α subunits of several distinct G proteins, as well as a common β subunit. The specificity and origin of these antibodies are described below.

II.0.1.4.1. Gsα

The Gsα antibodies used in these studies were obtained from either Dr. Graeme Milligan (University of Glasgow, United Kingdom), designated as CS/1, or purchased from NEN Dupont (Mississauga, ON), designated RM/1. Both of these antibodies were raised against the common sequence RMHLRQYELL, corresponding to the C-terminal end of the rat Gsα sequence (Simonds et al., 1989a, Simonds et al., 1989b). This C-terminal region is common for both Gsα and the Gs homologue known as Golf (Jones and Reed, 1989). As such, the non-specific RM/1 antiserum recognizes both Gsα and Golfα. These subunits can be distinguished on the basis of their apparent molecular weights, as Gsα isoforms are reported to appear at 45 and 52 kDa (Simonds et al., 1989a, Simonds et al., 1989b, Marcotte and Mishra, 1997), and Golfα at approximately 42 kDa

(Gupta and Mishra, 1992, Hervé et al., 1993, Marcotte et al., 1994). However, it is possible that the Gsα antibody may also recognize lower molecular weight species of Gs in some tissues (Rius et al., 1994), thus making an exact determination of Golfα with this antibody is problematic.

II.0.1.4.2. Golfα

Since the non-specific Gsα antisera desribed above recognized both Gsα and Golfα isoforms, a specific Golfα antibody was developed for use in the studies presented here. The anti-peptide Golfα antibody was synthesized by Research Genetics (Huntsville, AL), according to the method of Hervé (Hervé et al., 1993). Specifically, rabbits were immunized against the synthetic peptide CKTAEDQGVDEKERREA, derived from the N-terminal region of the published sequence for rat Golfα (Jones and Reed, 1989). The N-terminal region differs significantly from that of Gsα. The anti-peptide antibodies were subsequently purified by affinity chromatography using Sulfolink gel columns (Pierce, Brockville, ON) according to the method of Girault et al. (Girault et al., 1989). The apparent molecular weight of Golfα using these purified antibodies was 42 kDa (Hervé et al., 1993, Marcotte et al., 1994), in keeping with its previous identification (Jones and Reed, 1989).

II.0.1.4.3. Giα

Giα G proteins occur in several major isoforms, including $Gi\alpha_1$, $Gi\alpha_2$, $Gi\alpha_3$, and retinal transducin (Gt). The Giα antibodies used in these studies were obtained from either Dr. Graeme Milligan, designated SG/1, or purchased from NEN Dupont (Mississauga, ON), designated AS/7. Both of these antibodies were raised to the common sequence KENLKDCGLF, corresponding to the C-terminal regions of $Gi\alpha_1$, $Gi\alpha_2$, and transducin (Goldsmith et al., 1987, Goldsmith et al., 1988). This antibody recognized a single band of apparent molecular weight 41 kDa (Gupta and Mishra, 1992, Marcotte et al., 1994).

II.0.1.4.4. Goα

Goα antibodies were obtained from either Dr. Graeme Milligan, designated IM/1, or purchased from NEN Dupont (Mississauga, ON), designated GC/2. Given the high degree of homology between the C-terminal regions of Goα and $Gi\alpha_3$, antibodies used in these studies were raised against the specific N-terminal sequence of rat Goα, NLKEDGISAAKDVK (Simonds et al., 1989a). These antibodies recognized a single band of apparent molecular weight 39 kDa (Gupta and Mishra, 1992, Marcotte et al., 1994).

II.0.1.4.5. Gβ

The common Gβ G protein subunit used in these studies corresponds to the N-terminal sequence of rat Gβ (MSELDQLRQUE). Gβ antibodies were obtained from either Dr. Graeme Milligan, designated BN/2, or purchased from NEN Dupont (Mississauga, ON), designated MS/1. These antibodies recognized a single band of apparent molecular weight 36 kDa (Gupta and Mishra, 1992).

II.0.1.5. Western Blotting

Polyacrylamide gels were transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using either the Biorad wet tank transblotter (Biorad, Mississauga, ON), the Millipore Milliblot electroblotter system (Millipore, Etobicoke, ON), or the Helix mini-wet tank transblotter (Helix, Mississauga, ON). In all cases, polyacrylamide gels were placed opposite a nitrocellulose membrane in the presence of half-strength Towbin buffer (0.012M Tris, 0.096M glycine, 10% methanol, approximate pH 8.5 at room temp) (Towbin et al., 1979) and an electrical current was passed across the assembly. Current settings and duration varied among the different electroblotters, but never exceeded 2.5 mA/cm^2 of gel. Subsequently, nitrocellulose blots were blocked for non-specific labeling with 5% skim milk powder in Tris-buffered saline (0.05M Tris, 0.15M NaCl, pH 8.5) with 0.2% Tween 20 (Sigma, St. Louis, MO).

The degree of protein transfer varied among the different electroblotters, as estimated by the amount of residual proteins detected in the gels by Coomassie Blue staining (0.1% Coomassie Blue, 10% acetic acid, 40% methanol), or by the total protein content detected on nitrocellulose blots using Ponceau S (0.2% Ponceau S, 10% acetic acid, 20% methanol). Moreover, given the high sensitivity of the chemiluminescence detection system (described below) and the variability between transfers for the same apparatus, reliable measures of the differences between samples were limited to those run on the same polyacrylamide gel.

II.0.1.6. Immunodetection

Nitrocellulose blots were blocked for non-specific binding sites by shaking incubation for 1 hr in 5% skim milk powder in Tris-buffered saline with 0.2% Tween 20 (TBS-T; 0.05M Tris, 0.15M NaCl, 0.2% Tween 20, pH 8.5). Blocked nitrocellulose blots were used immediately in immunodetection studies, or stored for up to 3 days wet-wrapped in saran wrap at 4°C. Blots were incubated with a primary G protein antibody (1:15,000 to 1:30,000 dilution) in the presence of TBS-T in a sealed plastic pouched and left gently shaking overnight at 4°C. Prior to second antibody incubation, blots were washed 3 times in approximately 250 ml of TBS-T for 10 min each time.

For human post-mortem studies, blots were subsequently labeled with a [^{125}I]goat-antirabbit IgG antibody (1:1,000 dilution) in TBS-T for 3 hours at room temperature with gentle shaking. Immunolabeled blots were subsequently washed 3 times in 250 ml of

TBS-T for 10 min each time as before, and wet-wrapped in saran wrap. Immunoblots were placed apposed to Kodak X-OMAT film in the presence of fluorographic intensifying screens and stored at -80°C for 1-3 days, depending on the intensity of the [^{125}I] antibody. Photographic films were subsequently developed to reveal the presence of immunoreactive bands and quantified using an image analysis system.

Given the relatively poor resolution and sensitivity of this radio/fluorographic detection system, all subsequent studies were performed using an enhanced chemiluminescence system (ECL, Amersham, Oakville, ON). Following primary labeling and washing, immunoblots were labeled with an HRP-conjugated (horse radish peroxidase-conjugated) donkey-antirabbit secondary antibody (1:4,000 dilution, Amersham, Oakville, ON) in TBS-T for 1 hr at room temperature with gentle shaking. Immunoblots were subsequently washed 4 times in 250 ml of TBS-T for 10, 5, 5, and 5 min each time. Immunoblots were gently blotted to remove excess TBS-T and incubated with a chemiluminescence substrate (luminol) in the presence of hydrogen peroxide and enhancing agents (ECL, Amersham, Oakville, ON, or BCL, Boehringer Mannheim, Laval, QC) for 1 min. Subsequently, immunoblots were gently blotted to remove excess substrate and wrapped in saran wrap. The protein/antibody face of the immunoblots were exposed to Kodak X-OMAT film for 30 sec to 2 min, depending on the intensity of the chemiluminescent reaction. Photographic films were subsequently developed to reveal the presence of immunoreactive bands and quantified using an image analysis system.

As a positive control for labeling specificity, detection of secondary antibody in the absence of primary was performed for both methods. No significant labeling was

observed in either case. As a negative control for sample loading variability, immunoblots were stripped and sequentially reprobed for each of the G protein antibodies. Immunoblots were stripped of their antibodies in a stripping solution (0.063M Tris, 0.1M β-mercaptoethanol, 2% SDS, pH 6.7) at 50°C for 30 min with gentle shaking. Blots were subsequently washed 4 times in a large volume of TBS-T to remove all traces of the stripping solution. Stripped blots were then blocked in 5% skim milk powder in TBS-T for 3 hrs with gentle shaking as described previously. The sequential reprobing of individual immunoblots allowed for the direct comparison of the differences between samples with each G protein primary antibody. Since Goα G protein levels were not significantly altered by any of the treatments performed for this research project, this antibody was used as an internal control for loading variability. Moreover, each sample was run in duplicate and compared to a matched control in the same gel to verify the consistency of the response.

II.0.1.7. Image Analysis

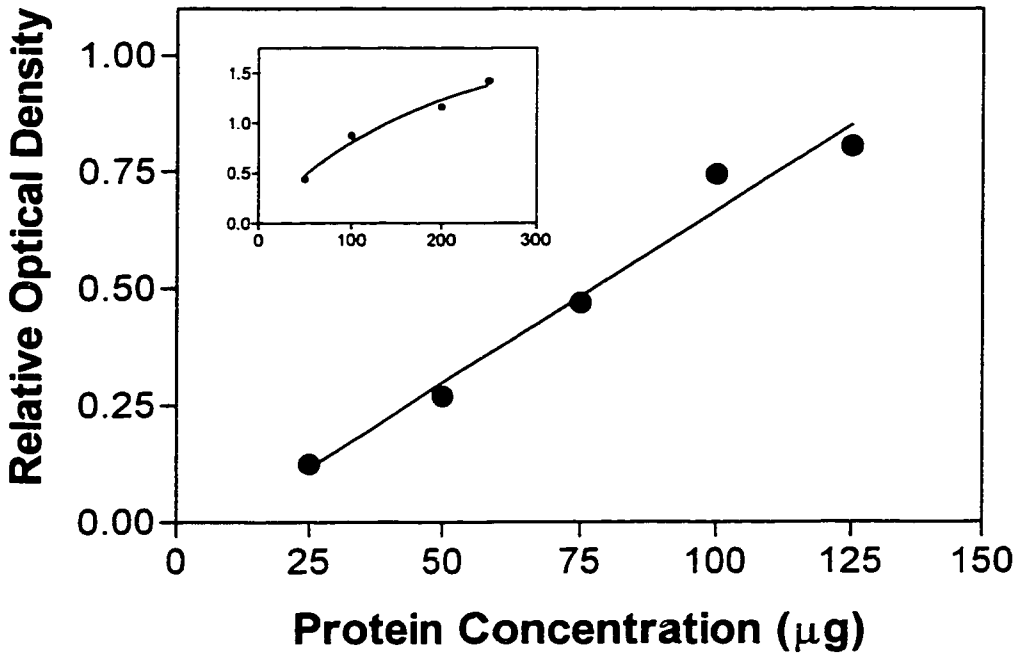
Immunoblots were quantified by densitometric analysis of band intensity on Kodak X-OMAT film. Quantitation was carried out using either the Northern Exposure image analysis system (EMPIX Imaging, Mississauga, ON) or the MCID image analysis system (Imaging Research, St. Catherines, ON). All comparisons between samples were made within the same gel. Given that non-linear effects could occur at many steps during the Western blot process (e.g. during sample preparation, gel loading, electrophoretic

transfer, immunolabeling and detection, and film quantitation), standard curves were constructed using known protein concentrations. The results of these studies are presented in Figures 16 and 17 for both densitometric techniques. As can be seen in Figure 16, a linear change in G protein labeling was observed over the concentration range of 25-125 μg using relative optical density (ROD) measures. A similar result, over the range 20-100 μg, was also observed for total grain density measurements (Figure 17). Thus, even a 3 fold increase in the labeling of the 35μg samples used in these studies would still be within the linear range for densitometric analysis by either method.

II.0.1.8. Statistical Analysis

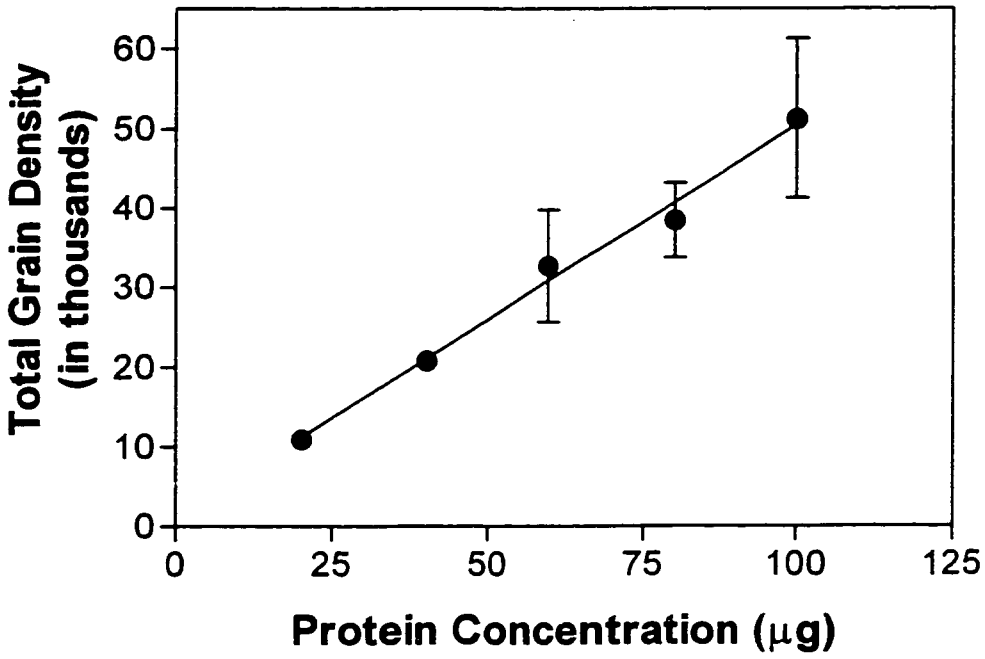
The statistical measures used to analyze results presented in this thesis and in published articles arising from this work (see Appendix C), were limited to Student's t test and one-way analysis of variance (ANOVA) for comparison of multiple groups. Unless otherwise stated, two-tailed t tests with pooled or equal variances were performed in all cases, either paired or unpaired as required. Similarly, standard or repeated measures ANOVA were performed as indicated. Depending on the nature of the comparisons required following ANOVA, various post-hoc tests were employed. Specifically, the Dunnett's post-test was performed when comparison to a single control group was required. The Bonferroni selected pair post-test was performed when only selective pairs of comparisons between groups was required, as determined by the experimental design. For all remaining comparisons, the Tukey post test was employed

Figure 16. Standard curve for protein estimation by MCID relative optical density (ROD) measurement



Relative amounts of total protein were prepared as previously described in the text. Samples were processed for Western blot immunodetection with Giα as a representative antibody. Densitometric analysis is presented in relative optical density units (ROD). As can be seen from the main figure, densitometric analysis of Western blots was linear across the range of 25 to 125 μg loaded protein. This indicates that the preceeding steps (e.g. sample preparation, gel loading, electrophoretic transfer, antibody labelling) were all performed in a linear fashion within this range. The inset figure shows that the response was curvilinear above this range.

Figure 17. Standard curve for protein estimation by Northern Exposure total grain density measurement



Relative amounts of total protein were prepared as previously described in the text and in Figure 16. Samples were processed for Western blot immuno-detection with Giα as a representative antibody. Data points represent mean ± SD of two separate experiments. Densitometric analysis is presented as total grain density (in thousands). As can be seen, densitometric analysis of Western blots was linear across the range of 20 to 100 μg loaded protein. This indicates that the preceeding steps were all performed in a linear fashion within this range.

to examine all possible comparisons between groups. For graphical purposes, error bars refer exclusively to standard deviation units throughout this thesis. Statistical analysis was performed using either GraphPad Prism (GraphPad, San Diego, CA), or MINITAB (Minitab Inc., PA) software.

II.1. POST-MORTEM HUMAN BRAIN TISSUE

II.1.1. Methods and Materials

Western blot analysis of human post-mortem tissue was carried out according to the methods described in Section II.0.1. The specific antibodies used in these studies consisted of the Gsα/Golfα, Giα, Goα, and Gβ subunits previously described.

II.1.1.1. Tissue Preparation

Coronal sections cut 1cm thick through the striatum of Parkinson's disease and control brain tissue were obtained from the National Neurological Research Specimen Bank, USA (VA Wadsworth Medical Center, Los Angeles, CA). Control donor tissue was matched for age with parkinsonian patient tissue. Caudate and putamen samples were isolated using a human brain atlas as a guide, and cut out of still-frozen post-mortem tissue slices. Caudate and putamen samples were homogenized as described in Section II.0.1.1. Samples were prepared for Western blot analysis and labeled with the primary G protein antibodies as described in Section II.0.2-7.

II.1.2. Results and Discussion

Alterations in G protein levels have previously been reported in a number of human diseases, including several neurological and neuropsychiatric disorders (see Table II). In particular, post-mortem brain tissue studies in schizophrenia patients have revealed reduced levels of several inhibitory G proteins in the putamen and hippocampus regions (Okada et al., 1990, Nishino et al., 1993). In addition to post-mortem studies, analysis of platelets and leukocytes from schizophrenics have revealed a variety of abnormalities in receptor/G protein/effector interactions (for a review, see (Nishino et al., 1997)). Similarly, a variety of studies of unipolar (depressed) and bipolar affective disorder patients have found alterations in stimulatory G proteins (for reviews, see (Manji, 1997, Wang and Young, 1997)). Although peripheral markers provide no direct evidence for alterations of G protein function in the CNS, they do support the hypothesis that G proteins can be modulated as a result of CNS disturbances.

In this study, we have examined G protein levels in post-mortem caudate and putamen samples of Parkinson's disease and control tissue. As can be seen in Figures 18 and 19, there was no significant change in the overall levels of any G protein examined, as determined by one-way analysis of variance (ANOVA) for both the caudate or putamen samples. However, the relative levels of the stimulatory G protein subunits appeared to be more variable than that of the inhibitory G protein subunits between PD and control groups (Figures 18, 19).

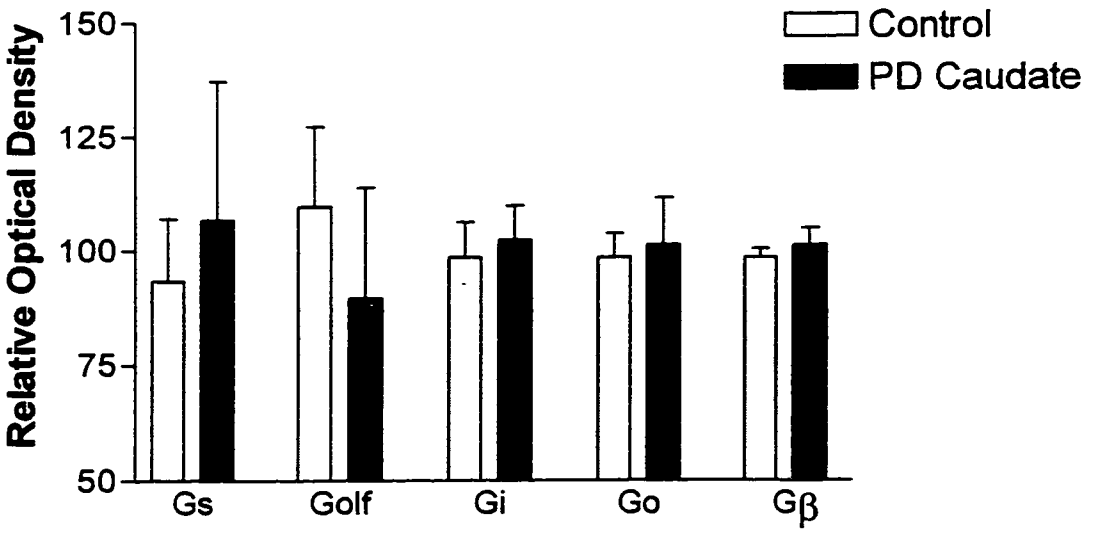
The RM/1 antibody used in these studies recognized two bands in the striatum that are believed to correspond to Gsα and Golfα, as previously described. Generally, these bands appeared at a relative ratio of approximately 40:60 (Gsα:Golfα) in both the

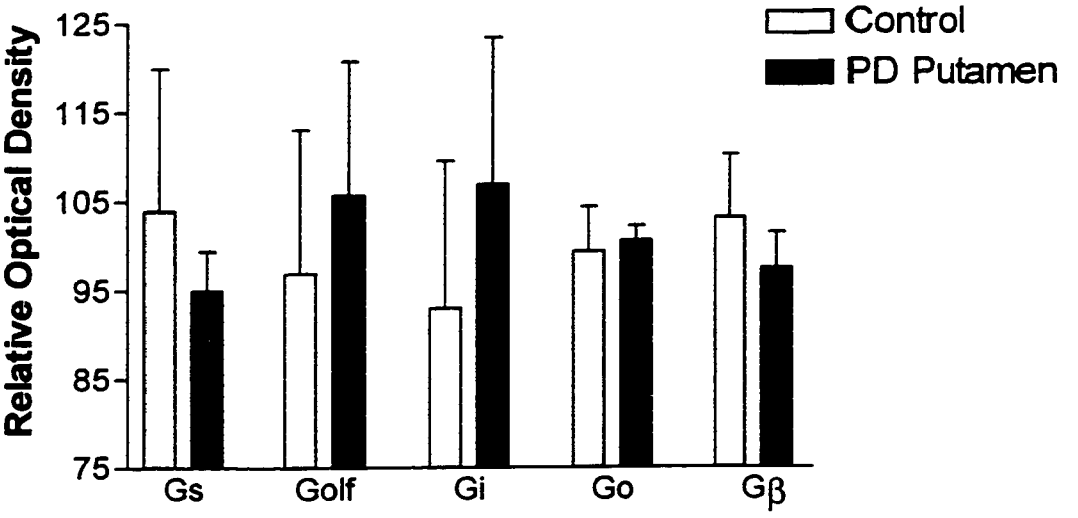
Figure 18. G protein levels in the caudate of human post-mortem tissue samples

This figure represents the change in each G protein level for the caudate tissue samples. Bars represent the relative optical density, expressed in arbitrary units, of Parkinson's disease (PD) and control (Control) samples. Error bars refer to the SD of each group (n=4 for PD, n=3 for Control). None of the alterations in G proteins reached statistical significance.

Figure 19. G protein levels in the putamen of human post-mortem tissue samples

This figure represents the change in each G protein level for the putamen tissue samples. Bars represent the relative optical density, expressed in arbitrary units, of Parkinson's disease (PD) and control (Control) samples. Error bars refer to the SD of each group (n=4 for PD, n=3 for Control). None of the alterations in G proteins reached statistical significance.


Relative Optical Density
150
125
100
75
50
Gs
Golf
Gi
Go
Gβ
Control
PD Caudate

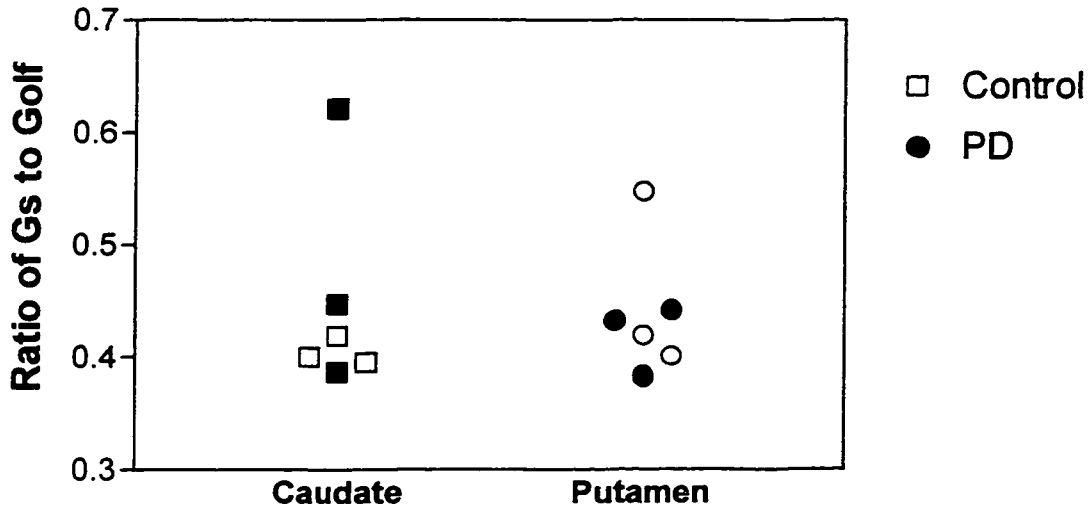

Relative Optical Density
125
115
105
95
85
75
Gs
Golf
Gi
Go
Gβ
Control
PD Putamen

caudate and putamen. However, a reversal of this ratio was observed in one of the Parkinson's disease caudate samples and one of the control putamen samples, as shown in Figure 20. The reversal of the relative stimulatory G protein ratio in these two samples explains the increased variability seen in Figures 18 and 19. When both stimulatory G proteins are considered together, there was no obvious difference between Parkinson's disease and control groups. The relevance of this reversal of stimulatory G protein levels is unclear. It may be a natural polymorphism in the population that is not related to disease pathology. It may also be a result of sampling error during dissection of the caudate and putamen samples. Moreover, unequivocal identification of stimulatory G protein subunits in this tissue was not possible, as the specific Golfα antibody had not been developed when these studies were performed.

The lack of overall significant change in G protein levels in the available post-mortem tissue is not necessarily indicative of normal G protein function in these patients. The limited number of samples available for analysis prevents any definite conclusions from being drawn regarding G proteins and Parkinson's disease. Moreover, post-mortem tissue studies are limited by a number of factors, most notably the great difficulty in obtaining brain tissue of a uniform and controlled nature in sufficient quantities for analysis. Among the potential confounding variables are the incomplete case history of patients, pre-mortem agonal condition of the brain, interval before freezing, and duration and temperature of frozen storage, among others.

Alternatively, G protein function could be assayed in peripheral tissues or blood elements of patients with Parkinson's disease as previously described. For example,

Figure 20. Relative ratio of Gs to Golf in human post-mortem striatum



This figure represents the relative ratio of Gs (45kDa) to Golf (42kDa) in post-mortem human Parkinson's disease (●, PD) and control tissue (O, Control), as estimated using the non-specific RM/1 antiserum. The relative ratio of Gs:Golf for most samples was approximately 40:60, except for one PD caudate sample and one control putamen sample, where the ratio was reversed.

dopamine receptors have been shown to interact with G proteins in mononuclear leukocytes (MNLs). In a recent study, the 45kDa form of Gsα has been shown to be specifically reduced in MNLs from patients with Parkinson's disease (Avissar et al., 1997). Moreover, this decrease in Gsα levels was correlated with a decline in dopaminergic and β-adrenergic receptor-coupled Gsα function. Although this provides no direct evidence for the role of G proteins in mediating dopamine receptor supersensitivity in the brain of these patients, it does support the concept that alterations in G protein function can occur in Parkinson's disease.

Given the limitations of studying G protein function in Parkinson's disease patients, a variety of animal models have been developed. Most of these involve lesioning of the dopaminergic neurons of the substantia nigra to simulate the cell loss that occurs in Parkinson's disease. Clearly, however, these models do not reproduce or mimic all the features of this disorder. Nevertheless, they are useful in examining specific causative theories or limited aspects of neuronal degeneration. The remainder of this thesis will focus on the development and characterization of animal models of Parkinson's disease, and the potential role of G proteins in these animals.

II.2. 6-HYDROXYDOPAMINE (6-OHDA) LESIONED RATS

II.2.1. Methods and Materials

Male Sprague Dawley rats (300-350 g) were used exclusively in these studies. Animals were obtained from Charles River Canada (St. Constant, QC) and housed in the Central Animal Facility (CAF), McMaster University, according to Canadian Council for Animal Care (CAC) guidelines. All animals received free access to food and tap water, and were maintained on a 12 hour on/off light cycle, beginning at 8:00 AM daily. Animals were handled extensively during the course of these studies. All behavioural testing and handling was carried out in a quiet room at the same time of day.

6-Hydroxydopamine hydrobromide and desipramine hydrochloride were obtained from the Sigma Chemical Co. (St. Louis, MO). Atropine, buprenorphine, doxapram hydrochloride, ketamine hydrochloride, providodine, sodium pentobarbital, xylazine, and xylocaine were all obtained from the Central Animal Facility (CAF), McMaster University in injectable form.

II.2.1.1. Surgical Procedure

Prior to anaesthetization, all animals received pre-treatment with desipramine hydrochloride (15 mg/kg) intraperitoneally (i.p.). Desipramine is a noradrenergic uptake inhibitor that is believed to enhance the development of dopamine receptor

supersensitivity following 6-OHDA lesioning by the selective sparing of noradrenergic pathways (Ungerstedt, 1971). Animals were also pre-treated with the opiate analgesic temgesic (buprenorphine, 0.015 mg/kg, i.p.) for prophylactic pain relief prior to surgery. Animals were subsequently anaesthetized with somnotol (sodium pentobarbital, 50 mg/kg, i.p.). If additional anaesthesization was required, animals received either excess somnotol or a mixture of ketamine and xylazine (9:5 ratio). Alternatively, the local anaesthetic lidocaine (xylocaine) was administered in the scalp area to reduce sensation when needed. In cases of respiratory distress, animals were administered dopram (doxapram) sublingually (s.l.) and atropine (0.3 mg/kg, i.p.), as necessary.

Once anaesthetized, the cranial area was shaved and cleaned using a tamed iodine solution (providodine or betadine). Animals were mounted into a stereotaxic frame and the head immobilized for the surgical procedure. Opthalamologic ointment (duratears) was applied to eyes to prevent them from drying out during surgery. Animals were kept warm on a heating pad set on low or with a heating lamp throughout the procedure. An incision was made in the scalp large enough to locate both bregma and the target coordinates.

Stereotaxic coordinates for 6-OHDA lesioning were determined from the atlas of Paxinos and Watson (Paxinos and Watson, 1986) as relative to bregma: -4.8P, ±1.6L, -7.8V to skull. A small hole was made in skull at the posterior and lateral coordinates using a hand drill or small dremel tool and a 30 gauge Hamilton syringe was lowered to the desired ventral depth. 6-hydroxydopamine hydrobromide (8 μg) was dissolved in 4 μl of vehicle (0.1% ascorbate in 0.9% saline) and injected stereotaxically over a 4 min

period with an additional 4 min to allow for drug diffusion before removal of the lesioning cannula. Sham lesioned rats received an equal volume of vehicle over the same time period.

The scalp incision was closed with wound clips and an antibiotic ointment (vetropolycin) was applied to the area. Animals were kept warm and closely monitored during recovery. For hydration, animals received 5 - 10 ml of warm sterile saline subcutaneously (s.c.) immediately prior to wakefulness. Animals were subsequently handled and examined twice daily for 3 days and once a day for the following week. Animals were also monitored for weight loss and dehydration.

II.2.1.2. Animal Handling and Tissue Preparation

Acutely following the lesion, several animals demonstrated an ipsilateral (same side as the lesion) bias in locomotion both spontaneously and in response to handling, as previously reported (Ungerstedt, 1971). This bias was generally self-limiting and diminished with time, though often correlated with the degree of supersensitivity observed during behavioural testing. Similarly, the degree of ipsilateral rotation following lesioning has previously been correlated with the degree of dopamine depletion (Matsuda et al., 1995). In this study, G protein levels were examined in animals at the following times post-lesion: 1, 4, 8, 16, 21 and 28 days. Animals were sacrificed by decapitation. Striata were quickly dissected out on ice and stored at -85°C until analyzed. Samples were isolated and homogenized as described in Section II.0.1.1. Western blots

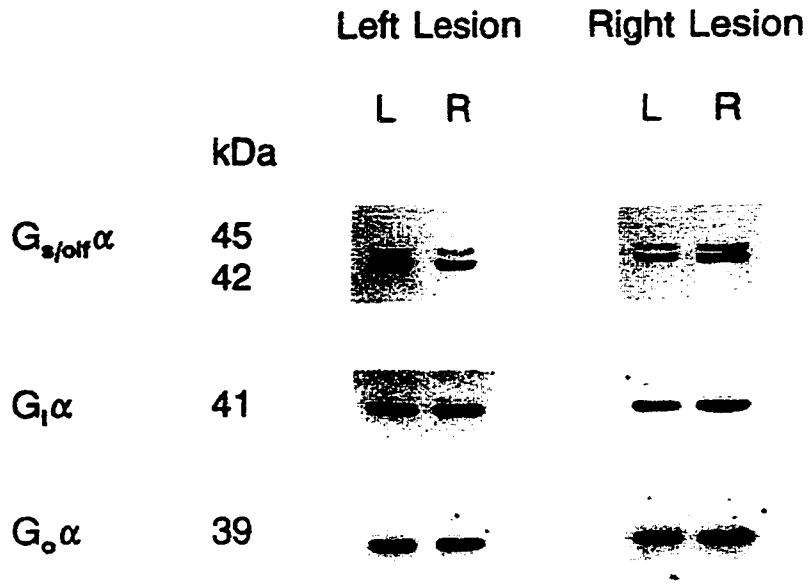
were prepared and analyzed by densitometry as previously described in Section II.0.1.2-7.

The G-protein antisera examined in this study consisted of the α subunits of Gsα/Golfα (RM/1 antiserum), Giα, and Goα. As shown in the representative immunoblots (Figure 21), antiserum RM/1 detected two bands of approximate molecular weight 45 and 42 kDa. These have recently been identified as Gsα (higher molecular weight species, ~45 kDa) and Golfα (~42 kDa species) (Hervé et al., 1993). Inhibitory G proteins antibodies consistently recognized a single band for Giα (~41 kDa) and Goα (~39 kDa). The specificity of these antibodies has been described in Section II.0.1.4.

II.2.2. Results

The results presented in this chapter have been published previously (Marcotte et al., 1994, Marcotte and Mishra, 1997). All 6-OHDA lesion results are reported as the percent change in optical density units of the lesioned striatum relative to the non-lesioned hemisphere for each animal (mean ± SD). Comparison of left and right striata from unoperated control and vehicle sham lesioned rats revealed no hemispheric differences for any of the G proteins assayed, indicating that sham lesioning did not alter striatal G protein levels. Both left and right hemisphere lesions were performed, with no apparent difference on G protein levels. Accordingly, both lesion groups were combined for presentation purposes and statistical analysis. For statistical analysis, 6-OHDA lesion groups were compared to sham lesion groups by one-way analysis of variance (ANOVA) followed by either Dunnett's post-hoc test for comparison to a control group or Tukey's

Figure 21. Representative immunoblots from 6-OHDA lesioned rats



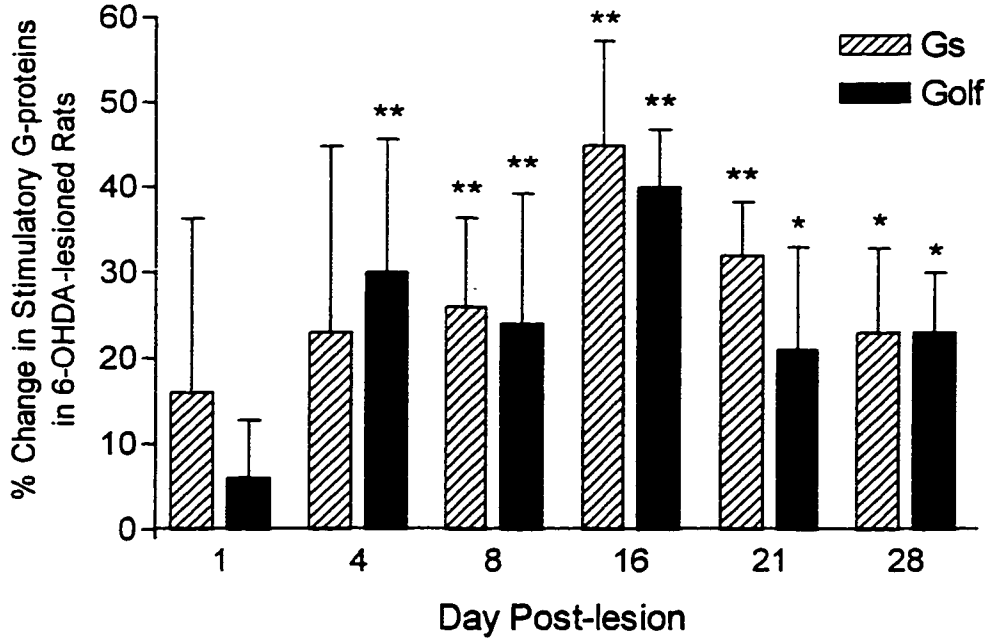
Representative immunoreactive bands for left and right hemispheres of left and right 6-OHDA lesioned animals (Day 16 for $G_s\alpha$, Day 4 for $G_i\alpha$, and $G_o\alpha$). Immunoreactivity was determined by peroxidase/chemiluminescence method (ECL, Amersham, Oakville, ON). Approximate molecular weights are indicated in kilodaltons (kDa).

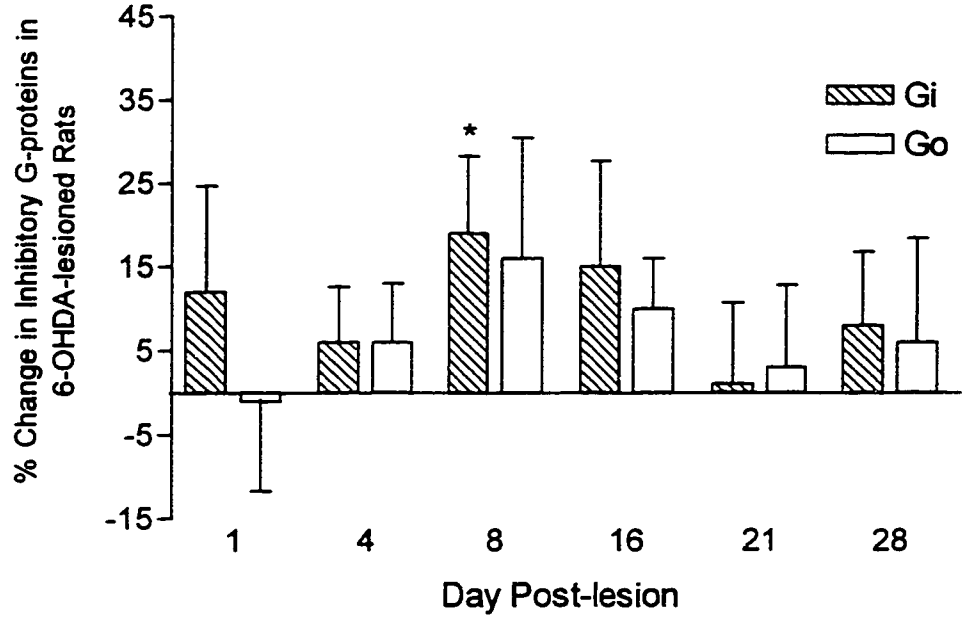
Figure 22. Stimulatory G protein levels in 6-OHDA lesioned rats

Effects of 6-OHDA lesions on striatal stimulatory G protein levels. Bars refer to the percent change in G protein levels in the lesioned hemisphere relative to the non-lesioned hemisphere for each animal. Both Gsα (~ 45 kDa) and Golfα (~ 42 kDa) levels were measured at various days post-lesion. Data are presented as the mean ± SD for each day (n=7 for Days 1,8,16,28, n=4 for Day 4, and n=6 for Day 21). Statistical significance based on one-way analysis of variance (ANOVA) followed by Dunnett's comparison to sham-lesioned control groups, * $p<0.05$, **, $p<0.01$.

Figure 23. Inhibitory G protein levels in 6-OHDA lesioned rats

Effects of 6-OHDA lesions on striatal inhibitory G protein levels. Bars refer to the percent change in G protein levels in the lesioned hemisphere relative to the non-lesioned hemisphere for each animal. Both Giα (~ 41 kDa) and Goα (~ 39 kDa) levels were measured at various days post-lesion. Data are presented as the mean ± SD for each day (n=7 for Days 1,21, n=6 for Days 4,8,16, and n=5 for Day 28). Statistical significance based on one-way analysis of variance (ANOVA) followed by Dunnett's comparison to sham-lesioned control groups, * $p<0.05$.


% Change in Stimulatory G-proteins in 6-OHDA-lesioned Rats
60
50
40
30
20
10
0
Gs
Golf
**
**
**
**
**
**
*
*
*
1
4
8
16
21
28
Day Post-lesion


% Change in Inhibitory G-proteins in 6-OHDA-lesioned Rats
45
35
25
15
5
-5
-15
Gi
Go
*
1
4
8
16
21
28
Day Post-lesion

post-test for all possible comparisons between groups.

II.2.2.1. Stimulatory Striatal G proteins

The stimulatory G-protein subunits Gsα and Golfα showed a similar pattern of alteration over the four-week time course (Figure 22). By day 8, both subunits were significantly increased in the 6-OHDA lesioned group as compared to the sham lesioned group (Dunnett's post-hoc test). Both Gsα and Golfα levels remained elevated up to day 28. However, there appears to be a biphasic effect, with the peak increase occurring at day 16 (40-45% increase). However, statistical analysis using ANOVA with Tukey's post-hoc comparison test did not reveal any significant difference for either Gsα or Golfα levels between days 8 and 28.

II.2.2.2. Inhibitory Striatal G proteins

In contrast to the stimulatory striatal G proteins, inhibitory G protein levels were not generally affected by 6-OHDA lesioning (Figure 23). Although Giα levels were significantly increased on day 8 (20% increase) as determined by Dunnett's post-hoc comparison, this increase did not persist over the time course. Goα levels were not significantly altered on any day post-lesion (Figure 23). Nevertheless, it is possible that physiologically relevant alterations in the level or function of inhibitory G proteins may occur below the level of detection by Western blot analysis.

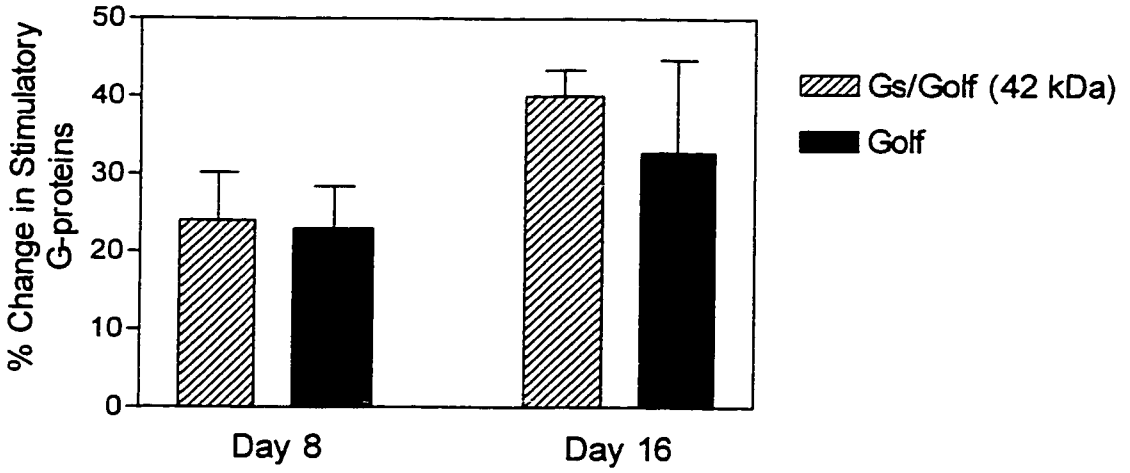
II.2.2.3. Comparison of Golfα Antibodies

The initial studies of G protein levels following 6-OHDA lesions were performed prior to the development of the specific Golfα antibody described in Section II.0.1.4.2. Given the lack of certainty surrounding the identification of the lower molecular weight band (~ 42kDa) recognized by the common Gsα antiserum, Golfα levels were subsequently measured at 2 time points following development of this antibody. As shown in Figure 24, there was no significant difference in the magnitude of the increased immunolabeling shown on days 8 and 16 using the specific Golfα antiserum or the lower band detected with the Gsα antiserum believed to correspond to Golfα. Golfα levels will continue to be measured by both antisera throughout this thesis.

II.2.2.4. Cortical G protein Levels

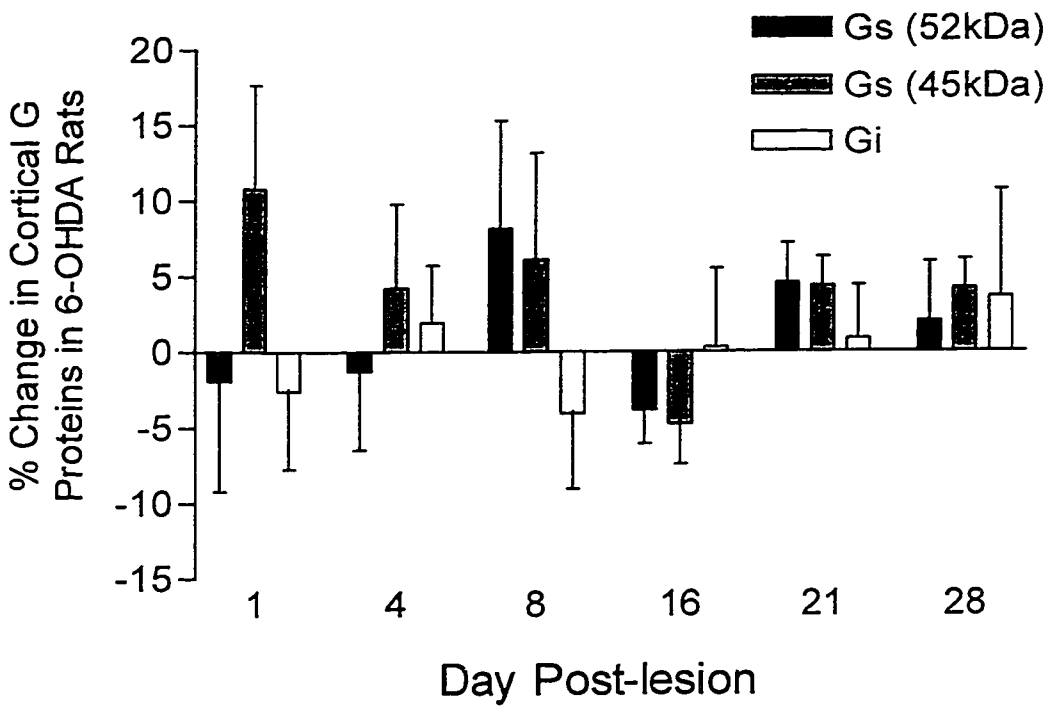
In addition to striatal tissue, prefrontal cortex samples were also examined to confirm the relative specificity of the effects of 6-OHDA lesions on G protein levels (Figure 25). The Gsα antiserum recognized two bands of approximate molecular weight 52 and 45 kDa in cortical tissue (see Section II.0.1.4.1). As shown in Figure 25, both Gsα and Giα levels were measured as representative examples of stimulatory and inhibitory G proteins. No significant differences in the levels of either G protein were observed at any time post-lesion (ANOVA followed by Tukey's post-hoc comparison). Golfα levels were not measured as no immunolabeling was detected using this antibody

Figure 24. Comparison of Golfα antibodies in 6-OHDA lesioned rats



Golf G protein levels were compared using both the non-specific RM/1 antiserum and the specific Golf antiserum developed in our laboratory. The results presented in Figure 22 and 23 involved only the lower 42kDa band of the non-specific antibody RM/1. To verify the selectivity of this band, Golf levels were also estimated using a specific Golf antibody. There was no significant difference in the percent change of Golf levels measured with the specific antibody as compared to the lower band of RM/1.

Figure 25. Cortical G protein levels in 6-OHDA lesioned rats



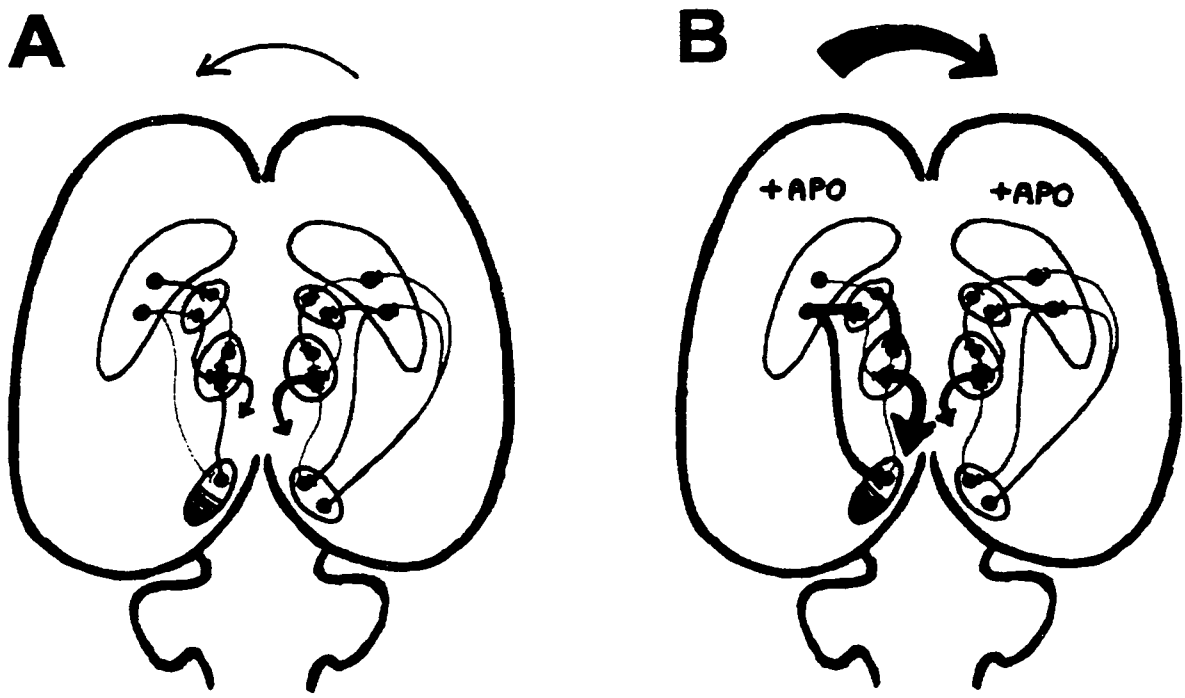
Effects of 6-OHDA lesions on cortical G protein levels. Bars refer to the percent change in G protein levels in the lesioned hemisphere relative to the non-lesioned hemisphere for each animal. Both Gs (~ 45 kDa) and Gi (~ 41 kDa) levels were measured at various days post-lesion. Data are presented as the mean ± SD for each day (n=3). Statistical significance based on one-way analysis of variance (ANOVA) followed by Fisher's comparison of treatment day groups.

in this region, consistent with previous reports (Hervé et al., 1993, Jones and Reed, 1989, Rius et al., 1994).

II.2.3. Discussion

In 1971, Ungerstedt reported that dopamine receptor agonists could induce contralateral rotations in 6-hydroxydopamine (6-OHDA) lesioned rats (Ungerstedt, 1971). This behaviour was interpreted as resulting from either increased receptor activity in the lesioned hemisphere or downstream alterations in neuronal circuits. Subsequently, unilateral 6-OHDA lesions have been one of the standard animal models of Parkinson's disease (Schwarting and Huston, 1997). In fact, this model has greatly stimulated the examination of basal ganglia neurotransmitter systems and the identification neuronal circuits such as those presented in Figures 10 and 11 (Schwarting and Huston, 1996). Specifically, the loss of dopaminergic innervation to the striatum is believed to increase the sensitivity of post-synaptic dopamine receptors. In the presence of a dopamine agonist, this supersensitivity results in the enhanced output of the lesioned hemisphere. A schematic diagram of the differential effects of lesioning in this model is presented in Figure 26. The resulting motor imbalance in these animals is manifested as rotational behaviour away from the side of the lesion (i.e. contralateral rotation). Furthermore, the number of rotations produced by a given concentration of a dopamine agonist is highly consistent and can be easily quantified. Alternatively, administration of a dopamine releasing agent such as amphetamine would be expected to produce a greater effect in the

Figure 26. Apomorphine-induced rotations in 6-OHDA lesioned rats



Effects of 6-OHDA lesioning on basal ganglia output and rotational behaviour. The neurochemical and neuroanatomical connections presented in this figure were described previously in Figures 10 and 11. **A.** A left 6-OHDA lesion results in decreased "thalamic drive" in the left hemisphere, producing mild ipsilateral rotations. **B.** Apomorphine treatment (+APO) stimulates both hemispheres, but produces a greater effect in the lesioned striatum due to dopamine receptor supersensitivity in this region. As a result, thalamic drive is increased in the same hemisphere, and contralateral rotations are produced. Adapted from Ungerstedt, 1971, Carlsson and Carlsson, 1990, Marsden and Obeso, 1994, Albin, Young et al, 1995, and Graybiel, 1996.

unlesioned hemisphere due to the larger number of dopaminergic terminals in this hemisphere, resulting in ipsilateral rotations.

The increased responsiveness of dopamine receptors to dopamine agonists following 6-OHDA lesioning has generally been referred to as dopamine receptor supersensitivity (Kostrzewa, 1995). Historically, this responsiveness to dopamine agonists has been measured in two distinct ways, behaviourally (e.g. enhanced behavioural response, such as number of rotations) and biochemically (e.g. increased second messenger production, such as cAMP). As previously mentioned, most studies focused on dopamine receptor levels or expression for the potential explanation of this supersensitivity phenomenon. Although dopamine D_2 receptor upregulation has been consistently observed following 6-OHDA lesions, there are conflicting reports on the effects of these lesions on D_1 receptors. In general, although some early reports observed a transient or persistent increase in D_1 receptor levels, the vast majority of studies have found either no change or a decrease in D_1 receptors (Table IV).

The reasons for this discrepancy in D_1 receptor studies are unclear. Taken together, however, they suggest that dopamine receptor upregulation alone is not sufficient to explain all aspects of dopamine receptor supersensitivity following 6-OHDA lesioning. These findings have helped to create an important distinction between dopamine receptor supersensitivity and receptor upregulation. As previously mentioned in the Introduction, the term dopamine receptor supersensitivity has also been used to describe increased sensitivity of dopamine receptors following chronic antagonist treatment (Kostrzewa, 1995). In fact, chronic dopamine receptor blockade with either

selective D_1 or D_2 antagonists has consistently been reported to increase striatal D_1 and D_2 receptor levels (for a review, see McGonigle et al., 1989). As such, the presence or absence of receptor proliferation alone is not considered a sufficient indicator of supersensitivity status (Kostrzewa, 1995).

A characteristic of dopamine receptor supersensitivity following 6-OHDA lesioning is the loss of D_1/D_2 synergism (LaHoste and Marshall, 1992). As previously mentioned, D_1/D_2 synergism refers to the observation that concomitant stimulation of both receptor subtypes is often required to elicit many of the behavioural and electrophysiological effects of dopamine. The underlying physiological basis of this interaction is unclear, and may occur at various levels of organization (Robertson, 1992b). Biochemically, the interaction between dopamine D_2 receptors and D_1 receptors on adenylyl cyclase activity appears to be altered or "uncoupled" in the striatum following 6-OHDA lesions (Thomas et al., 1992). Ultimately, however, the specificity of this effect is uncertain, as chronic dopamine receptor antagonist treatment has also been reported to prevent D_1/D_2 synergism (Hu and White, 1994). Moreover, D_1/D_2 synergism appears to persist in the striatum of MPTP-treated monkeys (Gagnon et al., 1995, Domino, 1997, Luquin et al., 1994), raising doubts about the usefulness of this particular measure in assessing dopamine receptor supersensitivity.

Given the lack of significant D_1 receptor upregulation following 6-OHDA lesioning, it is reasonable to examine other aspects of the receptor signaling cascade. The conceptual framework for G protein signaling has already been described in Section I.2.2 (Figures 6-8). As previously described, increased G protein levels would be expected to

result in enhanced sensitivity to receptor agonists (Figure 9) (Ross, 1992). G protein upregulation also offers the potential for increased convergent signaling of multiple receptors through a common G protein (Figure 6B). Consistent with this hypothesis, the striatum possesses high levels of a large number of neurotransmitter systems, the most prominent being dopaminergic, glutamatergic, cholinergic, and GABAergic (Gilman and Newman, 1992). Moreover, many of the other major neurotransmitter systems are also well represented, particularly purinergic, adrenergic, opiate, and serotonergic systems (Kawaguchi, 1993, Selden et al., 1994). The striatum also contains high concentrations of several neuroactive peptides, including substance P, dynorphin, met-enkephalin, cholecystokinin, neurotensin, somatostatin and neuropeptide Y (Gerfen et al., 1991, Graybiel, 1990, Kawaguchi et al., 1995, Angulo and McEwen, 1994). Many of these neuropeptides bind to specific seven transmembrane-domain receptors that are coupled to G proteins. As result, it is possible that G protein upregulation may offset reduced dopaminergic transmission through the enhanced signaling of multiple neurotransmitter systems.

As mentioned in the Introduction, alterations in G protein levels have been implicated in a variety of disorders, including several neurological and psychiatric illnesses (see Table II). Evidence for G protein alterations following denervation or chronic drug treatment have also been described previously (Table III). Of particular relevance, upregulation of G protein subunits has been reported following reserpine-induced dopamine depletion (Butkerait and Friedman, 1993), stimulus deprivation (Babila and Klein, 1992), and chronic opioid treatment (Eriksson et al., 1992a, Van Vliet

et al., 1993, Eriksson et al., 1992b) (see Table III). This latter example is suggestive, as behavioural supersensitivity can be demonstrated following chronic morphine treatment even though opiate receptor expression remains unaltered (Reddy et al., 1993). Moreover, there is direct evidence for increased stimulatory G protein levels in the absence of dopamine D_1 receptor upregulation in the renal proximal tubules of aged rats (Kansra et al., 1997). In this case, however, G protein upregulation did not appear to be associated with increased D_1 receptor-mediated signaling.

One of the main findings from the current studies is that the stimulatory G proteins Gsα and Golfα are specifically increased shortly after 6-OHDA lesioning (4 days for Golfα, 8 days for Gsα). Moreover, this elevation persists until the end of the examined time course (28 days post-lesion). These findings are suggestive given the lack of D_1 receptor upregulation previously described in this model. Thus, it is possible that the increase in stimulatory G proteins observed here may be responsible for maintaining D_1 receptor supersensitivity in the absence of increased D_1 receptor density. While increased levels of striatal Gsα and Golfα have previously been reported 6 weeks post-lesion (Hervé et al., 1993), our results indicate that increased levels of these G proteins occur within days of a 6-OHDA lesion. Thus, stimulatory G proteins may play a role in both the development as well as the maintenance of D_1 receptor supersensitivity following 6-OHDA lesioning, as we previously discussed in (Marcotte et al., 1994, Marcotte and Mishra, 1997).

Whether the observed increases in G protein levels are due to increased translation of mRNA or some post-translational modification is unknown. Studies of G protein

mRNA expression following 6-OHDA lesion have been less consistent than studies of G protein levels. Northern blot analysis has indicated increased expression of Gsα mRNA, but not Golfα mRNA, at 6 weeks post-lesion (Hervé et al., 1993). However, increased levels of Golf mRNA have been observed as early as one week post-lesion using in situ hybridization (Dr. S. Vincent, personal communication). Moreover, a lack of change in Gsα mRNA expression following 6-OHDA lesions has been reported at 28 days post-lesion using reverse transcriptase polymerase chain reaction (RT-PCR) amplification (Valerio et al., 1992, Valerio et al., 1993). The significance of these findings is unclear. G proteins are also regulated at various levels post-translationally, and it is thus possible that the observed increase in protein levels is maintained following mRNA translation. G proteins undergo many types of post-translational modifications that alter their function and stability, including ADP ribosylation, myristoylation, farnesylation, and isoprenylation (Spiegel et al., 1992).

Although both stimulatory G proteins have been observed to increase following denervation, it remains to be determined which subtype is primarily responsible for maintaining D_1 receptor supersensitivity. Golfα is believed to be the main stimulatory G protein subtype involved in coupling dopamine D_1 receptors to adenylyl cyclase in the striatum, and as such is the most likely candidate. This hypothesis is based on several lines of evidence. Firstly, protein levels and mRNA expression of Golfα is considerably greater than Gsα in this region (Hervé et al., 1993). Developmental studies have indicated that Golfα, but not Gsα, develops rapidly post-natally (Rius et al., 1994). Further, the developmental pattern of Golfα correlates with the onset of several isoforms

of adenylyl cyclase and the increased responsiveness to stimulatory agents such as Gpp(NH)p (5'-guanylyl imidodiphosphate), Mn^{2+}, and forskolin (Rius et al., 1994). Immunocytochemical studies have demonstrated that most of the striatal output neurons express Golfα, in particular the GABAergic neurons that project to the substantia nigra (Hervé et al., 1993). As demonstrated in Figure 10, this "direct" output pathway is known to be particularly rich in dopamine D_1 receptors.

The most direct support, however, for the role of Golfα in mediating dopamine D_1 receptor signal transduction comes from neurotoxin lesion studies (Hervé et al., 1993). In these studies, intranigral injections of the neurotoxic plant lectin volkensin have been used to retrogradely destroy striatonigral neurons. Consequently, both D_1 receptor binding and Golfα protein levels and mRNA expression were markedly decreased in the striatum (Hervé et al., 1993). In contrast, Gsα levels were elevated following volkensin-induced degeneration of striatonigral neurons, suggesting that Gsα may be located predominantly in non-neuronal cells such as glia that proliferate following lesioning. Alternatively, the majority of Gsα may be located on pre-synaptic terminals in the striatum. In support of this hypothesis, quinolinic acid lesions of the striatum, which produce significant cell loss, also greatly reduced Golfα levels without affecting Gsα (Hervé et al., 1993). Taken together, these findings suggest that Golfα is responsible for coupling dopamine D_1 receptors to adenylyl cyclase in the striatum.

In terms of the inhibitory G proteins, a significant increase in Giα levels was also observed at 8 days following a lesion in this study (Figure 23). However, unlike the stimulatory G proteins, this effect was transitory and not maintained over the time course.

This finding thus suggests that inhibitory G-proteins may play a role in the initial development of D_2 supersensitivity, but not in its long-term maintenance. This supposition needs to be interpreted with caution, however. Although the results reported here appear to show that 6-OHDA lesioning preferentially affects striatal stimulatory G proteins, it is possible that physiologically relevant alterations in inhibitory G proteins may occur below the level of detection by Western blot analysis. For example, supersensitivity of D_2 receptor-mediated GTPase activity has been reported to occur in 6-OHDA lesioned animals in the absence of significant Giα upregulation (Inoue et al., 1994).

Gene expression studies have similarly detected no change in inhibitory G proteins following 6-OHDA lesions using either Northern blots (Hervé et al., 1993) or RNase protection assays (Inoue et al., 1994). However, increased expression of $Gi\alpha_1$, $Gi\alpha_3$, and Goα subunits at 28 days post-lesion have been reported using RT-PCR amplification (Valerio et al., 1992). This latter finding needs to interpreted with caution, however, as the PCR amplification technique can easily lead to the production of false positives. For example, later studies by this same group have reported decreased levels of $Gi\alpha_3$ following 6-OHDA lesioning using this same technique (Valerio et al., 1993). The discrepancy in RT-PCR studies has not been addressed by the authors of these studies, and raises important questions about the specificity and consistency of their results.

In comparison to the transitory increase in Giα levels observed here, increased D_2 receptor density has consistently been reported two weeks post-lesion (see for example Angulo et al., 1991, Feuerstein et al., 1981, Stauton et al., 1981), and even as early as 7

days (Mishra et al., 1980). As such, it is possible that although upregulation of both inhibitory G proteins and D_2 receptors may be involved in the initial development of D_2 supersensitivity, long-term maintenance is due to an increase at the receptor level. A similar observation has also been noted in reserpine-treated rats (Butkerait and Friedman, 1993), where both inhibitory G protein and dopamine D_2 receptor mRNA were elevated 7 days following dopamine depletion, but only D_2 receptor mRNA remained elevated at 14 days.

The main finding from the studies presented in this chapter is that while there is a significant increase in the levels of both stimulatory and inhibitory G proteins acutely following 6-OHDA lesioning, only the stimulatory G proteins remain consistently elevated. Thus, both stimulatory and inhibitory G proteins may be involved in the initial adaptive response of striatal neurons to denervation, but not the long-term maintenance of that response. This potential differential regulation of D_1 and D_2 receptor supersensitivity may be explained in terms of spare receptor theory. Spare receptors refer to an available pool of receptors that are not linked to signal transduction systems but can be "recruited" under specific circumstances. In fact, the theoretical effects of receptor and G protein upregulation presented in Figure 9 are based on the assumption that there is an available pool of spare receptors. This assumption has been found to be generally valid for most of the hormonal and neurotransmitter systems examined to date. In particular, a large reserve of D_1 receptors that are uncoupled from adenylyl cyclase has been reported in the striatum (Battaglia et al., 1986, Hess et al., 1987, Watts et al., 1995, Trovero et al., 1992). Thus, activation of adenylyl cyclase by stimulatory G proteins constitutes the rate

limiting step of D_1 receptor signal transduction in the striatum. Accordingly, increased D_1 receptor number would be ineffective in increasing the maximum physiological response to stimulation (i.e. adenylyl cyclase production), but would increase the potency of D_1 receptor agonists, as outlined in Figure 9A. Finally, increased stimulatory G proteins following 6-OHDA lesioning would be expected to couple to spare D_1 receptors and lead to an increase in D_1 receptor-induced activation of adenylyl cyclase, as presented in Figure 9B.

In terms of D_2 receptors, however, no significant receptor reserve has been observed in the normal striatum (Bohmaker et al., 1992, Herdon, 1988, Enz et al., 1990). However, one study did observe the generation of a small post-synaptic D_2 receptor reserve following 6-OHDA lesioning (Enz et al., 1990). The lack of a dopamine D_2 receptor reserve would mean that increased D_2 receptor levels would increase the maximum physiological response to stimulation (i.e. inhibition of adenylyl cyclase) in addition to increasing the potency of D_2 receptor agonists. In other words, the theoretical model of receptor function presented in Figure 9A would not apply in this case. In fact, the effects of D_2 receptor upregulation would more closely resemble the model of G protein upregulation presented in Figure 9B. Thus, an increased physiological response to D_2 receptor stimulation could be achieved without G protein upregulation. This is in contrast to the D_1 receptor-mediated pathway where stimulatory G protein upregulation would be required to increase the maximal response to receptor stimulation. In support of this conclusion, there is at least one report suggesting that inactivation of either D_2 receptors or their associated inhibitory G proteins produced similar effects on functional

responsiveness in this system (Bohmaker et al., 1992).

If the initial studies on the presence of spare dopamine receptors in the striatum presented here are confirmed, they would provide a potential explanation for the results presented in this chapter. Thus, stimulatory G proteins are elevated in order to increase the maximal response to D_1 receptor stimulation following denervation. Increased dopamine D_1 receptors alone would be insufficient to produce this effect. In contrast, D_2 receptor upregulation without alterations in inhibitory G proteins would be sufficient to increase the maximal response to D_2 receptor stimulation.

The end result of this differential modulation of dopamine receptor and G protein levels would thus be expected to produce comparable effects on the responsiveness to dopamine receptor agonists.

II.3. 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) LESIONED MICE

II.3.1. Methods and Materials

Male C57 BL/6 strain of black mice were obtained from Charles River Canada (St. Constant, QC) for all MPTP treatment studies. All animals were extensively handled for at least one week prior to drug administration. Animals were housed in the Central Animal Facility (CAF), McMaster University, according to Canadian Council for Animal Care (CAC) guidelines. Specifically, all animals received free access to food and tap water, and were maintained on a 12 hour on/off light cycle, beginning at 8:00 AM daily. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride was obtained from RBI (Natick, MA).

II.3.1.1. Treatment Procedure

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride was dissolved in 0.9% saline for injection. MPTP was administered at a dose of 30 mg/kg subcutaneously (s.c.) once a day for 10 days (total cumulative dose: 300 mg/kg). Paired control animals were treated with vehicle solution. Following MPTP administration, animals were carefully monitored for weight loss and body temperature.

II.3.1.2. Animal Handling and Tissue Dissection

For G protein studies, animals were sacrificed at 10 days or 10 months post-treatment by cervical dislocation. Striatum and prefrontal cortex tissue samples were quickly dissected on ice using a dissecting microscope, and stored at -85°C until analyzed. Samples were prepared as described in Section II.0.1.1. Western blot analysis was performed as described in Section II.0.1.2-7. G protein antisera used in these studies consisted of the non-specific Gsα/Golfα, the specific Golfα, Giα, and Goα.

II.3.1.3. HPLC Analysis

To verify the extent of the dopaminergic lesion, dopamine and its metabolite levels were also measured by high performance liquid chromatography (HPLC). For HPLC studies, animals were killed at 21 days post-treatment and tissue dissected as described previously. Levels of neurotransmitters and their metabolites were measured in 0.1 N perchloric acid striatal tissue extracts by HPLC with electrochemical detection, according to the method of Felice et al. (Felice et al., 1978). Specifically, levels of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) were measured. Also measured were serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and noradrenaline (NA) and its metabolite 3-methoxy-4-hydroxy-phenethyeleneglycol (MHPG) to confirm the specificity of this neurotoxin on

dopaminergic systems.

II.3.2. Results

The results presented in this chapter have been published previously (Marcotte et al., 1998b, Marcotte et al., 1998a). All MPTP G protein results are presented as the percent change in optical density units in MPTP treated animals compared to matched controls (mean ± SD). For dopamine and metabolite levels, statistical significance was based on one-way ANOVA for each level group followed by Tukey's post-test for multiple comparisons. For G protein studies, statistical analysis is based on repeated measures one-way ANOVA followed by Tukey's post-test for multiple comparisons between groups.

II.3.2.1. Dopamine and Metabolite Levels

Levels of dopamine and its metabolites were specifically reduced in the striatum of MPTP treated C57 BL/6 mice (Table VII), consistent with previous reports (Gerlach and Riederer, 1996). Serotonin and noradrenaline levels were unaffected by MPTP treatment, as were their respective metabolites. MPTP produced the greatest effect on striatal dopamine levels, which were reduced by 91% compared to vehicle controls. This depletion is greater than that generally observed in most studies on mice (65-80 %) (Gerlach and Riederer, 1996), but is not unprecedented (Sheng et al., 1987). Dopamine

Table VII

Dopamine and its metabolite levels in the striatum following MPTP administration

Treatment	DA	DOPAC	HVA	3-MT	5-HT	5-HIAA	NA	MHPG
Control	59.8 ± 2.54	11.1 ± 1.08	9.7 ± 1.55	4.9 ± 0.46	4.9 ± 0.80	2.5 ± 0.55	2.9 ± 0.52	1.1 ± 0.21
MPTP	5.6 ± 2.12 # (-91 %)	3.4 ± 0.94 # (-69 %)	2.1 ± 0.52 # (-78 %)	1.5 ± 0.29 # (-70 %)	5.1 ± 0.64 (+6 %)	3.1 ± 0.42 (+23 %)	3.0 ± 0.65 (+1 %)	1.5 ± 0.43 (+34 %)

Values are expressed as ng/mg protein content, mean ± SD (n=8 per group). Values in parentheses depict the percent change from Control. Statistical significance based on one-way ANOVA for each level group followed by Tukey's multiple comparison test, and depicted as follows; # $p<0.001$ compared to Control.

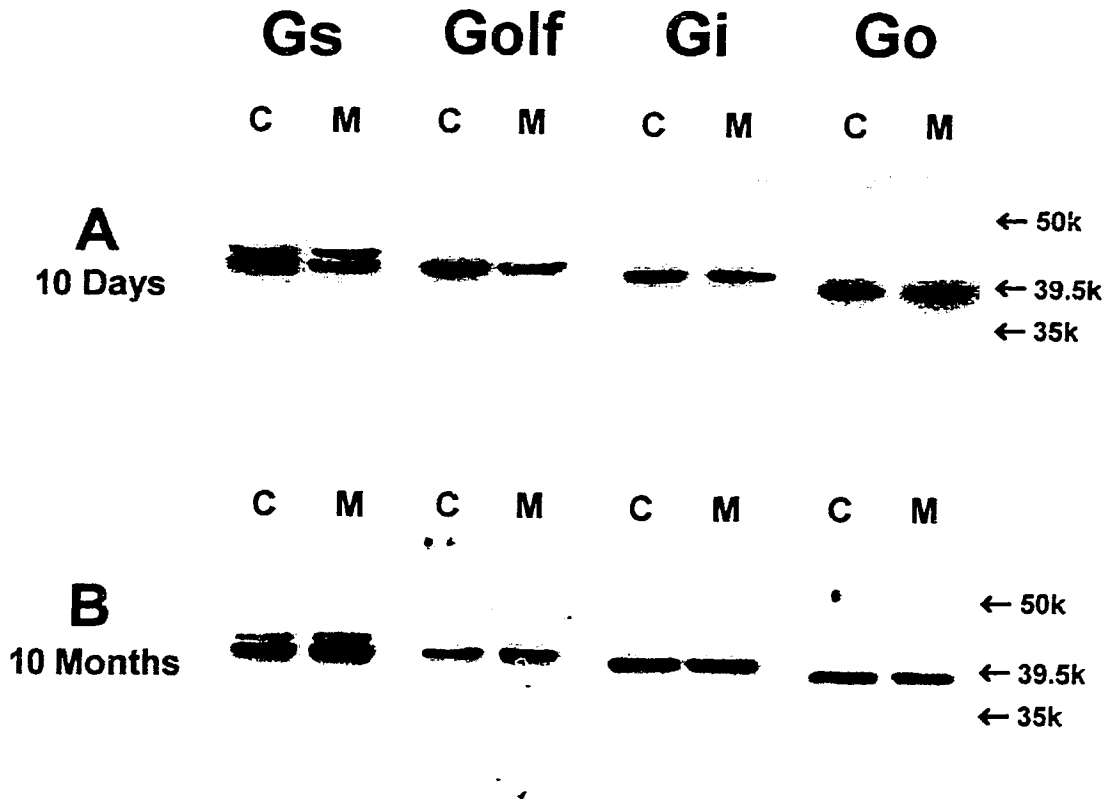
metabolite levels were also reduced by MPTP, but to a lesser extent than dopamine; 69 % for DOPAC and 3-MT, and 78 % for HVA. Accordingly, dopamine turnover, as measured by the ratio of dopamine metabolite levels to dopamine, was greatly enhanced in MPTP-treated mice (> 300% increase).

II.3.2.2. G protein Levels Acutely Following MPTP

Representative immunoblots for both acute (10 days post-treatment) and long-term recovery (10 months) striatal tissue samples are shown in Figure 27. The Gsα antiserum recognized two bands of apparent molecular weight 45 and 42 kDa in the striatum and 52 and 45 kDa in the cortex, consistent with our previous results in 6-OHDA lesioned rats. The specific Golfα G protein antibody recognized a single band of apparent molecular weight 42 kDa in the striatum, consistent with its identification as the main constituent detected by the non-specific Gsα antibody in this region (Hervé et al., 1993). As expected, Golfα levels were not detected in cortex. The inhibitory G proteins Giα and Goα each recognized a single band of 41 kDa and 39 kDa, respectively. The specificity of these antibodies has been described previously in Section II.0.1.4 (Marcotte et al., 1994, Gupta and Mishra, 1992).

Striatal and cortical tissue samples were examined by Western blot analysis using specific G protein antisera. Levels of the stimulatory G proteins Gsα and Golfα were specifically reduced by 20% and 25% in the striatum of acutely MPTP-treated animals, compared to control litter mates (Figure 28). This is in contrast to our previously

Figure 27. Representative immunoblot of G protein levels in MPTP treated animals



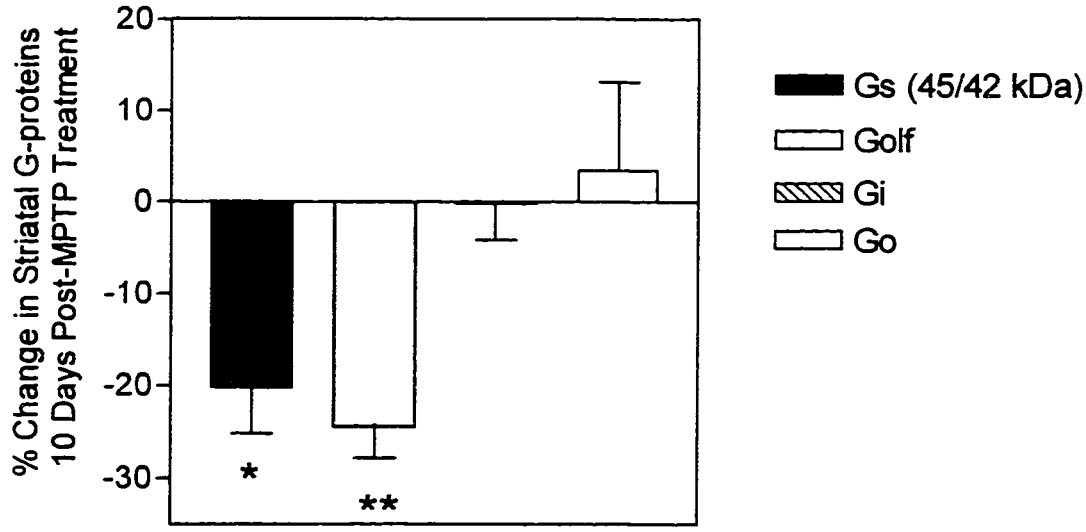
Representative immunoblots of Gsα, Golfα, Giα, and Goα G proteins in the striatum, (**A**) acutely following MPTP treatment, and (**B**) after long-term recovery. C and M refer to Control and MPTP treated animals, respectively. The location of molecular weight standards, in Daltons, are indicated by the arrows on the right.

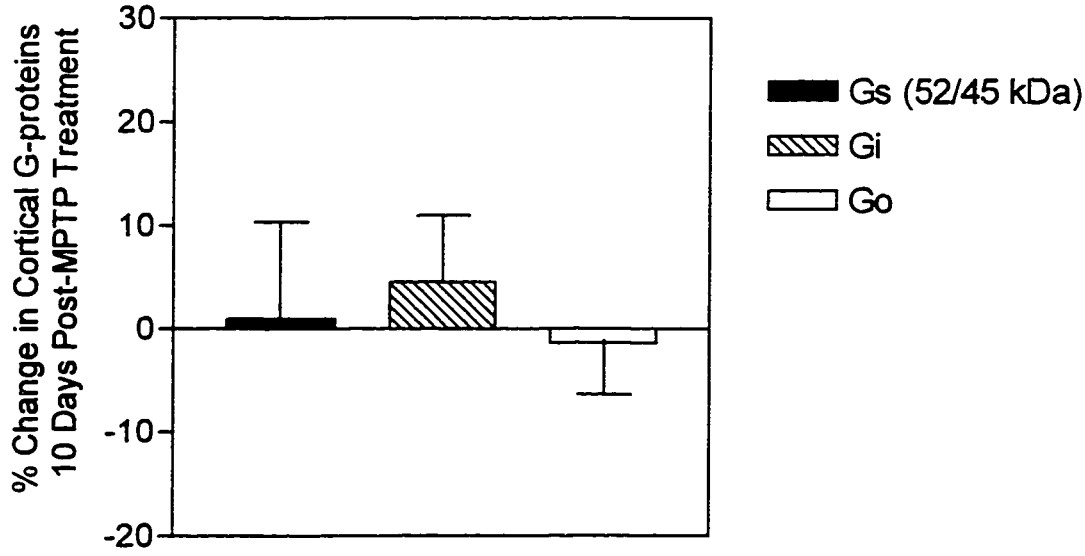
Figure 28. Striatal G protein levels acutely following MPTP treatment

Bars represent the percent change in G protein levels in MPTP treated mice compared to matched controls (n=5, mean ± SD) as measured by immunoblotting. Gs refers to the combined 45/42 kDa bands. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test. Statistical significance was based on comparison to Go, as this G protein was not altered by any treatment in this study. *$p<0.05$, **$p<0.01$.

Figure 29. Cortical G protein levels acutely following MPTP treatment

Bars represent the percent change in G protein levels in MPTP treated mice compared to matched saline controls (n=5, mean ± SD) as measured by immunoblotting. Gs refers to the combined 52/45 kDa bands detected in this tissue. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test for all groups.


% Change in Striatal G-proteins
10 Days Post-MPTP Treatment
20
10
0
-10
-20
-30
*
**
Gs (45/42 kDa)
Golf
Gi
Go


% Change in Cortical G-proteins
10 Days Post-MPTP Treatment
30
20
10
0
-10
-20
Gs (52/45 kDa)
Gi
Go

reported studies in 6-OHDA lesioned rats that found increased levels of both stimulatory G proteins within 8 days following a lesion (Marcotte et al., 1994) (see Section II.2). Consistent with 6-OHDA lesion studies, no change was observed in the levels of the inhibitory G proteins Giα and Goα in the striatum of MPTP-treated mice. Similarly, no change was observed in either stimulatory or inhibitory G proteins in the cortex of animals acutely following MPTP treatment (Figure 29).

II.3.2.3. G protein Levels During Recovery from MPTP

Based on the functional recovery observed in MPTP-treated mice, G protein levels were also measured 10 months following MPTP withdrawal (Figures 30, 31). Behaviourally, these animals appeared indistinguishable from vehicle-treated controls (data not shown). However, in contrast to the acute treatment group, the stimulatory G proteins Gsα and Golfα were increased by 15% and 30% respectively, in the striatum (Figure 30). Levels of the inhibitory G proteins Giα and Goα were not altered in the striatum following long-term recovery. Similarly, there was no change in cortical levels of any of the G proteins measured in this group (Figure 31), consistent with the acute treatment results.

II.3.3. Discussion

In recent years, the discovery that the relatively selective dopaminergic neurotoxin

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) reproduces many of the clinical and pathophysiological features of Parkinson's Disease has renewed interest in animal models of this disorder (Tipton and Singer, 1993, Gerlach and Riederer, 1996). In particular, the MPTP-treated C57 BL/6 mouse model has been proposed to represent a simple, reliable model of PD (Sundstrom et al., 1990). However, as previously mentioned, not all of the structural and motor abnormalities of PD are reproduced by MPTP treatment (Gerlach et al., 1991). Significantly, most MPTP-treated animals show behavioural recovery with time, in sharp contrast to the persistent and progressive degeneration observed in humans. Although the regrowth or collateral sprouting of nerve terminals may play a role (Hallman et al., 1985), the underlying biochemical basis of this phenomenon remains unclear.

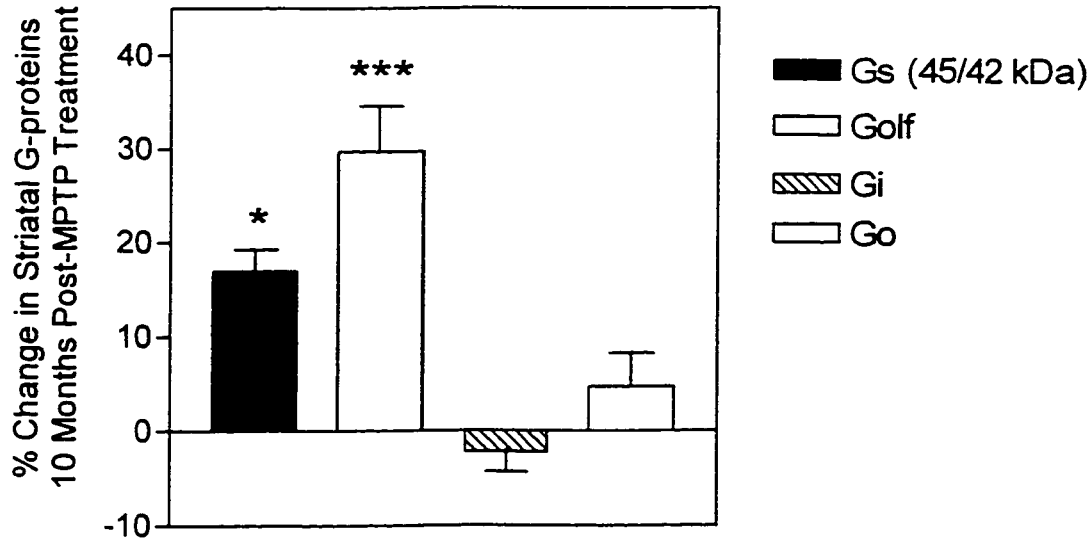
In an attempt to better understand the potential role of G proteins in Parkinson's disease, we have investigated G protein levels in MPTP-treated C57 BL/6 mice acutely following MPTP treatment, and after long-term recovery. As demonstrated in Table VII, the dosing regimen of MPTP used in these studies (30 mg/kg, s.c., once a day for 10 days) produces a greater than 90% depletion of striatal dopamine within 10 days after the last injection of MPTP (Marcotte et al., 1998b, Marcotte et al., 1998a). Consequently, animals were sacrificed by cervical dislocation 10 days following the last injection of MPTP for acute studies. This time point is consistent with earlier acute studies of MPTP-treated and 6-OHDA lesioned animals, thereby allowing direct comparison of G protein levels. For long-term recovery, animals were sacrificed 10 months following MPTP treatment. This time point is greater than most studies, and was chosen to allow the

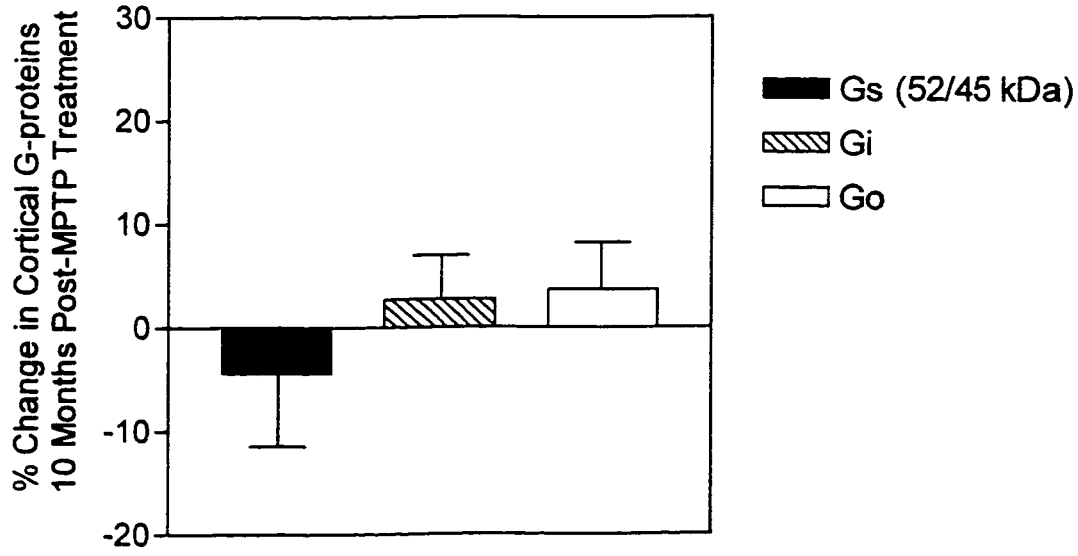
Figure 30. Striatal G protein levels during recovery from MPTP treatment

Striatal G protein levels following long-term MPTP withdrawal (10 month). Bars represent the percent change in G protein levels in MPTP-treated mice compared to matched controls (n=5, mean ± SD) as measured by immunoblotting. Gs refers to the combined 45/42 kDa bands. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test. Statistical significance based on comparison to Go. *$p<0.05$, ***$p<0.001$.

Figure 31. Cortical G protein levels during recovery from MPTP treatment

Bars represent the percent change in G protein levels in MPTP treated mice compared to matched saline controls (n=5, mean ± SD) as measured by immunoblotting. Gs refers to the combined 52/45 kDa bands detected in this tissue. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test for all groups.


% Change in Striatal G-proteins
10 Months Post-MPTP Treatment
40
30
20
10
0
-10
*
***
Gs (45/42 kDa)
Golf
Gi
Go


% Change in Cortical G-proteins
10 Months Post-MPTP Treatment
30
20
10
0
-10
-20
Gs (52/45 kDa)
Gi
Go

maximum possible time for recovery.

Initially, we anticipated an increase in stimulatory G proteins in the striatum acutely following MPTP treatment, commensurate with the previously reported increase in 6-OHDA lesioned rats (see Section II.2). In contrast, the stimulatory G proteins Gsα and Golfα were reduced by 20% and 25% acutely following MPTP treatment (Figure 28). No change was observed in the levels of striatal inhibitory G proteins or any cortical G protein in these animals (Figures 28, 29), consistent with the previous 6-OHDA studies. These results suggest that MPTP treatment preferentially affects the stimulatory G proteins of the nigrostriatal pathway. However, it is possible that physiologically relevant alterations in Giα or Goα may occur below the level of detection by Western blot analysis.

The reduction in stimulatory G protein levels observed in MPTP treated mice was unexpected given the generally comparable effects of these two animal models on other dopaminergic markers. For example, most studies on MPTP treated mice have reported no change in D_1 dopamine receptors, with either no change (Ogawa et al., 1987, Camps et al., 1989, Lange, 1990, Bhargava and Perlow, 1988) or a small and transient increase (Lau and Fung, 1986, Peroutka et al., 1985) in D_2 receptors. A comparable pattern in MPTP-lesioned monkeys has also been reported, with either no change or a decrease in D_1 receptors (Pifl et al., 1992, Alexander et al., 1991, Graham et al., 1990a, Morissette et al., 1996b, Graham et al., 1993, Morissette et al., 1996a) and an increase in D_2 receptors (Alexander et al., 1991, Graham et al., 1993, Graham et al., 1990a, Falardeau et al., 1988, Joyce et al., 1986, Todd et al., 1996, Herrero et al., 1996,

Morissette et al., 1996b, Gomez-Mancilla et al., 1993, Morissette et al., 1996a, Gagnon et al., 1995, Przedborski et al., 1991) frequently reported. However, small increases in D_1 receptors in the posterior region of striatum have been reported (Gnanalngham et al., 1993, Gomez-Mancilla et al., 1993, Gagnon et al., 1995). Taken together, however, these findings are not dissimilar to those frequently reported for 6-OHDA lesioned rats (see Table IV).

The reasons for this discrepancy in the direction of G protein change in these two animal models are unclear. They may reflect species differences, or differences in the lesioning paradigms. For example, although the mechanism of action of both agents is believed to involve oxidative stress and free radical production (Kumar et al., 1995, Gerlach et al., 1991), 6-OHDA is locally administered unilaterally into the substantia nigra, whereas MPTP is administered systemically over a period of days to weeks (Gerlach and Riederer, 1996). Moreover, a single infusion of 6-OHDA is believed to routinely result in a greater than 90% depletion of striatal dopamine, in contrast to the frequently less robust reduction (65-80%) observed in MPTP mice (Gerlach and Riederer, 1996). To help minimize this potential source of variability, we have used a dosing schedule of MPTP (30 mg/kg i.p., once a day for 10 days) that produces a greater than 90% depletion of striatal dopamine within 10 days after the last injection of MPTP (Table VII) (Marcotte et al., 1998b, Marcotte et al., 1998a).

These results call into question the applicability of previous studies of dopaminergic activity shortly following MPTP treatment in C57 BL/6 mice. For example, changes in dopamine receptors have generally been reported acutely following

MPTP treatment, with no measure of dopamine levels or receptor supersensitivity. This later point presumably reflects the difficulty in measuring dopamine receptor supersensitivity following MPTP. It may also help to explain the continuing popularity of the unilateral 6-OHDA lesion model, despite the potential confounding effects of the contralateral unlesioned hemisphere. Unlike the bilateral MPTP model, dopamine receptor supersensitivity can easily be demonstrated by both pharmacological and behavioural means in 6-OHDA lesioned rats (Kostrzewa, 1995). However, an approximate measure of dopamine receptor supersensitivity in MPTP-treated mice has been developed by Ogawa et al., (Ogawa et al., 1985) using the latency of animals to complete a motor task. Preliminary studies from our laboratory indicate that dopamine receptor supersensitivity may not be present for at least several weeks following MPTP treatment using this measure, despite the immediate reductions in striatal dopamine levels (Dr. Anita Chugh, personal communication). Although these results remain speculative at present, they raise the possibility that the acute measures of dopaminergic function used in this and other studies may in fact precede the development of true dopamine receptor supersensitivity. Interestingly, some studies of 6-OHDA lesioned rats have reported an acute decrease in D_2 dopamine receptors followed by long-term upregulation that may correlate with the development of receptor supersensitivity (see for example Mishra et al., 1980).

In addition to the uncertainty surrounding the onset of dopamine receptor supersensitivity in MPTP-treated animals, the point at which this supersensitivity may decline is also unknown. As a result, G protein levels were also examined after a long

post-treatment interval to allow maximum recovery from MPTP treatment. Behaviourally these animals appeared indistinguishable from vehicle-treated controls. Correspondingly, we had initially expected a normalization of G protein levels in the long-term recovery group. In contrast, Gsα and Golfα levels were significantly increased by 15% and 30% in the striatum following long-term recovery (Figure 30). This response is directly opposite to the decreased stimulatory G protein levels observed acutely following MPTP treatment, and is reminiscent of the acute increase observed in 6-OHDA lesioned rats (see Section II.2). Consistent with both acute 6-OHDA and MPTP treatment results, no change in striatal inhibitory G proteins or in any cortical G protein was observed (Figures 30, 31).

This differential regulation of stimulatory G proteins in the striatum following acute and long-term recovery from MPTP was unexpected, and its significance is not clear. The obvious possibility raised by this data is that the increased levels of stimulatory G proteins observed at 10 months may be involved in mediating some aspects of functional recovery following MPTP treatment. As previously postulated for the 6-OHDA lesion model, increased stimulatory G proteins may enhance dopamine D_1 receptor-mediated signaling in the striatum. This feature may in turn contribute to the observed recovery of function in these animals, in addition to other potential compensatory changes such as the regrowth of dopaminergic terminals (Hallman et al., 1985). If correct, this interpretation would provide further support for the hypothesis that acute changes in G proteins, and other dopaminergic markers, may precede the development of dopamine receptor supersensitivity in MPTP-treated animals. To date,

only relatively short time courses have been examined in MPTP-treated C57 BL/6 mice. Thus, with virtually no studies of long-term recovery in these animals, verification of this hypothesis is difficult.

In summary, these findings reveal that G protein levels are differentially regulated in MPTP-treated mice acutely following MPTP treatment and during long-term recovery. The acute increase in stimulatory G proteins observed in 6-OHDA lesioned rats does not appear to occur until much later in MPTP-treated mice. Further, the acute decrease observed in the MPTP-treated animals raises the possibility that the development of dopamine receptor supersensitivity may be regulated differently in these two models, with a delayed expression following MPTP treatment. Further studies examining a wider range of dopaminergic markers and signal transduction elements over a prolonged time course following MPTP treatment are warranted. Clear behavioural measures are also required to provide unequivocal evidence for the onset of dopamine receptor supersensitivity in this model. To date, there is insufficient evidence to make a clear determination of what role, if any, dopamine receptor supersensitivity plays in MPTP-induced parkinsonism.

CHAPTER III

FUNCTIONAL MEASURES OF G PROTEIN ACTIVITY

In the previous chapter, G protein subunit levels were characterized in Parkinson's disease and two animal models, the 6-OHDA lesioned rat and the MPTP-treated mouse model. However, levels of protein expression alone are insufficient to unequivocally establish alterations in these signaling pathways. Functional indicators of G protein activity are also required to determine the role of G proteins in dopamine receptor supersensitivity. A variety of functional measures of G protein activity were performed for this thesis, and are presented in this chapter. Given the difficulties in obtaining post-mortem human brain tissue suitable for biochemical analysis (see Section II.1), studies of functional measures of G protein activity were by necessity restricted to the use of animal tissues exclusively. Moreover, given the uncertainty surrounding the onset and nature of dopamine receptor supersensitivity in MPTP-treated mice, functional measures of activity also were restricted to rat striatal tissue exclusively. The two functional G protein measures attempted for this research project are the GTPase assay and a [α-^{32}P]-GTP binding assay.

III.1. THE GTPase ASSAY

Activation of G protein-coupled receptors is associated with increased activation of a membrane-bound high affinity GTPase activity (Onali and Olianas, 1987). This GTPase activity is an intrinsic property of all known G proteins (Hepler and Gilman, 1992, Kaziro et al., 1991). As presented in Figure 4, G protein-coupled receptor activation initiates the binding of GTP to the G protein α subunit, resulting in the dissociation of α and $\beta\gamma$ subunits. Subsequently, G protein activation is terminated by hydrolysis of the bound α subunit GTP by high affinity GTPases, with the consequent formation of the inactive GDP-bound α subunit. Displacement of bound GDP by free GTP reinitiates the signal transduction cycle of G protein activation (Ross, 1992). Since all G proteins cycle in the same manner, the intrinsic GTPase activity of any G protein-coupled system would be expected to increase upon receptor activation, regardless of the effect of these receptors on second messenger production. Thus, neurotransmitter-induced increases in GTPase activity can be taken as an indirect measure of receptor activation through the enhanced turnover of GTP (Onali and Olianas, 1987). GTPase activity can further be used as a index of receptor activation using specific receptor agonists.

III.1.1. Methods and Materials

Male Sprague Dawley rats (300-350 g) were used in these studies. Animals were maintained as described previously in Section II.2.1. [γ-^{32}P]GTP (10-50 Ci/mmol) was purchased from ICN Pharmaceuticals (Montreal, QC). 5'-Adenylyl-imidodiphosphate (App(NH)p), adenosine triphosphate (ATP), and guanosine triphosphate (GTP) were obtained from Boehringer Mannheim (Laval, QC). The remaining reagents for GTPase assay, including Tris base, bovine serum albumin (BSA), magnesium chloride ($MgCl_2$), EDTA, EGTA, DTT, phenylmethylsulfonyl fluoride (PMSF), benzamidine, sucrose, sodium chloride (NaCl), activated charcoal, phosphocreatine, and creatine kinase were obtained from Sigma Chemical co. (St. Louis, MO). Receptor agents such as carbachol, N-propylnorapomorphine (NPA), SCH 23390, dopamine, SKF 38393, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronapthalene (ADTN), and spiroperidol were obtained from RBI (Natick, NA). Quinpirole was a gift from Eli Lilly & Co. Drugs were dissolved in distilled water, dilute acetic acid, or ethanol immediately prior to the beginning of the enzyme assays. Control samples were incubated in the presence of equal amounts of solvent.

III.1.1.2. Sample Preparation

The standard sample preparation used in these studies is a modification of that developed by Cassel and Selinger (Cassel and Selinger, 1976) and later altered by Onali and Olianas (Onali and Olianas, 1987). Specifically, all tissue samples were homogenized in Tris buffer containing 50 mM Tris, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM

DTT, 0.1 mM PMSF, 0.1 mM benzamidine, and 10% sucrose. Tissue samples were homogenized by hand in a 2.5 ml glass homogenizer, with 25-30 strokes per tissue sample in approximately 10 volumes of buffer. Homogenized samples were centrifuged at 1000 x g for 10 min in an Eppendorf table top centrifuge. The resulting pellet was washed by resuspension in sample buffer and recentrifugation at 1000 x g for 10 min. Supernatant samples from both centrifugations were combined and centrifuged at 105,000 x g for 1 hour. The resulting pellet was resuspended in 20 volumes of sample buffer without sucrose and centrifuged at 30,000 x g for 20 min. The resulting pellet was then washed with sample buffer and centrifuged again at 30,000 x g for 20 min. The final pellet was resuspended in 2 volumes of sample buffer.

Protein content of sample membrane preparations were estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the protein standard. Biorad protein reagent (Biorad, Mississauga, ON) was added to each sample and BSA protein standard. Absorbance readings were taken at wavelengths of 595nm using a Beckman DU-20 spectrophotometer. Membrane preparation concentrations (mg / ml total protein content) were estimated by linear regression analysis of the standard curve constructed from absorbance readings.

III.1.1.3. Reaction Conditions

The standard reaction mixture (total volume 100 µl) contained 75 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM EGTA, 100 mM NaCl, 50 µg BSA, 0.5

mM App(NH)p, 5 mM phosphocreatine, 50 Units/ml creatine kinase, 0.5 mM ATP, 0.1 μCi [γ-^{32}P]GTP, 0.5 - 30 μM GTP. Reaction was started with the addition of 20 μl of membrane preparation (2 - 4 μg membrane protein) and carried out at 30 °C for 4 min. The reaction was stopped by the addition of 0.5 ml of 5% (w/v) ice-cold activated charcoal in 20 mM phosphoric acid (pH 2.5). Samples were centrifuged at 7000 x g for 10 min and 200 μl aliquots of the supernatant were used for counting ^{32}P released from [γ-^{32}P]GTP. Free ^{32}P was counted by liquid scintillation spectrometry using a Beckman LS 5000TA (Beckman, Mississauga, ON). The high affinity GTPase activity was calculated according to the method of Cassel and Selinger (1976) by subtracting the amount of [γ-^{32}P]GTP hydrolyzed in samples containing 30 μM of unlabeled GTP. This correction is necessary to account for the activity contributed by low affinity GTPases. Similarly, the presence of App(NH)p and ATP in the reaction mix are required to reduce the possible hydrolysis produced by non-specific ATPases.

III.1.2. Results

In an attempt to provide a functional measure of stimulatory G protein activity in the striatum, various modifications of the GTPase assay were performed. For example, the effects of differing concentrations of salts on GTP hydrolysis were examined by varying the concentrations of $MgCl_2$ and NaCl in the reaction mixture. The presence of $MgCl_2$ was required to detect significant GTPase activity, but increasing concentrations above 2mM $MgCl_2$ had no effect on the magnitude of the GTPase response. NaCl

concentration had a less marked effect on GTPase activity, with optimal concentrations in the 50 - 100 mM NaCl range. Similarly, varying incubation times over a range of 2 to 10 min had a negligible effect on GTPase activity (4 min was chosen as standard). By far the greatest effect on GTPase activity was observed with varying concentrations of protein (2 - 4 μg protein) prepared according to different protocols. Specifically, crude extracts evidenced little increased GTPase activity in response to receptor stimulation. As such, partially purified membrane preparations were used exclusively in these studies. Modifications of the membrane preparation included differing concentrations of $MgCl_2$ and sucrose, with varying number and duration of washes. However, no consistent effect on GTPase activity was observed with these modifications.

In these studies, the ability of dopamine to stimulate GTPase activity in partially purified rat striatal membranes was somewhat variable, but similar in magnitude to previously reported studies (Onali et al., 1983, Onali and Olianas, 1987, Odagaki and Fuxe, 1995, Treisman et al., 1985) (see Table VIII). Under various assay conditions, dopamine was able to increase GTPase activity by approximately 5 - 15 %. A greater increase in GTPase activity was observed in the presence of specific dopamine D_2 receptor agonists such as NPA, but the maximum observed increase was still no greater than 25% (Table VIII). As previously mentioned, these receptors are believed to be coupled to inhibitory G proteins. Attempts to increase the specific D_2 receptor-mediated response by the application of selective D_1 receptor antagonists were unsuccessful. Despite the modifications to the assay and sample preparation conditions attempted in these studies, stimulatory G protein-mediated GTPase activity was undetectable using

Table VIII

Summary of GTPase results

Ligand (100μM)	Observed Increase in GTPase activity (n = 20 expts.)	Reported Increase (Treisman et al., 1985)	Reported Increase (Onali & Olianas, 1987)	Reported Increase (Odagaki & Fuxe, 1995)
Dopamine	5 - 15 %	10 - 20 %	20 - 30 %	9 %
NPA	15 - 25 %	N/A	N/A	N/A
SKF 38393	No Effect	N/A	No Effect	2 %
Carbachol	20 - 45 %	N/A	65 %	12 %

Effect of various agents on GTPase activity in the rat striatum under various assay conditions. Dopamine-mediated increased GTPase activity was detected, but the consistency and magnitude of this effect varied in this and other studies. The dopamine D_2 receptor agonist NPA produced a greater response, but this agent was not assayed by other groups. As previously reported, the D_1 specific ligand SKF 38393 had no effect on GTPase activity. Carbachol, a non-hydrolyzable form of acetylcholine, is believed to stimulate GTPase activity through muscarinic receptors, and is included here as a control for assay function.

dopamine D_1 receptor agonists. This finding is consistent with previous reports on this tissue (Onali and Olianas, 1987, Odagaki and Fuxe, 1995).

III.1.3. Discussion

Increased GTPase activity has previously been reported in the rat striatum following administration of dopamine (Onali et al., 1983, Onali and Olianas, 1987, Treisman et al., 1985). However, the magnitude of this effect has varied among studies, with relatively small (Odagaki and Fuxe, 1995) or non-significant increases (Treisman et al., 1985) reported. The purpose of the present study was to determine if modification of the assay or membrane preparation conditions could improve the magnitude and specificity of the GTPase response. As expected, the presence of $MgCl_2$ in the assay buffer was required for GTPase activity to be observed. However, the magnitude of GTPase activity detected was independent of $MgCl_2$ concentration above a minimum threshold of 2mM. In contrast, the effect of NaCl concentration on enzyme activity was less pronounced. Indeed, its presence was not an absolute requirement for GTP hydrolysis to occur.

The agonist-induced increase in high affinity GTPase activity in the present studies was not remarkable when compared to the relatively high basal activity observed under all conditions. The percent increase in dopamine-stimulated activity above basal was only in the range of 5 - 15 % in these studies. This finding is consistent with several previous studies (Treisman et al., 1985, Hadman et al., 1995), although slightly lower

than some (Onali et al., 1983, Onali and Olianas, 1987) (see Table VIII). This discrepancy may reflect differing sample preparations among the published studies. For example, higher dopamine and carbachol-induced increases in GTPase activity were observed in studies using partially purified membrane preparations (Onali and Olianas, 1987, Onali et al., 1983) than in ones where relatively crude homogenate was used (Odagaki and Fuxe, 1995, Treisman et al., 1985). This finding is not entirely surprising, as brain synaptic membranes contain abundant background GTP hydrolyzing activity that may mask any specific increase. The further purification of membrane protein may help to reduce these non-specific hydrolyzing effects.

At least in principle, increased GTPase activity should be detectable for both stimulatory and inhibitory G protein pathways under the proper conditions. In the case of isolated cell systems or in vitro cell culture models, this has been demonstrated (Cassel and Selinger, 1976, Kazmi and Mishra, 1987). However, increased GTPase activity has rarely been detected for stimulatory G proteins in synaptic membranes in vivo. In fact, the only system in which increased stimulatory GTPase activity has consistently been reported in vivo is β-adrenergic stimulation in turkey erythrocytes (Cassel and Selinger, 1976). In contrast, increased GTPase activity can be readily demonstrated for inhibitory G protein-coupled receptor systems. In particular, increased GTPase activity has been observed following dopaminergic (Onali and Olianas, 1987, Tirone et al., 1985), α-adrenergic (Villalobos-Molina et al., 1992), muscarinic (Onali et al., 1983), and opiate (Tirone et al., 1985, Parenti et al., 1983) receptor stimulation in the rat striatum.

The reasons for this discrepancy in detecting stimulatory and inhibitory G protein-

mediated GTPase activity are unclear. A potential explanation may involve the greater number of inhibitory G proteins in most tissues, along with their correspondingly greater enzymatic activity (Birnbaumer, 1990, Milligan, 1988). The greater number of inhibitory G proteins could result in the suppression of Gsα-mediated activation, as depicted in Figure 8. In this case, excess βγ subunits released by Giα stimulation may swamp Gsα subunits and suppress their activity. Alternatively, Gsα activation may simply occur at levels too low to be reliably detected using this technique in most tissues. As previously mentioned, background GTPase and ATPase activity, originating from both G protein and non-G protein components, may mask any changes in stimulatory G protein activation in vivo. The isolated cell culture model may allow the greater separation of these signaling pathways, however, the relevance of signaling activity under these in vitro assay conditions is unclear. In particular, the relative receptor / G protein stoichiometry in recombinant cell systems may differ significantly from native tissues, producing anomalous results (Kenakin, 1997).

Interestingly, the high levels of background basal GTPase activity in the striatum preferentially interfered with our ability to detect dopamine receptor stimulated GTPase activity. Stimulation of muscarinic receptors by carbachol consistently increased GTPase activity to a greater degree than dopaminergic agents over the range of attempted assay conditions (Table VIII). The significance of this finding is unclear, but the direct comparison of different neurotransmitter receptor ligands in the striatum is difficult given the complex nature of receptor signaling in this region. A variety of features, including location and number of receptors, potency and stability of receptor ligands, and stability

of the receptor-ligand complex may all contribute to the observed variation in GTPase activity. For example, dopamine D_2 receptors and muscarinic receptors may be segregated on different populations of neurons with differing intracellular constituencies (e.g. cholinergic interneurons versus GABAergic output neurons). In addition, even in cases where dopamine D_2 and muscarinic receptors are co-localized, their relative number and affinity for receptor agonists may differ considerably. Moreover, these receptors may differentially couple to different Giα subunits within the same cell. As shown in Figure 5, the Gi family of G proteins contains several distinct subunits, most notably Gi_1, Gi_2, Gi_3, Go_1, and Go_2. These subtypes may differ in their relative concentrations, receptor preference, and intrinsic activity.

Previously, it has been reported that concomitant receptor blockade in cell culture can reduce background GTPase activity (Kazmi and Mishra, 1987). Thus, several specific dopamine receptor antagonists were also administered in these studies in an attempt to overcome the relatively poor dopaminergic GTPase signal. However, neither D_1 nor D_2 receptor antagonists had a significant effect on GTPase activity induced by their opposing receptor agonists, or by dopamine itself. Similarly, alterations in the amounts and components of the reaction mixture were unsuccessful in increasing the specific GTPase activity in response to dopamine receptor agonists.

Given the unreliable nature of dopamine-mediated GTPase activity in the striatum, it appears that this technique is unsuitable for measurement of stimulatory G protein activity following dopamine receptor supersensitivity. The sensitivity of this assay in the rat striatum appears to be too low to yield useful information concerning G

protein activity following dopamine receptor stimulation. Moreover, the GTPase assay is also a rather indirect measure of G protein activation, as it actually measures the inactivation of the G protein cycle. Thus, factors that affect GTP hydrolysis may give misleading results in this assay. As such, other functional measures of G protein activity were examined for this thesis.

III.2. THE [α-^{32}P]-GTP BINDING ASSAY

As evidenced by the failure of the GTPase assay, it is frequently difficult to study receptor-G protein coupling in native biological membranes. As a result, a variety of other techniques have been developed to measure the effects of receptor stimulation on G protein function. Chief among these are GDP release (Cassel and Selinger, 1978) and [^{35}S]-GTPγS binding (Asano et al., 1984). This latter technique, involving a non-hydrolyzable analogue of GTP (GTPγS) has been used in biochemical studies of purified and reconstituted systems, as well as in recent in vitro autoradiographic studies of the brain (Sim et al., 1995). Like the GTPase assay, however, this technique yielded only inconsistent results for stimulatory G protein activity following dopamine D_1 receptor stimulation in our hands (data not shown).

In addition to the difficulty of successfully applying these techniques in native membrane preparations, they only provide an indirect measure of receptor-G protein function. Like the GTPase assay, they cannot discriminate between G protein subtypes, and even the identification of inhibitory or stimulatory G protein-coupling is circumstantial. Attempts to overcome these limitations and label specific G proteins directly, using for example photoaffinity probes (Wange et al., 1991), have been limited by their restriction to reconstituted systems. Development of a suitable technique for measuring specific receptor-G protein coupling in intact membrane preparations has thus far proven elusive. One potential solution has been the development of an [α-^{32}P]-GTP

binding assay that is sufficiently stable to allow protein separation by SDS-PAGE gel electrophoresis (Friedman et al., 1993). Both basal and receptor-mediated [α-^{32}P]-GTP binding to specific G proteins in native biological membranes have been reported using this technique. Accordingly, we have examined the effects of dopaminergic agents on [α-^{32}P]-GTP binding to G proteins.

III.2.1. Methods and Materials

Male Sprague Dawley rats (300-350 g) were used in these studies. Animals were maintained as described previously in Section II.2.1. [α-^{32}P]-GTP (3000 Ci/mmol) was purchased from ICN Pharmaceuticals (Montreal, QC). Leupeptin and soybean trypsin inhibitor were obtained from Boehringer Mannheim (Laval, QC). All reaction and buffer agents, such as HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), Tris, EGTA, PMSF, β-mercaptoethanol, sucrose, NaCl, $MgCl_2$, SDS, glycerol, and bromophenol blue were obtained from Scientific Stores, McMaster University, or Sigma Chemical Co. (St. Louis, MO). Receptor agents such as NPA, dopamine, SKF 38393, and cis-fluphenazine were obtained from RBI (Natick, NA). Drugs were dissolved in distilled water or dilute acetic acid immediately prior to the beginning of the enzyme assays. Control samples were incubated in the presence of equal amounts of solvent.

III.2.1.1. Sample Preparation

Tissue was homogenized by hand in a glass homogenizer in 10 volumes of ice-cold homogenization buffer, consisting of 0.025M HEPES (pH 7.4), 0.001M EGTA, 0.1 M sucrose, 50 μg/ml leupeptin, 0.04 mM PMSF, 2 μg/ml soybean trypsin inhibitor, and 0.2% β-mercaptoethanol. Homogenates were centrifuged at 600 x g for 10 min, and the resulting supernatant was collected and centrifuged at 40,000 x g for 30 min. The resulting membrane pellets were washed once and resuspended in 10 volumes of reaction buffer containing 0.025M HEPES (pH 7.4), 0.1M NaCl, 50 μg/ml leupeptin, 0.04 mM PMSF, and 0.2% β-mercaptoethanol. Protein content of membrane samples was estimated using the Lowry method, as described in section II.0.1.1.

III.2.1.2. Assay Conditions

Membrane samples (200 μg protein / assay tube) were incubated for 2 min at 30 °C in reaction buffer containing 1 mM $MgCl_2$ and 5 nM GTP. After addition of 50 nM [α-^{32}P]-GTP (5 - 15 μCi/assay) with buffer (control blank) or dopamine receptor agonist, the incubation continued for another 3 min at 30 °C with gentle shaking (total reaction volume: 250 μl). Reactions were terminated by dilution with 750 μl of ice-cold reaction buffer containing 1 mM EGTA and immediately centrifuged at 16,000 x g for 5 min at 4 °C. The supernatants were discarded and the resulting pellets were prepared for electrophoresis.

III.2.1.3. SDS-PAGE

The sample pellets obtained in the previous step were resuspended in 40 µl of gel-loading sample buffer consisting of 0.0625M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.2% bromophenol blue (see Section II.0.1.2). Samples were briefly sonicated (30 sec) to dislodge the pellets, and gently shaken at room temperature for 1 hour.

Samples were run on 12% polyacrylamide gels with 4% polyacrylamide stacking gels (see Section II.0.1.3). The gels were dried using a Biorad water-vacuum gel dryer (Biorad, Mississauga, ON) and exposed to Kodak XAR X-ray film with fluorographic intensifying screens at - 70 °C overnight. The densities of radioactive bands were determined using an optical image analyzer, Northern Exposure (EMPIX Imaging, Mississauga, ON) as described in Section II.0.1.7.

III.2.2. Results and Discussion

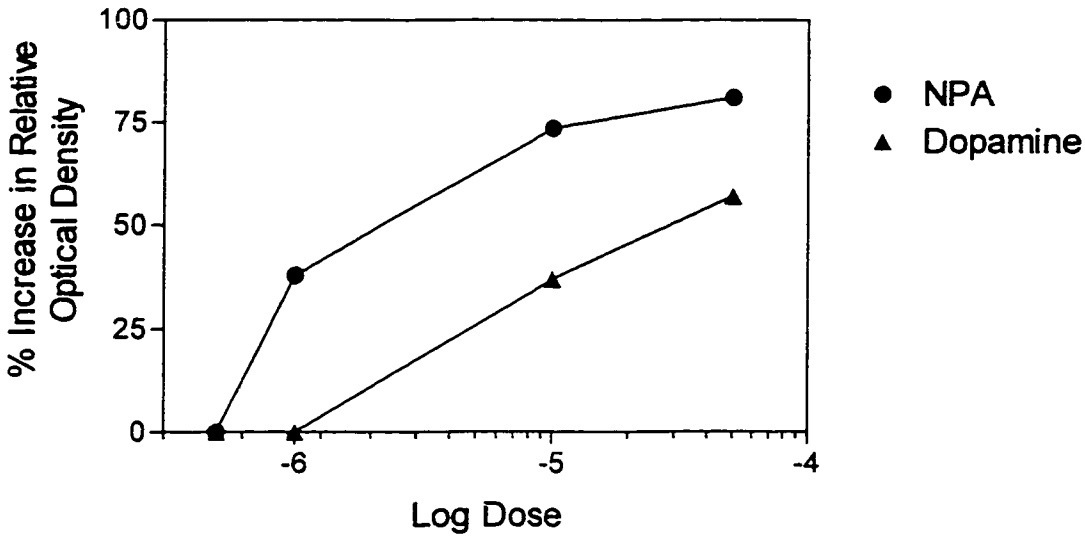
As expected, [α-^{32}P]-GTP labeled two distinct protein bands in striatal tissue, of apparent molecular weight 45 and 39 kDa (Friedman et al., 1993). Previous immunoprecipitation studies have identified these bands as corresponding to Gsα (45 kDa) and Giα/Goα (39 kDa) (Friedman et al., 1993). These bands are believed to represent predominantly [α-^{32}P]-GDP that remains bound to the inactive G protein following GTPase hydrolysis of [α-^{32}P]-GTP (Friedman et al., 1993). Interestingly,

Golfα (42 kDa) does not appear to be labeled using this technique.

Despite the initial promise of this technique, its application appears to be as limited as previous attempts at measuring receptor-G protein coupling. As in the case of the GTPase assay, increased labeling was frequently observed in the lower inhibitory G protein band in response to dopamine or specific D_2 receptor agonists, but the magnitude of this effect was variable. A representative experiment is presented in Figure 32. Blockade of dopamine receptors with cis-fluphenazine completely blocked the effect of dopamine, confirming the agonist-induced nature of this effect. Unfortunately, also as in the case of the GTPase assay, stimulatory G proteins were not affected by treatment with dopamine or the D_1 receptor agonist SKF 38393.

The failure of this assay to detect changes in agonist-induced stimulatory G protein activity is consistent with previous functional studies. As in the case of the GTPase assay, several modifications were made to the reaction mixture and reaction times, but to no effect. Increased basal binding of [α-^{32}P]-GTP to both subunits has been reported in 6-OHDA lesioned rats (Tenn and Niles, 1997), but agonist-stimulated binding was only observed for Gi/oα in this study. Sub-chronic treatment with the dopamine depleting agent reserpine has also been reported to increase basal [α-^{32}P]-GTP binding to Gsα (Butkerait et al., 1994), but this treatment also non-specifically enhanced both dopaminergic and non-dopaminergic binding to both Gsα and Gi/oα. The usefulness of this assay in detecting specific agonist-induced effects must be questioned. Specifically, agonist-stimulated [α-^{32}P]-GTP binding to Gsα appears to be difficult to detect with this technique.

Figure 32. Agonist-stimulated [α-32P]-GTP binding



Representative [α^{32}P]-GTP binding experiments with increasing concentrations of the dopamine D2 receptor agonists NPA and dopamine. Band intensity refers only to the inhibitory G protein band of approximate molecular weight 38-39 kDa, and is expressed as relative optical density units (ROD). Pre-treatment with 1 μM of the dopamine receptor antagonist cis-fluphenazine abolished agonist-stimulated increases in [α^{32}P]-GTP.

The lack of [α-^{32}P]-GTP labeling in the region of Golfα (42 kDa from immunoblot studies) is another problem that must be addressed. Initial studies by Wang et al. (Wang et al., 1995a), reported that dopamine D_1 receptors co-precipitate with Gsα antiserum. However, these authors used the non-specific antiserum RM/1, which recognizes both Gsα and Golfα, thus preventing the clear identification of the responsible G protein. Further studies by Wang et al., (1995a) using immunoprecipitation with specific Golfα antibodies, appear to have confirmed a lack of specific [α-^{32}P]-GTP binding to Golfα. One possible explanation for this finding is that Golfα is not involved in mediating dopamine receptor signaling in the striatum, and that this function is served solely by the 45 kDa form of Gsα (Wang et al., 1995a). This interpretation is unlikely, given the strong evidence in favour of the role of Golfα in mediating dopamine D_1 receptor signal transduction in this region. This evidence includes both indirect indicators, such as the immunocytochemical and developmental correlations of Golfα expression with D_1 receptors (Hervé et al., 1993) and other signal transduction elements (Rius et al., 1994), as well as direct measures of D_1 receptor-Golfα coupling from lesion and neurotoxin studies (Hervé et al., 1993) (see discussion in Section II.2.3). Given the evidence supporting Golfα coupling to dopamine D_1 receptors in the striatum, the failure of this technique to detect Golfα may be viewed as another indication of its limited applicability to stimulatory G proteins.

In summary, the GTPase assay, [^{35}S]-GTPγS binding, and [α-^{32}P]-GTP binding all proved ineffective in measuring stimulatory G protein function in the striatum. Although these techniques all demonstrated measurable increases in dopamine agonist-

stimulated inhibitory G proteins, this effect was not consistently observed. On the basis of these results, and the observation that only stimulatory G proteins were altered following 6-OHDA lesioning or MPTP treatment, an alternative functional measure of stimulatory G protein activity had to be developed.

CHAPTER IV

G PROTEIN ANTISENSE STUDIES

As introduced in Section I.5, one approach to examining the role of specific proteins is their selective blockade using locally administered antisense oligonucleotides. Theoretically, these agents have the ability to specifically reduce gene expression in a reversible manner. Given the inability of the GTPase, [^{35}S]-GTPγS, and [α-^{32}P]-GTP binding assays to detect functional alterations in stimulatory G proteins in the striatum, the use of antisense agents were subsequently examined. Specifically, antisense oligonucleotides were designed to selectively "knockdown" Golfα levels in the striatum of 6-OHDA lesioned rats. The effects of this treatment on G protein expression and rotational behaviour were examined and are described below.

IV.1. IN VIVO ADMINISTRATION OF OLIGONUCLEOTIDES

IV.1.1. Methods and Materials

IV.1.1.1. Design of Oligonucleotides

The oligonucleotides used in this study were synthesized as 20 mer (20 base) full phosphorothioate oligonucleotides corresponding to the region surrounding the initiation codon of the published rat Golfα mRNA sequence (-10 to +10) (Jones and Reed, 1989). Phosphorothioate modification (Figure 14) greatly increases oligonucleotide stability in vivo, although increased toxicity has also been reported (Crooke et al., 1995). Sense oligonucleotides were identical to the published rat Golfα sequence, while antisense oligonucleotides consisted of the complementary base sequence (see Appendix A). Missense oligonucleotides consisted of a scrambled, random order sequence of the antisense oligonucleotide. Both sense and missense oligonucleotides serve as controls for oligonucleotide infusion. However, both controls may potentially bind non-specifically to other endogenous nucleotides sequences or proteins. Although the likelihood of 20 bases in a random sequence showing a good homology match to a known protein is low, two separate missense oligonucleotides were constructed to further control for oligonucleotide effects (designated MS-1 and MS-2, see Appendix A).

All of the oligonucleotide sequences used in these studies were tested for lack of homology to other known proteins by BLAST NIH GenBank search, with an acceptable cutoff of no more than a 15/20 overall identity match with a maximum of 13 consecutive bases with other non-specific sequences for the each of the oligonucleotides. However, more recent GenBank searches of the missense oligonucleotide MS-1 have revealed a 17/19 identity match (13 consecutive bases) with a Bacillus gene encoding several nucleases and a 16/17 identity match (15 consecutive bases) with a portion of the human chromosome 11p15.5. The missense sequence MS-2 also showed a 15/15 identity match

(15 consecutive bases) with a N. crassa phytoene dehydrogenase (al-1) gene. Golf antisense and sense sequences continue to show specificity within pre-defined cutoff limits. Although these matches are still unlikely to account for the effects of missense oligonucleotides observed in these studies (see below), the increasing number of fairly selective matches observed with time in the available GenBank database suggests a potential area of concern.

IV.1.1.2. Synthesis of Oligonucleotides

All oligonucleotides were synthesized using an automated DNA sequencer by the McMaster Biotechnology Institute (MOBIX, McMaster University, Hamilton, ON). Full phosphorothioate modifications were performed for all oligonucleotides. Following synthesis, all oligonucleotides were desalted, ethanol washed, and purified by HPLC. All oligonucleotides were stored lyophilized at
-20 °C until needed. Oligonucleotides were dissolved in sterile saline for infusion on the first day of oligonucleotide infusion and additional aliquots were stored at - 20 °C until needed. The concentration of oligonucleotides used in these studies ranged from 2.5 - 4.0 nmoles in a final volume of 2 μl for infusion.

The purity of oligonucleotides was verified in several ways. Qualitatively, oligonucleotides all appeared as white crystalline residues with no evidence of contamination. One likely source of toxicity in early studies with phosphorothioate oligonucleotides was free sulfur contamination (Dr. Ian Creese, personal

communication). Contamination of this sort would be readably detectable as a change in colour (i.e. yellow-tinge in the residue) or reduced solubility, neither of which were observed in these studies. Work from many laboratories has shown that insufficient purity of oligonucleotides is a likely source of toxicity (Brysch and Schlingensiepen, 1994, O'Keefe et al., 1989, Mirabelli et al., 1991).

To directly verify the integrity of oligonucleotides, samples were run on a polyacrylamide gel with appropriate oligonucleotide sizing markers (Pharmacia Biotech, Baie D'Urfé, QC). Oligonucleotide samples and sizing markers were prepared in gel loading buffer consisting of 0.03% bromophenol blue and 8% sucrose in distilled water. 20% polyacrylamide gels consisting of 0.375 M Tris (pH 8.8), 0.1% SDS, 0.05% APS, and 0.5% TEMED, were cast between two glass plates separated by 1.5 mm. Lanes were formed by insertion of a plastic comb during polymerization. No stacking gel was required. Gels were run at 100 V for approximately 1 ½ hours to allow for maximal separation of oligonucleotide sizing markers. Electrophoresis runs were conducted with the Biorad Mini-Protean II gel apparatus (Biorad, Mississauga, ON). Following electrophoresis, gels were stained with ethidium bromide (Sigma Chemical Co, St. Louis, MO) and visualized on a U.V. light box. No contamination or significant degradation of oligonucleotides was observed. A representative ethidium bromide-stained gel is presented in Figure 33.

IV.1.1.3. In Vivo Oligonucleotide Animal Model

Figure 33. Oligonucleotide stability and purity

Representative example of several oligonucleotides run on a polyacrylamide gel stained with ethidium bromide. Lanes 1,4, Gs antisense; lanes 2,5, Golf missense; lanes 3,6, Golf sense; lane 7, oligonucleotide sizing markers. No contamination or significant degradation of oligonucleotides was observed, even in samples over one year old (lanes 1,4). The approximate molecular weight of 20 mer oligonucleotide is indicated by the arrow. Note that base composition affects the distance traveled for each of the three oligonucleotides examined here.

Male Sprague Dawley rats (300-350 g) were used in oligonucleotide studies. Animals were maintained as described in Section II.2.1. Similarly, all pharmacological agents were obtained as described in Section II.2.1. Behavioural studies were carried out in a quiet room at the same time of day.

IV.1.1.4. Surgical Procedure

6-OHDA lesions were performed as described in Section II.2.1.1. Immediately following 6-OHDA lesioning, a stainless steel permanent cannula guide (Plastics One, Roanoke, VA) was mounted onto a cannulation bracket on the stereotaxic frame, and bregma coordinates were redetermined. Stereotaxic coordinates for the permanent cannula guide were also determined from the atlas of Paxinos and Watson (Paxinos and Watson, 1986) as relative to bregma: +0.3P, ±2.8L, -4.5V to skull. The custom-made internal cannulas were designed to protrude 1 mm beyond the tip of the cannula guide. A small hole was drilled in the skull at these coordinates using a hand drill, with an additional three holes for anchoring screws arranged in a triangular pattern around the cannula hole for support. Following insertion of the anchoring screws, the permanent cannula guide was lowered to the desired ventral depth and held in place by the addition of dental cement (Dentec, Burlington, ON) within the incision site. Once the cement cured, the mounting assembly was removed and a temporary dummy cannula was inserted into the implanted cannula guide to prevent tissue entry. The skin surrounding the incision site was left to seal around the dental cement cap, and an antibiotic ointment

(vetropolycin) was applied to the area to prevent infection.

Animals were kept warm and closely monitored during recovery. For hydration, animals received 5 - 10 ml of warm sterile saline subcutaneously (s.c.) immediately prior to wakefulness. Animals were subsequently handled and examined twice daily for 3 days and once a day for the following week for signs of pain or dehydration. Biases in spontaneous motor activity were also monitored.

IV.1.1.5. Behavioural Testing

As introduced in Section I.4.3.1, lesioning of the substantia nigra with 6-OHDA results in the depletion of striatal dopamine levels, with the corresponding supersensitivity of post-synaptic dopamine receptors. This supersensitivity can be easily detected by the administration of the dopamine receptor agonist apomorphine (Ungerstedt, 1971). This results in contralateral rotational behaviour (i.e. rotation away from the side of the lesion) due to the hemispheric imbalance in dopamine receptor activity. Accordingly, animals were tested for apomorphine-induced rotations within 8 - 10 days following the lesion.

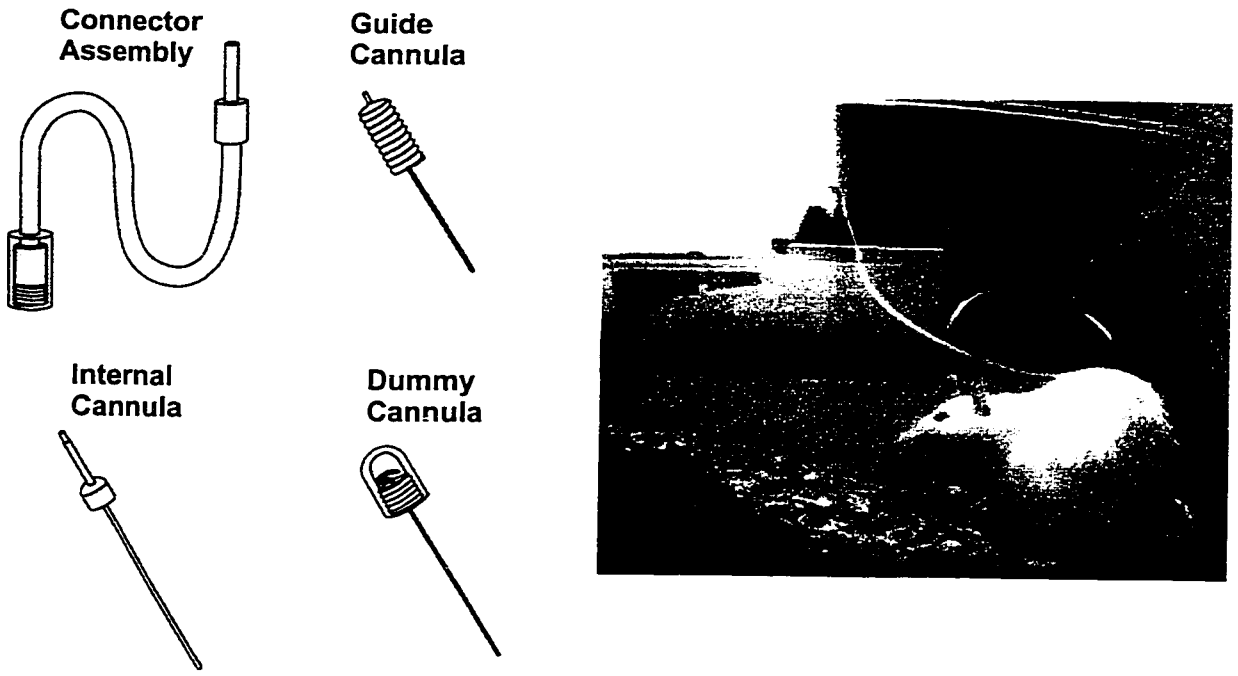
On the testing day, animals were given a 5 min habituation period to the testing environment, which consisted of a clear, round, flat-bottom bowl. Rats were subsequently injected with apomorphine (0.25 mg/kg, i.p.) and allowed a 5 min latency period to allow for distribution of the drug to its target sites of action. Only those rats exhibiting greater than 30 contralateral rotations over a 10 min period were included in

the study. Behavioural testing was performed at the same time of day on at least three separate occasions to obtain a stable baseline of control rotations for each animal. Animals were given 2 - 3 days in between injections of apomorphine to allow for drug washout. Oligonucleotide infusion began 2 days following the last injection of apomorphine. All animals thus began receiving oligonucleotides within 16 - 21 days following 6-OHDA lesioning, corresponding to the maximal observed increase in stimulatory G protein levels (Figure 22). All results are presented as the percent change in the number of rotations compared to the control baseline value previously recorded for each animal.

IV.1.1.6. Infusion of Oligonucleotides

Oligonucleotides were infused using a 30 gauge stainless steel internal cannula custom made to protrude 1 mm beyond the tip of the implanted cannula guide (Figure 34). The cannula was attached to a connector assembly consisting of polyethylene supply tube encased in thick vinyl tubing (Plastics One, Roanoke, VA) that could be screwed into place on the exterior surface of the dental cement-encased cannula guide (Figure 34). Infusion cannulas and tubing were sterilized with ethanol prior to infusion. Oligonucleotides were infused over a 4 min period at a rate of 0.5 μl/min (2 μl total volume) in freely moving animals using a Harvard Apparatus infusion pump (Harvard Apparatus, St. Laurent, QC). Following a 1 min diffusion period, cannulas were removed and the ethanol-sterilized temporary dummy cannulas were replaced.

Figure 34. Oligonucleotide infusion



Schematic diagram and representative photo of the infusion equipment (Plastics One, Roanoke, VA, USA). A stainless steel permanent cannula guide with a threaded cylindrical plastic top is implanted in the striatum at the time of surgery and held in place with dental cement. A dummy cannula, with a stainless steel wire cut to fit the length of cannula guide, is inserted and tightened into place to prevent tissue entry when the animal is not being infused. On the day of infusion, a stainless steel internal cannula is attached to the connector assembly, consisting of a polyethylene supply tube encased in vinyl tubing. Tightening the cap of the connector assembly with the internal cannula on the cannula guide hydrostatically seals the infusion assembly. Oligonucleotide solutions are infused using an infusion pump (Harvard Apparatus, St. Laurent, QC, Canada) while the animal moves freely in its cage.

Animals were carefully monitored following oligonucleotide infusion for signs of infection or non-specific oligonucleotide effects. Signs of infection could include behavioural changes, discharge around the cannula guide, rapid weight loss, or spontaneous ipsilateral rotations. All animal were monitored daily for weight loss prior to and during oligonucleotide treatment. Diagnosis of infection was confirmed by visual examination of the tissue surrounding the cannula guide during dissection, or through culture swab samples of the cannula guide tip.

IV.1.2. Results

The results presented in this chapter are currently in preparation for publication (Marcotte and Mishra, 1998). For rotational behaviour following Golf oligonucleotide infusion, all results are presented as the percent change from pre-infusion values for each animal (mean ± SD). Presentation of G protein results and statistical analysis were performed as described previously in Section II.2.2.

IV.1.2.1. General Findings

Acutely following the lesion, many of the animals demonstrated an ipsilateral (same side as the lesion) bias in locomotion both spontaneously and in response to handling. This bias was generally self-limiting and diminished with time. However, the magnitude of this ipsilateral bias served as a useful indicator of the extent of lesioning.

As a general rule, the more pronounced the acute ipsilateral bias, the more robust the number of contralateral rotations induced by apomorphine, as observed during behavioural testing.

In response to acute injections of apomorphine (0.25 mg/kg, i.p.), lesioned rats demonstrated a contralateral rotational response (i.e., rotations away from the side of the lesion). Although the number of rotations observed during the recording period varied among different animals, each animal was extremely consistent in its response to repeated injections. Generally, a slight increase in the number of rotations induced by apomorphine was observed for each animal over successive trials. By the third injection of apomorphine, however, animals appeared to reach a stable baseline. This baseline was used as the basis for comparison following oligonucleotide infusion.

In addition to rotational behaviour, other behavioural characteristics were occasionally manifested in these animals. The second most commonly observed behavioural consequence of apomorphine injection was repetitive grooming of the contralateral side. In some animals, excessive grooming reduced the overall number of rotations in a given time period. However, the effect of this behaviour on rotations was consistent for each animal, and thus did not adversely affect the results.

Following determination of the stable baseline for apomorphine-induced rotations in each animal, animals received daily oligonucleotide infusions. During initial oligonucleotide infusion, some animals exhibited mild ipsilateral rotational behaviour. In extreme cases, this required temporary manual restraint to prevent obstruction of the connector tubing assembly. Generally, this rotational behaviour was self-limiting, and

subsided within the infusion period (5 min). It was rarely observed during subsequent infusion periods, and appears to represent an acute response to cannula insertion and/or infusion.

Following oligonucleotide infusion, animals were carefully monitored for any abnormal behavioural activity. The only non-specific behavioural abnormality observed in a handful of animals was rotations around the longitudinal axis of the rat's body, a phenomenon known as "barrel rolling." This phenomenon has been described previously following the infusion of a variety of agents including: endothelin-1 (Nikolov et al., 1992, Gross et al., 1993, Chew et al., 1995, D'Amico et al., 1996, Sullivan et al., 1996), GABA receptor antagonists (Kelly et al., 1977, Zainos et al., 1984), galanin receptor antagonists (Crawley et al., 1993), opioid receptor antagonists (Long et al., 1988, Hyytia, 1993), dynorphin-A (Long et al., 1988, Shukla et al., 1992, Shukla et al., 1997), dynorphin-B (Nakazawa et al., 1989), met-enkephalin (Zadina et al., 1993), quaternary-chlorpromazine (Rotrosen et al., 1980), cholecystokinin (Morency et al., 1987), neuropeptide Y antibodies (Walter et al., 1994), L-aspartate (Toth and Lajtha, 1989), kainate (Kilpatrick and Starr, 1981), arginine vasopressin (Ehlers et al., 1985), pertussis toxin (Cavicchini et al., 1990), phorbol esters (Patishi et al., 1996), Staphylococcal alpha-toxin (Lipman and Harshman, 1985), mast cell degranulators (Lewis et al., 1989), and oligonucleotides (Weiss et al., 1997). Given the wide range of agents known to induce this behaviour, its significance is not clear, but it appears to be a non-specific effect of infusion. Barrel rolling has been likened to a form of dystonia (Gollasch et al., 1993) or epileptic seizure (Gross et al., 1993, Chew et al., 1995),

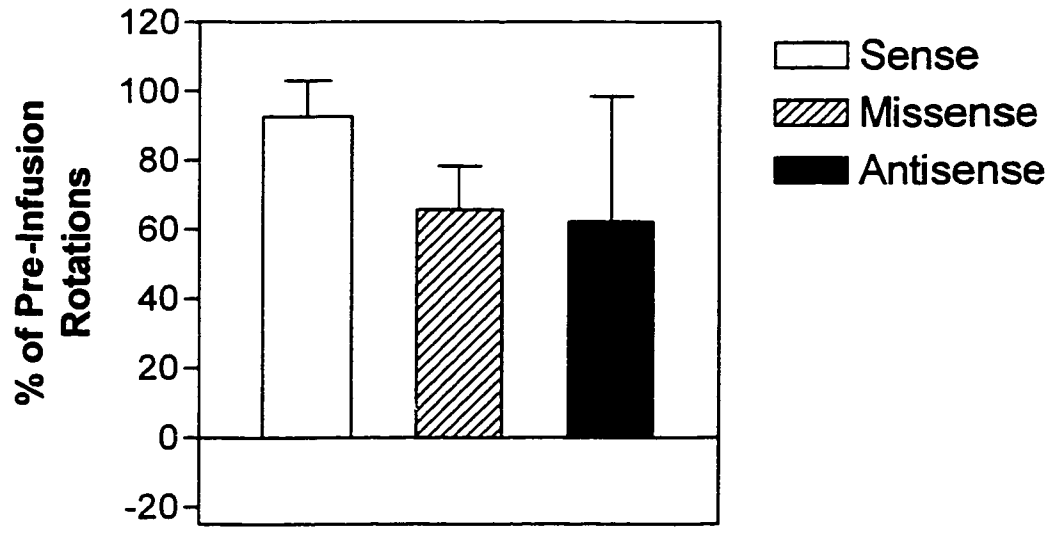
however seizure activity has not always been recorded (Ehlers et al., 1985). Although infrequently observed in these studies, this behaviour demonstrated a consistent pattern. The behaviour was short lasting, beginning approximately 10 min after infusion and lasting for no more than 30 min. The number of rotations varied among affected animals, but never exceeded more than 2 turns per minute and appeared to diminish with repeated injections. The presence of this behaviour did not appear to adversely affect outcome measures in affected animals, similar to previous reports with antisense oligonucleotides (Weiss et al., 1997).

A variety of dose regimens were examined in these studies. At all doses, both apomorphine-induced rotations and G protein levels were examined. A variety of G proteins (Golfα, Gsα, Giα, and Goα) were measured for each treatment group in order to determine the specificity of antisense oligonucleotide infusion on Golfα levels. G protein levels were assayed according to standard protocols using Western blots with specific antisera (Golfα, Gsα, Giα, and Goα) as previously described (see Section II.0.1).

IV.1.2.2. Very Low Dose Oligonucleotide Infusion

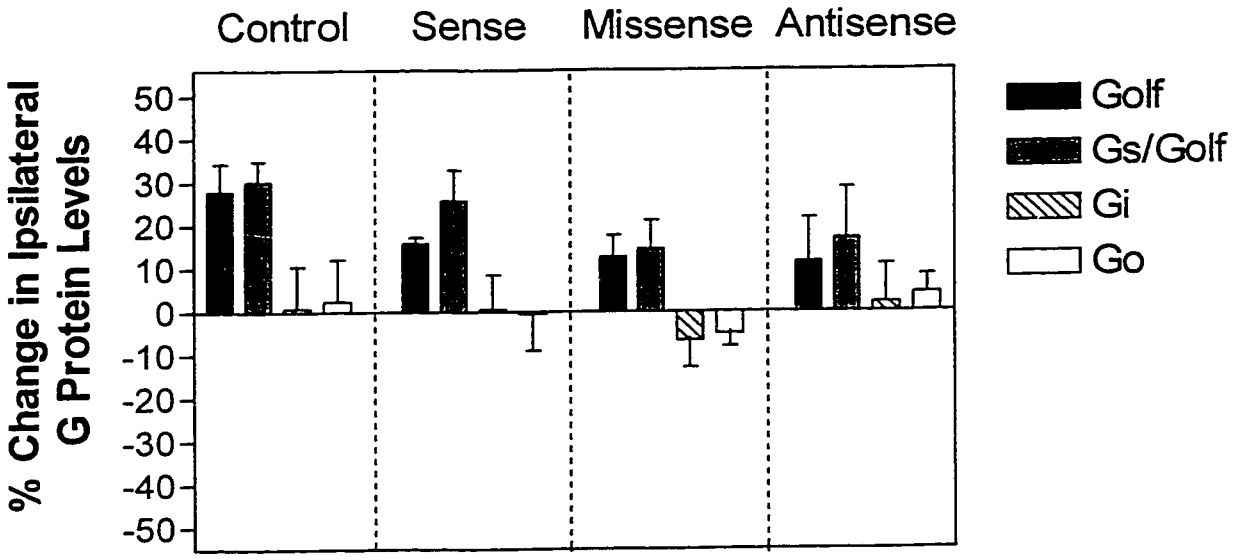
The effects of very low dose oligonucleotide infusion (2.5 nmoles / 2 µl / day for 2 days) are presented in Figure 35. This dose of oligonucleotide was chosen to correspond to the lower range of doses used for in vivo and in vitro G protein antisense studies (see Table V). The duration of treatment (2 days) was chosen to provide a lower limit to dose-response studies, as a two day treatment would be expected to have little

Figure 35. Effects of very low dose Golf oligonucleotide infusion on apomorphine-induced rotations



Data points represent the percent change in apomorphine-induced rotations following Golf oligonucleotide infusion in 6-OHDA lesioned rats at the very low dose regimen, as described in the methods (2.5 nmoles / day / 2 days). Data are presented as the mean ± SD (n=3 for all groups). Note the partial reduction in rotations produced by Golf antisense and missense but not control sense infusion. However, the effect of oligonucleotide infusion was not statistically significant (one-way ANOVA, Tukey post-hoc comparisons between all groups).

Figure 36. G protein levels following very low dose Golf oligonucleotide infusion



Bars represent the percent change in G protein levels in the infused/lesioned striatum relative to the contralateral hemisphere. Gs/Golf refers to the non-specific RM/1 antiserum described previously. Control refers to a lesioned but non-infused group of animals. Infused groups received the indicated Golf oligonucleotide at the very low dose regimen (2.5 nmoles / day for 2 days). Data are presented as the mean ± SD (n=3 for all groups). Note the slight reduction in stimulatory G protein levels in all groups. There was no significant difference in the levels of any of the G proteins in any of the treatment groups (ANOVA followed by Bonferroni's selected pair comparison of each treatment group to control for each G protein subtype).

effect on G protein levels or apomorphine-induced rotations. As can be seen in this figure, control Golf sense oligonucleotide infusion had no effect on apomorphine-induced rotations relative to pre-infusion values, as expected. However, both Golf missense and antisense oligonucleotides appeared to reduce apomorphine-induced rotations, although this effect was not statistically significant (one-way ANOVA, Tukey post-hoc comparison).

Analysis of G protein levels in very low dose oligonucleotide treated animals is presented in Figure 36. As expected, the very low oligonucleotide infusion regimen produced little change in G protein levels. There was a slight reduction in the levels of the stimulatory G proteins Golfα and Gsα in all three infusion groups, relative to non-infused 6-OHDA lesion controls, but this effect was not significant (one-way ANOVA, Bonferroni post-hoc multiple comparison test). As expected, oligonucleotide infusion had no effect on inhibitory G protein levels. These results are consistent with the behavioural data, and demonstrate that very low dose oligonucleotide infusion is relatively ineffective.

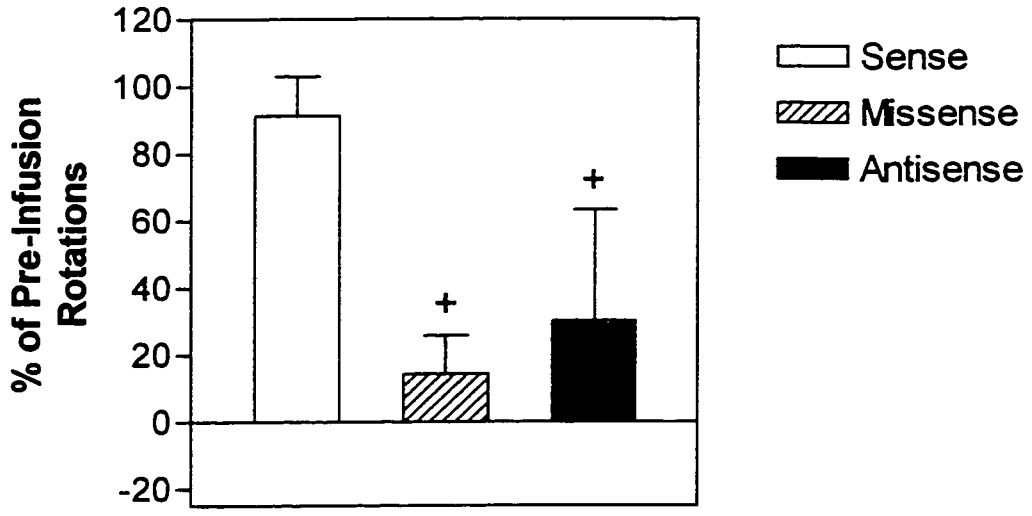
IV.1.2.3. Low Dose Oligonucleotide Infusion

To further characterize the effects of this relatively low dose of oligonucleotides (2.5 nmoles / day / 4 days), an additional two days of infusion were examined. The results of this low dose oligonucleotide infusion (2.5 nmoles / 2 µl / day for 4 days) are presented in Figure 37. As expected, control Golf sense infusion had no effect on

apomorphine-induced rotations. However, both Golf antisense and missense oligonucleotides significantly reduced rotational behaviour at this low dose (one-way ANOVA, Tukey post-hoc comparison). In fact, Golf missense appeared to have an even greater effect than Golf antisense, although the difference between these two groups was not significant. The response to oligonucleotide infusion was somewhat variable in these animals, particularly among the Golf antisense group (Figure 37). The partial reduction in apomorphine-induced rotations in Golf antisense treated animals was expected, but the significance of the Golf missense reductions is unclear. Neither the control sense or missense oligonucleotides would be expected to have any effect on apomorphine-induced rotations if these oligonucleotides were acting through an antisense mechanism.

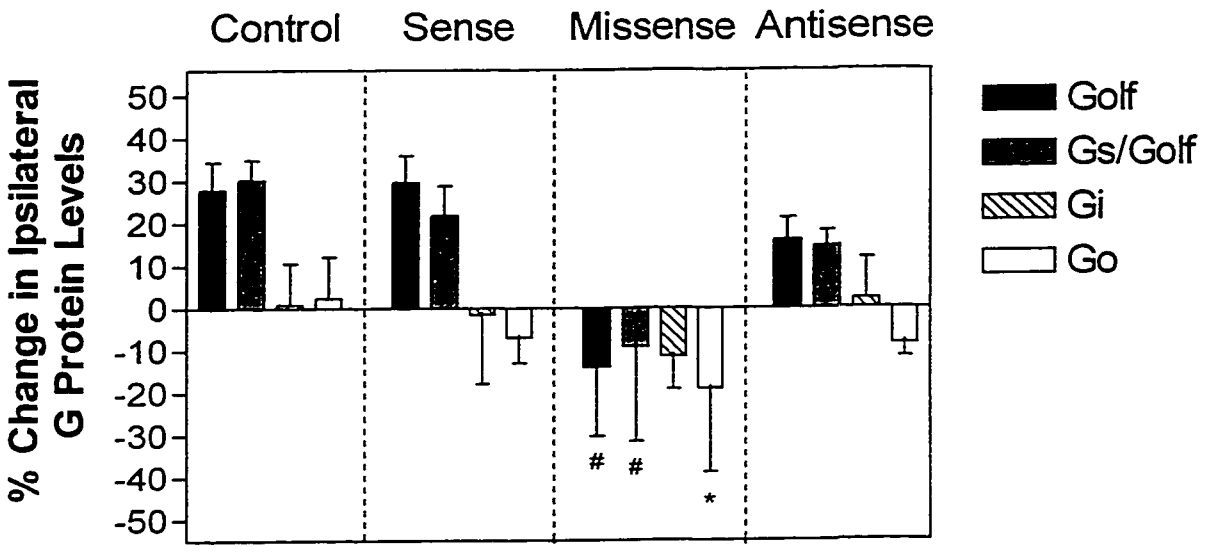
Analysis of G protein levels in the low treatment groups was performed to further explore the effects of oligonucleotide infusion at this dose (Figure 38). As expected, control sense infusion had no effect on G protein levels. Golf antisense infusion produced a partial reduction in stimulatory G protein levels similar to that observed in the very low treatment group (Figure 36). As before, however, this reduction was not statistically significant (one-way ANOVA, Bonferroni post-hoc multiple comparison test). Similarly, inhibitory G proteins were not affected by Golf sense or antisense infusion. However, a non-specific reduction in all G protein levels was observed in the control Golf missense group (Figure 38). This non-specific reduction in G protein levels presumably reflects the impaired rotational behaviour in these animals. However, the reasons for this Golf missense effect remain unclear.

Figure 37. Effects of low dose Golf oligonucleotide infusion on apomorphine-induced rotations



Data points represent the percent change in apomorphine-induced rotations following Golf oligonucleotide infusion in 6-OHDA lesioned rats at the low dose regimen, as described in the methods (2.5 nmoles / day / 4 days). Data are presented as the mean ± SD (n=6 for all groups). A significant reduction in apomorphine-induced rotations was observed for both Golf antisense and missense infusion compared to control sense infusion (+ $p<0.001$, one-way ANOVA, Tukey post-hoc comparisons between all groups). Golf sense infusion had no effect on rotations.

Figure 38. G protein levels following low dose Golf oligonucleotide infusion



Bars represent the percent change in G protein levels in the infused/lesioned striatum relative to the contralateral hemisphere. Gs/Golf refers to the non-specific RM/1 antiserum described previously. Control refers to a lesioned but non-infused group of animals. Infused groups received the indicated Golf oligonucleotide at the low dose regimen (2.5 nmoles / day for 4 days). Data are presented as the mean ± SD (n=6 for all groups). Note the non-specific reduction in all G protein levels observed following Golf MS infusion (* $p < 0.05$, # $p < 0.001$, ANOVA followed by Bonferroni's selected pair comparison of each treatment group to control for each G protein subtype). Golf AS infusion appeared to specifically reduce Gs/Golf levels, but this effect was not statistically different from control. Golf S infusion had no effect on any G protein level.

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IV.1.2.4. High Dose Oligonucleotide Infusion

Despite the non-specific effects of Golf missense at the low dose oligonucleotide infusion, we also examined the effects of a higher dose (4.0 nmoles / day / 4 days) in 6-OHDA lesioned rats. This dose was chosen to correspond to the higher end of doses found in the available literature on G protein oligonucleotides (see Table V). The effects of high dose oligonucleotide infusion (4.0 nmoles / 2 µl /day for 4 days) are presented in Figure 39. Similar to the low dose infusion data, both Golf antisense and missense reduced apomorphine-induced rotations. In fact, Golf antisense treatment virtually abolished apomorphine-induced rotations, while the net effect of Golf missense treatment was to increase ipsilateral rotations slightly (Figure 39). In contrast to earlier results, however, Golf sense infusion also reduced apomorphine-induced rotations at this dose.

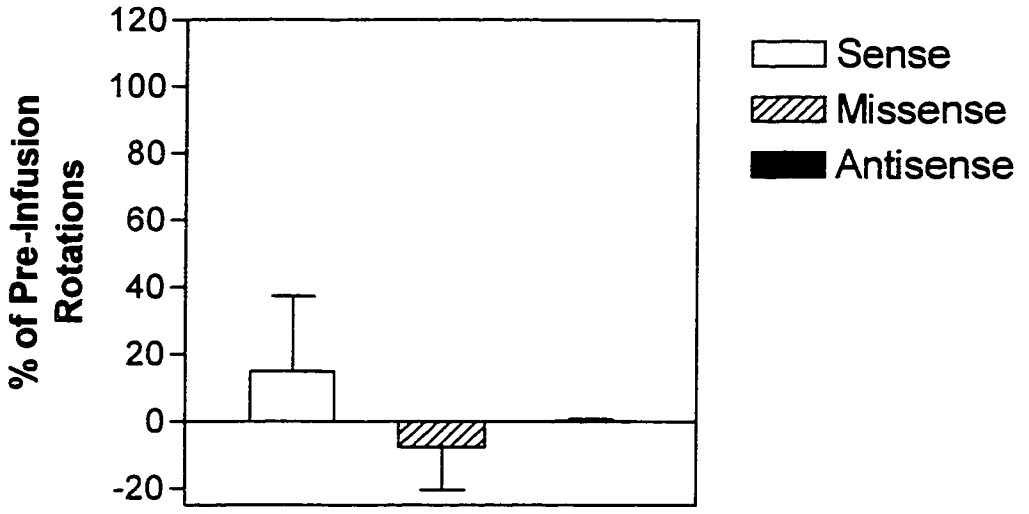
To further explore the underlying basis of this behavioural effect, G protein levels were measured as described previously, and are shown in Figure 40. Both Golf antisense and missense produced a non-specific reduction in all G protein levels, similar to the reduction observed following low dose Golf missense infusion (Figure 38). In the Golf sense infusion group, only a partial reduction in G protein levels was observed, corresponding to the partial reduction in apomorphine-induced rotations. These results suggest that at higher concentrations, Golf oligonucleotide infusion produces non-specific effects on apomorphine-induced rotations and G protein expression.

IV.1.2.5. Combined Dose Oligonucleotide Infusion

In an attempt to isolate a dose regimen of oligonucleotides that would show specific reductions in rotations and Golfα levels following Golf antisense infusion, we looked at the effects of combining the low and high treatment groups. The rationale for these experiments was to allow the animal time to adjust to a lower concentration of oligonucleotide prior to the introduction of higher concentrations. The results of combined oligonucleotide infusion (2.5 nmoles / day for 4 days + 4.0 nmoles / day for an additional 3 days) are presented in Figure 41. Interestingly, control Golf sense infusion had no effect on apomorphine-induced rotations, similar to the low dose response (Figure 37). In contrast, both Golf antisense and missense virtually abolished apomorphine-induced rotations, similar to the high dose response (Figure 39). Thus, it appears that initial low dose infusion can prevent the behavioural effects of high dose administration for Golf sense oligonucleotides, but not Golf antisense or missense.

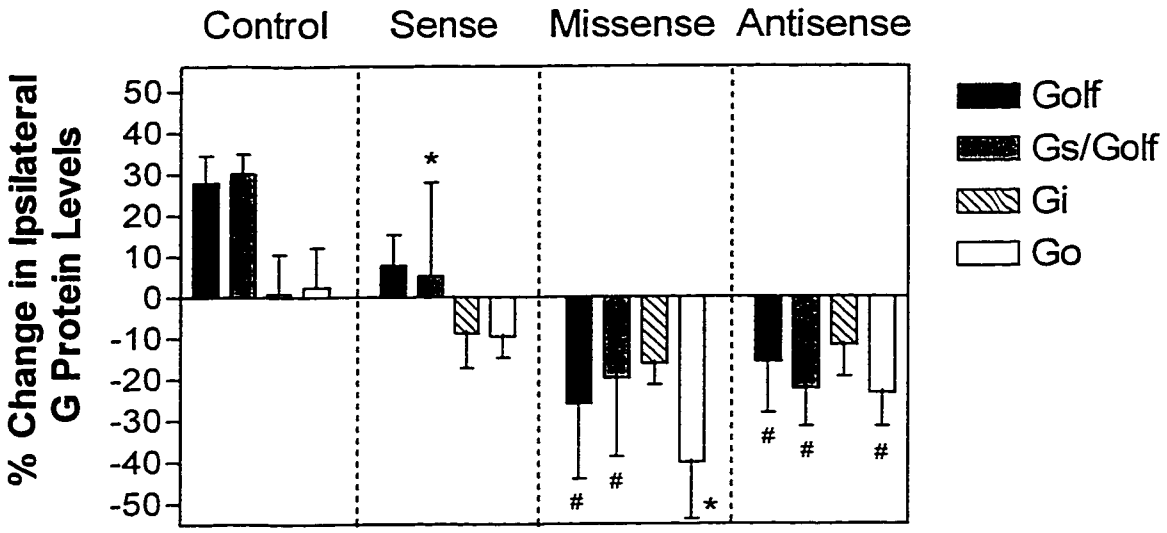
As before, G protein levels were examined following combined infusion (Figure 42). Golf sense infusion had no effect on G protein levels, consistent with its lack of effect on apomorphine-induced rotations. This lack of effect is the same as was observed following low dose administration. In contrast, both Golf missense and antisense oligonucleotides reduced all G protein levels in a non-specific manner (Figure 42). This finding is in keeping the effects of high dose administration. Thus, both the behavioural and G protein data indicate that pre-treatment with low dose oligonucleotides selectively prevents the effects of Golf sense high dose administration, but is ineffective against Golf missense or antisense infusion.

Figure 39. Effects of high dose Golf oligonucleotide infusion on apomorphine-induced rotations



Data points represent the percent change in apomorphine-induced rotations following Golf oligonucleotide infusion in 6-OHDA lesioned rats at the high dose regimen, as described in the methods (4.0 nmoles / day for 4 days). Data are presented as the mean ± SD (n=4 for all groups). Note the virtual abolition of apomorphine-induced rotations in all Golf oligonucleotide infusion groups. The effect of different oligonucleotides was not statistically significant (one-way ANOVA, Tukey post-hoc comparisons between all groups).

Figure 40. G protein levels following high dose Golf oligonucleotide infusion



Bars represent the percent change in G protein levels in the infused/lesioned striatum relative to the contralateral hemisphere. Gs/Golf refers to the non-specific RM/1 antiserum described previously. Control refers to a lesioned but non-infused group of animals. Infused groups received the indicated Golf oligonucleotide at the high dose regimen (4.0 nmoles / day for 4 days). Data are presented as the mean ± SD (n=4 for all groups). Note the non-specific reduction in G protein levels following Golf MS or AS infusion (* $p < 0.05$, # $p < 0.001$, ANOVA followed by Bonferroni's selected pair comparison of each treatment group to control for each G protein subtype). Golf S infusion produced a partial reduction that was only statistically significant for the non-specific Gs antibody.

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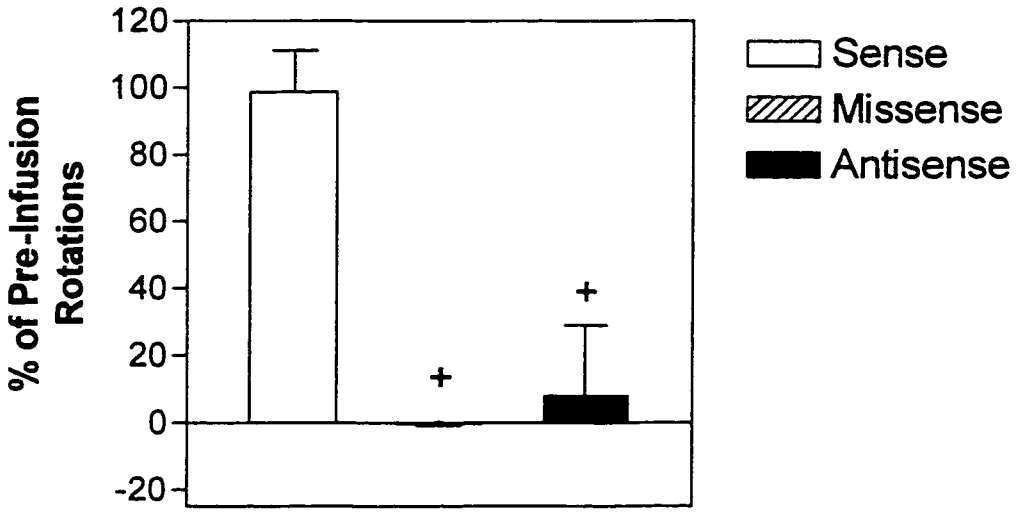
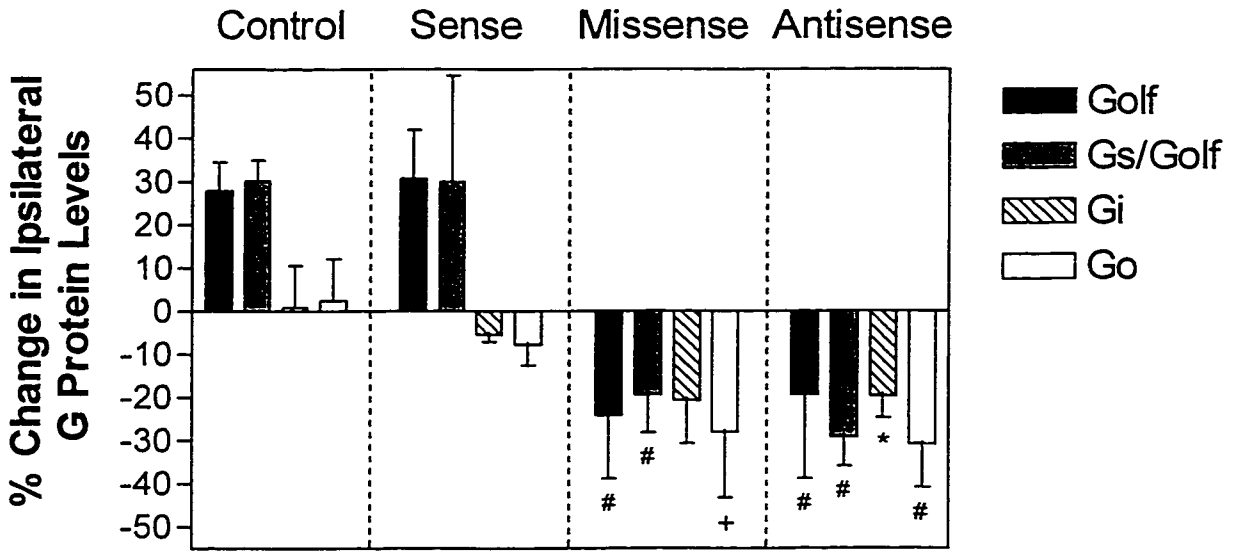
Figure 41. Effects of combined dose Golf oligonucleotide infusion on apomorphine-induced rotations



Data points represent the percent change in apomorphine-induced rotations following Golf oligonucleotide infusion in 6-OHDA lesioned rats at the combined dose regimen, as described in the methods (2.5 nmoles / day for 4 days + 4.0 nmoles / day for 3 days). Data are presented as the mean ± SD (n=5 for all groups). Note the virtual abolition of apomorphine-induced rotations in Golf antisense and missense infusion groups compared to control sense infusion (+ $p<0.001$, one-way ANOVA, Tukey post-hoc comparisons between all groups). As previously observed, Golf sense infusion had no effect on rotations.

Figure 42. G protein levels following combined dose Golf oligonucleotide infusion

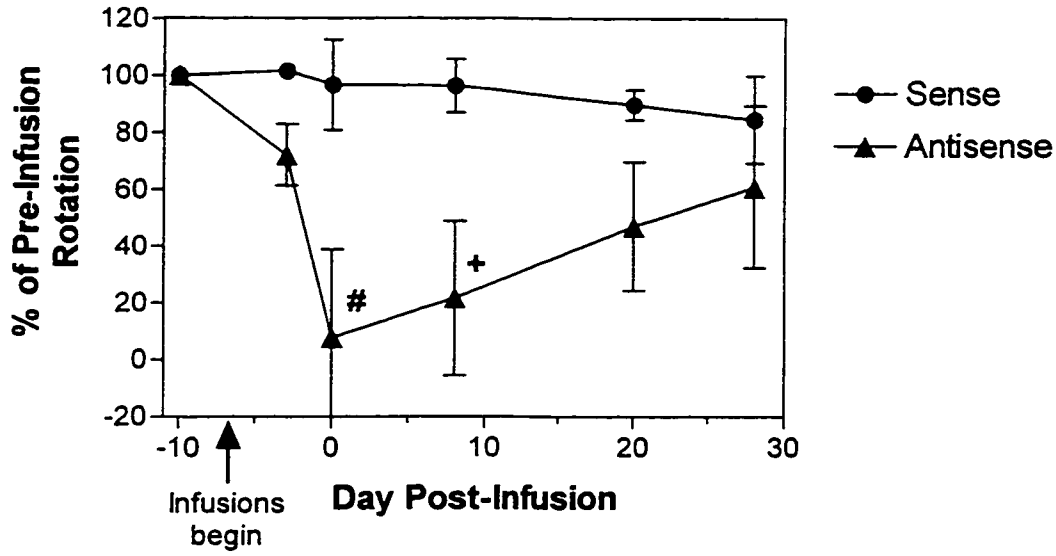


Bars represent the percent change in G protein levels in the infused/lesioned striatum relative to the contralateral hemisphere. Gs/Golf refers to the non-specific RM/1 antiserum described previously. Control refers to a lesioned but non-infused group of animals. Infused groups received the indicated Golf oligonucleotide at the combined dose regimen (2.5 nmoles / day for 4 days + 4.0 nmoles /day for 3 days). Data are presented as the mean ± SD (n=5 for all groups). Note the non-specific reduction in G protein levels produced by Golf MS and AS treatment (* $p < 0.05$, + $p < 0.01$, # $p < 0.001$, ANOVA followed by Bonferroni's selected pair comparison of each treatment group to control for each G protein subtype). As observed previously, Golf S infusion had no effect on G protein levels.

IV.1.2.6. Recovery of Rotational Behaviour

One of the hallmarks of antisense oligonucleotide knockdown is that the effects of oligonucleotide infusion should be reversible. To test whether oligonucleotide infusion was reversible, a pilot study using combined dose Golf sense and antisense treated animals was performed (Figure 43). Recovery of apomorphine-induced rotations was monitored in these animals by additional apomorphine injections over a four week period (0.25 mg/kg apomorphine, i.p.). As can be seen in Figure 43, low dose (2.5 nmoles / day / 4 days) Golf antisense infusion resulted in only a partial reduction in apomorphine-induced rotations. The additional three days of higher concentration treatment (4.0 nmoles / day) resulted in the virtual abolition of rotations in the Golf antisense group, with Golf sense oligonucleotides continuing to produce no effect. These findings are consistent with the behavioural data presented in Figure 41. By eight days post-infusion, rotations were still severely depressed in Golf antisense treated animals. However, by 21 days post-infusion, rotations had recovered to 50% of pre-infusion values, which was not statistically significant from Golf sense treated animals due to the high variability of the Golf antisense treated group (one-way ANOVA followed by Bonferroni's selected pair comparison between infusion groups). Interestingly, Golf sense infusion animals showed a slight decline in the number of rotations over time, but this trend was not significant. These results suggest that the animals recover from oligonucleotide treatment with time, although the time course of this response is slower than expected.

Figure 43. Recovery from combined dose Golf oligonucleotide infusion



Plot of recovery of apomorphine-induced rotations following combined dose Golf oligonucleotide infusion. Data points represent the percent change in apomorphine-induced rotations in Combined dose animals as compared to their pre-treatment values. Infusions begin at the arrow mark on Day -7, and end on Day 0 (n=3 for both groups). By Day 28, antisense-infused animals had returned to approximately 60% of baseline, with no significant change in control sense animals. Statistical significance based on one-way ANOVA followed by Bonferroni's selected pair comparison of sense and antisense groups at each time post-infusion. + $p < 0.01$, # $p < 0.001$.

IV.1.3. Discussion

The goal of in vivo antisense oligonucleotide infusion is to specifically reduce the expression of a target mRNA of interest. As previously mentioned in the Introduction, the method(s) by which exogenously applied oligonucleotides may prevent protein translation are not fully understood at present. In fact, there is little evidence to support many of the proposed mechanisms of action, and some are only theoretical (Robinson et al., 1997). Translational arrest, perhaps involving steric hindrance, is the most likely mechanism by which protein synthesis may be blocked (Figure 13). Alternatively, antisense oligonucleotides may act through the activation of RNase H by cleaving DNA:RNA hybrids (Baertschi, 1994). However, there are several reports showing that antisense oligonucleotides can down-regulate specific gene products without causing degradation of their respective mRNA (Brysch and Schlingensiepen, 1994, Wahlestedt et al., 1993, Chiang et al., 1991), thus casting doubt on this proposed mechanism. In addition, phosphorothioate oligonucleotides have been reported to exert a biphasic effect on RNase H activity (Gao et al., 1992).

One of the main theoretical advantages of oligonucleotides over traditional pharmacological agents is their potential high affinity and specificity. Exogenously applied oligonucleotides are expected to hybridize to their target mRNA in a highly sequence-specific manner without appreciable binding to other mRNA species. It has previously been determined that the optimal length of applied oligonucleotides is 15 - 30 bases (Brysch and Schlingensiepen, 1994). Oligonucleotides shorter than this would be

more prone to potential multiple matches (Helene, 1991). Longer sequences of oligonucleotides would have a higher probability that a portion of the sequence would be homologous to other mRNA species, and thus prove more non-specific.

The specificity of oligonucleotides also depends on their base composition. In particular, oligonucleotides with a high G-C content are more likely to produce non-specific effects due to the higher stability of these bonds (i.e. three hydrogen bonds for G-C pairing versus two for A-T). Thus, hybridization of even a short portion of the oligonucleotide may be stable enough to cause inhibition. It has been suggested that many of the non-sequence-specific effects of oligonucleotides may actually be due to sequences that bind too well to multiple targets (Brysch and Schlingensiepen, 1994). This is a potential source of concern given the 65% G-C content of oligonucleotides used in these studies (see Appendix A). To minimize this potential problem, oligonucleotide sequences have been limited to 20 bases (mer) in these studies, consistent with the literature reports. The selectivity of oligonucleotides is also a function of how well they bind to non-RNA targets such as proteins. Unfortunately, relatively little is known at present on the nature or specificity of these interactions. Interestingly, the charge conferred by phosphorothioate modification (Figure 14) has been implicated in increasing the likelihood that these molecules will interact with proteins in a non-selective fashion (Perez et al., 1994).

In addition to specificity, oligonucleotide administration also faces several potential difficulties in regard to cellular uptake, nuclease stability, pharmacodynamics, and toxicity. Surprisingly, oligonucleotides appear to be taken up readily by cells

through both adsorptive endocytosis (pinocytosis) and receptor-mediated transport systems (Crooke and Bennett, 1996). Nuclease stability is a far greater concern since unmodified oligonucleotides are rapidly degraded by endogenous nucleases. Accordingly, a variety of modifications to the sugar-phosphate backbone and nucleotide bases of DNA has been explored in order to produce more nuclease resistant oligonucleotide analogues. As previously described, phosphorothioate oligonucleotides are the most popular of these modifications, involving the replacement of a non-bridging oxygen atom in the DNA backbone is by a charged sulfur atom (see Figure 14). In addition to being more nuclease resistant, these phosphorothioate analogues show many of the same characteristics of regular phosphodiester oligonucleotides, including water solubility, potency at nanomolar concentrations, activation of RNase H, and efficient cellular uptake (Baertschi, 1994). However, a greater degree of non-sequence-specific effects has also been attributed to this modification.

In addition to their high affinity and potency, oligonucleotides share the ability of many pharmacological agents to produce non-specific and toxic effects. Several studies have suggested that insufficient purity of phosphorothioate oligonucleotide preparations may be one of the main reasons for toxicity (O'Keefe et al., 1989, Mirabelli et al., 1991, Reed et al., 1990)(Personal communication, Dr. Ian Creese). However, many of these studies were performed using in vitro cell culture models, which may not be entirely applicable to in vivo situations. In particular, it is possible that other factors may be involved in mediating toxicity in vivo. It has been suggested that some of the toxicity of phosphorothioate oligonucleotides may be species-dependent (Akhtar and Agrawal,

1997). Moreover, some of the toxicological effects have been found to be sequence-specific. For example, several control sense oligonucleotides, but not their respective antisense counterparts, have been reported to cause massive splenomegaly in mice following systemic infusion (McIntyre et al., 1993, Branda et al., 1993). A detailed list of the non-antisense effects of oligonucleotide infusion, including both sequence-specific and sequence-independent effects, is presented in Table IX. As can be seen in this table, non-antisense effects have been reported in a large number of experimental systems. These results are particularly alarming considering the likely bias against reporting negative findings in the literature.

Many of the limitations of in vitro toxicology studies are also applicable to the pharmacokinetic analysis of oligonucleotides. Although pharmacokinetic characteristics of oligonucleotides have been established in vitro, there is ample evidence that these may not be comparable to in vivo situations (Crooke et al., 1995). Direct in vivo experiments have generally revealed that systemic administration results in the uptake of oligonucleotides predominantly in peripheral tissues (Brysch and Schlingensiepen, 1994). Under physiological conditions, there is virtually no uptake of oligonucleotides from the circulation into the CNS (Akhtar and Agrawal, 1997), suggesting that exogenously applied oligonucleotides cannot penetrate the blood-brain barrier. As such, neuroscience studies of oligonucleotides require direct administration into the brain parenchyma or ventricles. These methods are particularly advantageous as the brain seems to have a lower nuclease activity than most peripheral tissues (Wahlestedt, 1994, Whitesell et al., 1993). Based on in vitro studies, it has also been suggested that neuronal cells may

Table IX

Examples of non-antisense effects of oligonucleotides

Type of Activity	Sequence Specific	Reference
Inhibition of thrombin	Yes	Bock et al., 1992
Inhibition of gp120-CD4 interaction	Yes	Wyatt et al., 1994
Inhibition of type II phospholipase A_2	Yes	Bennet et al., 1994a
Inhibition of protein kinase C	No	Stein et al., 1993
Inhibition of DNA polymerases	No	Gao et al., 1989, Gao et al., 1992
Inhibition of RNase H	No	Gao et al., 1992
Inhibition of cell proliferation	Yes, No	Yaswen et al., 1993, Clark et al., 1994,, O'Brien et al., 1994, Burgess et al., 1995, Smetsers et al., 1997
Inhibition of herpes simplex virus growth	Yes, No	Gao et al., 1990, Ecker et al., 1993, Zelphati et al., 1994
Inhibition of cell adhesion	Yes	Watson et al., 1992, Narayanan et al., 1993, Maltese et al., 1995, Hertl et al., 1995
Inhibition of in vitro translation	No	Ghosh et al., 1992
Inhibition of transferrin receptor expression	No	Ho et al., 1991
Inhibition of interferon-λ receptor binding	Yes	Ramanathan et al., 1994
Inhibition of interferon-λ production	Yes	Halpern and Pisetsky, 1995
Inhibition of PDGF receptor binding	No	Guvakova et al., 1995
Inhibition of Sp1 binding	No	Perez et al., 1994
Inhibition of electrical potentials	No	Abraham et al., 1997
Inhibition of globin synthesis	No	Larrouy et al., 1995
Binding to laminin	No	Benimetskaya et al., 1995
Binding to CD4	No	Yakubov et al., 1993, Benimetskaya et al., 1995
Binding to bFGF	No	Guvakova et al., 1995, Benimetskaya et al., 1995
Binding to NF-κB	No	Ho et al., 1991

Type of Activity	Sequence Specific	Reference
Expression of CD40 antigen	No	Chen et al., 1996
Proliferation of lymphocytes	Yes	Pisetsky and Reich, 1993a, Pisetsky and Reich, 1993b, Krieg et al., 1995, Chen et al., 1996
Uptake of ^{3}H-thymidine	No	Jessop et al., 1997
Splenomegaly	Yes	McIntyre et al., 1993

possess more efficient uptake than other tissues (Walker et al., 1995).

One of the first in vivo CNS applications of oligonucleotide technology was the specific reduction of dopamine D_2 receptors in 6-OHDA lesioned mice (Weiss et al., 1993, Risby et al., 1991). These studies showed that intracerebroventricular (I.C.V.) administration of D_2 antisense oligonucleotides specifically reduced dopamine D_2 receptor agonist-induced rotations while having no effect on dopamine D_1 receptor agonist-induced rotations (Zhou et al., 1994). As a control, D_2 receptor missense oligonucleotides had no effect on rotational behaviour. Further, this effect of D_2 receptor antisense oligonucleotides was dose- and time-dependent (Zhou et al., 1994, Qin et al., 1995). Subsequently, these authors have also shown that D_1 receptor antisense oligonucleotides selectively produce comparable effects on D_1 receptor mediated behaviours (Weiss et al., 1997). Similarly, other groups have also confirmed the ability of centrally-administered dopamine receptor antisense agents to reduce dopaminergic behaviours in vivo (Zhang and Creese, 1993, Silvia et al., 1994, Tepper et al., 1997, Nissbrandt et al., 1995).

Given the increase in stimulatory G proteins observed in 6-OHDA lesioned rats in these studies (Section II.2), we adopted a similar approach to examine the effects of Golf antisense oligonucleotides on dopamine receptor supersensitivity. Relatively low dose oligonucleotide infusion at 2.5 nmoles / day for 2 days (very low dose, Figure 35) had little effect on apomorphine-induced rotations. This suggests that oligonucleotide infusion per se does not produce immediate non-specific or toxic effects in these animals. However, an additional 2 days of infusion (low dose, Figure 37) resulted in the significant

reduction of apomorphine-induced rotations for both Golf missense and antisense oligonucleotides. Golf sense oligonucleotides continued to have no effect.

The differential response of control oligonucleotides at low dose infusion is puzzling. To further clarify the effects of oligonucleotides in these studies, a higher concentration of 4.0 nmoles / day for 4 days (high dose, Figure 39) was also examined. This dose resulted in the complete abolition of rotations in both antisense and missense groups, with a partial reduction in Golf sense treated animals. Thus, it appears that Golf oligonucleotides produce non-specific dose-dependent effects on apomorphine-induced rotations. However, this response is at least partially sequence-specific, as Golf sense had no effect in the very low, low, or combined dose groups. To rule out some potential unknown sequence-specific interaction of the Golf missense sequence used in these studies, a second random-order missense oligonucleotide was also tested at the low dose (see Appendix A). This oligonucleotide produced identical effects to the first missense sequence (data not shown).

To further characterize this non-specific effect, additional groups of animals received combined low and high concentrations of oligonucleotides to determine if pre-treatment at the low dose can prevent the non-specific effects of high dose administration. Animals received 2.5 nmoles / day for 4 days followed immediately by 4.0 nmoles / day for an additional 3 days (7 days total, combined group, Figure 41). Interestingly, while Golf missense and antisense infusion severely reduce apomorphine-induced rotations, Golf sense treatment had no effect. In this regard, these results more closely resemble the low dose infusion group than the high dose group. The significance of this finding is

unclear, but it suggests that pre-treatment with lower concentrations of oligonucleotides may, in some cases, prevent the expression of non-specific effects.

In addition to behavioural data, functional measures of any specific oligonucleotide effect are also needed to unequivocally demonstrate an antisense mechanism of action. These are required to confirm that any observed biological effect is due to the specific inhibition of a particular mRNA (Brysch and Schlingensiepen, 1994). Given our previous detailed time course studies of G protein levels in 6-OHDA lesion rats using Western blot analysis, a similar strategy was employed here. Any specific antisense effect should result in the reduction of Golfα without affecting other protein levels. To confirm any specific effect, we also examined the effects of oligonucleotides on related G proteins (Gsα, Giα, and Goα).

Very low dose infusion had minimal effects on G protein levels (Figure 36), consistent with its general lack of effect on apomorphine-induced rotations. Slight reductions in stimulatory G proteins in all three oligonucleotide groups were observed, although this trend was not significant. At the prolonged low dose exposure, Golf oligonucleotides produced differential effects on G protein expression (Figure 38). Specifically, Golf sense infusion had no effect on any G protein level, while Golf antisense produced the same mild reduction in stimulatory G proteins observed at the very low dose. However, Golf missense infusion produced a non-specific reduction in all G protein levels at this dose that was statistically significant. This effect was most pronounced on stimulatory G protein levels (Figure 38). Interestingly, Golf missense oligonucleotides also appeared to produce a more pronounced reduction in rotational

behaviour at this dose (Figure 37). These results suggest that behavioural measures per se do not provide an unequivocal indication of G protein status. Treatment with higher concentrations of oligonucleotides (high dose) produced a partial reduction in stimulatory G protein levels in Golf sense treated animals (Figure 40). Both Golf missense and antisense infusion resulted in the non-specific reduction of all G protein levels.

G protein levels were also examined in the combined treatment group (Figure 42). Golf sense infusion had no effect on G protein levels, consistent with the previously observed lack of effect on apomorphine-induced rotations. Similarly, both Golf antisense and missense non-specifically reduced G protein levels, consistent with their effect on apomorphine-induced rotations. As previously stated, this result suggests that in at least one instance, namely Golf sense infusion, pre-treatment with a lower concentration of oligonucleotide can prevent development of non-specific effects observed at higher concentrations. The significance of this finding is uncertain, as the reduction in G protein levels and apomorphine-induced rotations in the high dose group could be anomalous.

It thus appears that each of the three oligonucleotides produces a similar pattern of reduced rotations and G protein levels, but at differing concentrations. Golf missense produced the greatest effect at the lowest concentration, non-specifically reducing G proteins and rotations following low dose infusion. Golf antisense also reduced rotations at this dose, but had only mild effects on G protein levels. Subsequently, high dose Golf antisense treatment produced non-specific effects on both G protein levels and rotations similar to that observed for Golf missense at the low dose. Finally, Golf sense had no

effect on G protein levels or rotations except at the high dose, where it produced a partial reduction. A rank order potency of non-specific effects can thus be attributed to Golf oligonucleotides as follows: missense > antisense > sense. However, the ability of antisense oligonucleotides to reduce apomorphine-induced rotations at the low dose with only a small and non-significant effect on Golf levels is encouraging. This particular finding is comparable to that previously reported for D_2 receptors in 6-OHDA lesioned animals following antisense oligonucleotide infusion (Zhou et al., 1994, Qin et al., 1995). In both cases large reductions in dopamine mediated behaviours were observed despite small decreases in receptor or G protein levels. These findings are intriguing given the relatively small increases reported for both D_2 receptors (see Table IV) and stimulatory G proteins following 6-OHDA lesioning (see Section II.2).

The non-specific effects observed here may be a result of several possible mechanisms, including toxicity and insufficient specificity as previously described. We did not specifically look for histological signs of cell damage in these experiments, as we expected that any abnormal functional changes in neuronal activity would be manifested at concentrations lower than those required to induce cell death. In this regard, general toxicity is an unlikely explanation for the results observed in these studies for several reasons. For example, the effects of various oligonucleotides at different doses were extremely consistent. In particular, Golf sense had no effect on rotations or G protein levels except at the highest dose, while Golf missense consistently reduced both features at lower doses. All oligonucleotides were obtained from the same supplier (MOBIX, McMaster Biotechnology Institute), and purified in the same fashion. There was no

evidence of degradation or contamination on polyacrylamide gels (Figure 33). Similarly, there was no obvious difference in the appearance or solubility of any of the oligonucleotides. Animals treated at the highest dose (combined group) showed no adverse behavioural effects to prolonged oligonucleotide treatment. Moreover, recovery studies indicated that rotational behaviour could recover in these animals (Figure 43). Specifically, over a period of several weeks, rotational behaviour recovered in Golf antisense treated animals, up to approximately 60% of pre-infusion control values by 4 weeks following cessation of infusion (Figure 43). This recovery of function would be unlikely if the reduction in apomorphine-induced rotations were due to neuronal cell loss.

The nature of the non-specific effects observed in these studies remains to be determined. As previously mentioned, use of phosphorothioate oligonucleotides has been associated with increased toxicity. In support of this contention, phosphorothioate oligonucleotides have been reported to dose-dependently elevate body temperature following CNS infusion (Schobitz et al., 1997, Pezeshki et al., 1996). There is also preliminary evidence for a significant inflammatory response following central phosphorothioate oligonucleotide infusion, including the induction of interleukin-6, (Yaida and Nowak, 1995, Pezeshki et al., 1996). Antisense, sense, and missense vasopressin and oxytocin phosphorothioate oligonucleotides have also been shown to suppress food and fluid intake and inhibit locomotor activity when infused into the rat hypothalamus (Schobitz et al., 1997). Interestingly, mock-synthesized products, which would presumably contain the putative by-products of phosphorothioate modification, were not biologically active when infused into this system (Schobitz et al., 1997).

Moreover, native salmon sperm DNA, which was not chemically synthesized, also produced fever and sickness behaviour in infused animals. These results call into question the role of phosphorothioate modification in the development of immunopathological signs following oligonucleotide infusion. Unmodified phosphodiester oligonucleotides may also produce non-specific effects through toxic mechanisms independent of phosphorothioate modification.

Apart from oligonucleotide-mediated toxicity, the generalized reduction in G protein levels observed in several of the infusion groups in these studies may be the indirect result of downstream regulatory or compensatory effects. It is possible that the persistent knockdown of proteins integral for cellular function, such as G proteins, may have long-lasting effects on cell function and membrane physiology. This possibility is supported by the relatively slow recovery from antisense infusion in the combined group. In comparison, studies using D_2 receptor antisense oligonucleotides in 6-OHDA lesioned mice have demonstrated recovery of behavioural supersensitivity within 2-4 days following cessation of antisense treatment (Weiss et al., 1993, Zhou et al., 1994). This quick recovery of function seems somewhat surprising given that the half-life of the D_2 receptor has been reported to be approximately 3-4 days in vivo (Qin et al., 1995). Although the turnover of stimulatory G proteins in vivo is unknown, studies in astroglia cell cultures have reported that the half-life of $Gs\alpha$ is biphasic, with an early phase at 5-6 hr and a slower second phase at 20-25 hr (Dib et al., 1994). In vitro studies of G protein antisense have observed an effective antisense block of various G protein-mediated behaviours between 1 and 2 days after injection (for a review, see Kleuss et al., 1994).

This time course did not appear to differ significantly for α, β, or γ subunits. Thus, it appears that proteins with a relatively slow turnover, such as cell surface receptors and G proteins, require at least 2 days of treatment before a biological effect can be observed.

Finally, the non-specific reduction in G protein levels and apomorphine-induced rotational behaviour in missense treated animals may reflect the specific sequences employed in this study. As previously described, both missense sequences have shown increasing homology to recently identified mRNAs and gene sequences in the GenBank database. Although no highly specific match has yet been observed that would account for the effects observed here, the increasing number and specificity of homology matches is a source of concern. These raise the possibility that large portions of the sequences used in this study may be widely distributed throughout the genome, although as yet undetected. In light of the relatively low number of identified genes and gene products currently available, the potential specific interactions of these control oligonucleotide with as-yet undetermined endogenous nucleotide sequences cannot be ruled out.

In addition to the potential confound variables discussed above, there are several potential modifications of the experimental protocol that may yield more consistent data. Due to the concerns over phosphorothioate toxicity and specificity, partial phosphorothioate analogues are now beginning to be widely used. These "ends-only" analogues feature phosphorothioate-modified nucleotides at either end of the oligonucleotide sequence, while maintaining normal phosphodiester linkages along the majority of the sequence. This modification should be sufficient to protect against endogenous endonucleases, which are believed to account for the majority of

oligonucleotide degradation in vivo. Thus, use of partial phosphorothioate analogues may reveal more specific effects on apomorphine-induced rotations and G protein levels. As previously discussed, however, the significance of phosphorothioate modification in terms of specificity and toxicity is unclear.

In addition to the form of oligonucleotide used, the mode or frequency of administration are other features that could be altered. A revised dosing schedule, with a greater range of concentrations at different intervals may prove helpful in delineating the effects of oligonucleotide treatment. In one study of phosphorothioate oligonucleotides, administration every fifth day instead of daily infusions (4 doses in total) resulted in minimal toxicity (Chiasson et al., 1994). However, every day or every third day infusion produced significant signs of toxicity in that study. The feasibility of such a delayed infusion time course in 6-OHDA lesioned rats is unclear, as a detailed time course of G protein change has not been performed past six weeks post-lesion.

Alternatively, oligonucleotides could be infused using mini-osmotic infusion pumps into the lateral ventricles. This approach has been used extensively in CNS oligonucleotide studies. Despite the lack of anatomical selectivity, this method of administration reduces the risk of infection compared to multiple once-daily infusions. Moreover, unmodified phosphodiester oligonucleotides could also be examined by this method using a higher infused concentration.

Although most studies, including those performed here, have targeted the start codon and surrounding bases for oligonucleotide design, it is possible that other sequences may be better suited for suppression of stimulatory G proteins. Several groups

have reported that mRNA expression can be more efficiently blocked by oligonucleotides which target regions farther downstream in the coding region (Brysch and Schlingensiepen, 1994, Bennet et al., 1994b, Jachimczak et al., 1993). In one study of oligonucleotides targeted to 10 different sites of the intracellular adhesion molecule-1 mRNA, only certain oligonucleotides were effective in reducing expression (Chiang et al., 1991). The mechanism by which these oligonucleotides inhibit mRNA expression is not entirely clear, but presumably involves steric hindrance of the ribosome during translation. RNase H activity would still be maintained in these oligonucleotide preparations, although the importance of RNase H in eukaryotic cells has been questioned, as previously mentioned (Brysch and Schlingensiepen, 1994, Wahlestedt et al., 1993, Chiang et al., 1991).

An additional oligonucleotide control group could consist of randomly mismatched bases. Substitution of one or more bases in the antisense sequence to create mismatch oligonucleotides has been proposed as an alternate control to missense oligonucleotides (Chiasson et al., 1994). However, the number of mismatches required to eliminate antisense action has not been clearly established. For example, 2- or 3-base mismatches of c-fos antisense have been demonstrated to retain significant biological action (Chiasson et al., 1994). In any case, additional control groups would not address the non-specific effect of missense controls raised in the studies presented here.

In summary, Golf oligonucleotide infusion produced largely consistent results on apomorphine-induced rotations and G protein levels in these studies, although several control oligonucleotides produced non-specific effects. At the low dose, Golf antisense

infusion appeared to specifically reduce rotational behaviour and Golfα levels. Control Golf sense infusion had no effect on either of these measures, as expected. However, control Golf missense also reduced apomorphine-induced rotations and non-specifically reduced G protein levels at this dose. At a higher dose, greater non-specific effects for both Golf antisense and missense were also observed. These studies raise the possibility that a narrow dose-response window may exist where Golf antisense oligonucleotides are effective in reducing functional and behavioural measures of stimulatory G protein activity. However, the non-specific effect of two separate Golf missense sequences calls into doubt the general applicability of this technique in this system.

CHAPTER V

CONCLUSIONS

There is growing evidence that factors other than cell-surface receptors are involved in regulating the sensitivity of cells to external signals. A classical example is receptor desensitization as a result of prolonged receptor activation (Hadcock and Malbon, 1993, Freedman and Lefkowitz, 1996). In this case, both short- and long-term regulation of receptor sensitivity is known to be under the control of a variety of signal transduction elements, including G proteins. G proteins have also been implicated in the increased sensitivity of receptor function in a number of experimental models (see Table II). Together, these studies have provided the impetus for the examination of G protein function in denervation-induced dopamine receptor supersensitivity presented in this thesis.

The decision to specifically examine dopamine receptor supersensitivity in Parkinson's disease was taken for several reasons. This debilitating motor syndrome is the second most common neurodegenerative disorder after Alzheimer's disease. Unlike other neurodegenerative or neuropsychiatric conditions, however, the proximal cause of Parkinson's disease is well established: degeneration of the dopamine-containing neurons of the substantia nigra. This in turn results in the depletion of striatal dopamine and the

disruption of basal ganglia function. Dopamine receptor supersensitivity, resulting from loss of dopaminergic tone and fluctuations in dopamine replacement therapy, is believed to account for many of the long-term adverse effects of L-DOPA treatment (Nutt, 1990, Jeste and Caligiuri, 1993, Chase, 1998a). Clearly, a better understanding of the underlying cellular responses to dopamine denervation can only serve to improve our ability to manage this disorder.

Studies of G protein levels or functional activity in humans are difficult for many reasons. There are currently no direct in vivo measures of G protein activity in human subjects. Post-mortem human brain tissue studies are fraught with difficulties, as outlined in Section II.1. Analysis of G protein levels in a small sample of post-mortem parkinsonian brain tissues in this thesis was inconclusive. Although potential alterations in the relative ratio of stimulatory G proteins was observed in some cases, the relevance or replicability of this finding is unknown at present. As such, animal tissues and models were used exclusively in subsequent studies.

A time course of alterations in G protein levels was performed for the unilateral 6-OHDA rat lesion model of Parkinson's disease (Section II.2). The results of these studies indicated that there was a significant increase in the levels of both stimulatory and inhibitory G proteins acutely following 6-OHDA lesioning (Marcotte et al., 1994, Marcotte and Mishra, 1997). However, the magnitude of this effect was much greater for stimulatory G proteins than inhibitory ones. Moreover, inhibitory G proteins quickly returned to baseline levels while stimulatory G proteins remained elevated at the end of the time course (4 weeks post-lesion). Other studies have shown similar findings, and

have extended the time course up to 6 weeks post-lesion (Hervé et al., 1993). Thus, both stimulatory and inhibitory G proteins may be involved in the initial adaptive response of striatal neurons to denervation, but only stimulatory G proteins are involved in the long-term maintenance of that response. These results are consistent with the spare receptor concept for this receptor system, and support the hypothesis that G protein upregulation may play a pivotal role in mediating dopamine receptor supersensitivity.

Attempts to replicate these findings in the MPTP mouse model of Parkinson's disease were somewhat inconclusive. G protein levels were differentially regulated in MPTP-treated mice, with decreased stimulatory G proteins observed acutely following MPTP treatment, and increased stimulatory G proteins following long-term recovery (Marcotte et al., 1998a). The acute decrease in stimulatory G proteins in MPTP-treated animals is in sharp contrast to the early and persistent upregulation observed in 6-OHDA lesioned rats, suggesting that the development of dopamine receptor supersensitivity may be different in these two models. Similarly, the significance of the increased stimulatory G protein levels following long-term recovery is unclear. Given our lack of understanding of the mechanisms that mediate functional recovery in these animals, it is possible that G protein upregulation may play a contributory role, perhaps through enhanced dopaminergic neurotransmission at D_1 receptors. These results provide a further impetus for the examination of postsynaptic compensatory mechanisms following dopaminergic denervation.

In addition to protein levels, it is also important to characterize functional changes in G protein activity in any discussion of the role of these proteins. Toward this end, a

variety of functional G protein assays were attempted, including the GTPase assay and [α-^{32}P]-GTP binding. Despite extensive modification of the assay, stimulation of GTPase activity by dopaminergic agents proved unreliable in the striatum, particularly for the dopamine D_1 receptor pathway. These findings are generally consistent with the available literature (Onali et al., 1983, Onali and Olianas, 1987, Odagaki and Fuxe, 1995, Treisman et al., 1985). Part of this difficulty with this assay may stem from the fact that it is an indirect measure of G protein activation. GTPase activity is actually a measure of the inactivation of the G protein cycle, and is thus susceptible to factors that affect GTP hydrolysis. Accordingly, this technique may be best suited to in vitro situations where greater control over potential confounding variables is possible.

Development of a suitable technique for measuring specific receptor-G protein coupling in native membrane preparations has proven elusive. The [α-^{32}P]-GTP binding assay was developed as a potential measure of both basal and receptor-mediated GTP binding to specific G proteins (Friedman et al., 1993). Although increased basal [α-^{32}P]-GTP binding has been reported in 6-OHDA lesioned rats (Tenn and Niles, 1997), dopamine D_1 receptor-induced increases in stimulatory G protein binding have not been observed. Similar results were obtained in this research project, where only D_2 receptor-mediated changes in inhibitory G protein binding could be detected. Thus, the applicability of this technique in measuring stimulatory G protein activity in the striatum appears to be as limited as the GTPase assay.

Given the failure of these various functional assays to detect alterations in stimulatory G protein activity in the striatum, we proceeded to investigate the effects of

antisense agents on G protein expression and function. In theory, antisense oligonucleotides should be able to specifically reduce stimulatory G protein gene expression in a reversible manner (Helene, 1991, Crooke et al., 1995). The effects of this treatment on G protein expression and dopamine agonist-induced rotational behaviour could then be examined to verify the specificity and efficacy of this technique. For this research project, Golf antisense oligonucleotides were administered to selectively knockdown Golfα levels in the striatum of 6-OHDA lesioned rats (Marcotte and Mishra, 1998).

Generally, Golf oligonucleotide infusion produced consistent results on both apomorphine-induced rotations and G protein levels in these studies. At a relatively low dose, Golf antisense infusion appeared to specifically reduce rotational behaviour and Golfα levels as expected. However, control Golf missense (random-order sequence) oligonucleotides also reduced apomorphine-induced rotations and non-specifically inhibited G protein levels at this dose. Interestingly, the control sense strand oligonucleotide was ineffective at this dose. At higher doses, greater non-specific effects for all Golf oligonucleotides were observed.

Clearly, a non-specific mechanism of action was present for the control missense oligonucleotides examined in these studies. However, this non-specific response to oligonucleotides was at least partially dose- and sequence-dependent. Moreover, infused animals showed behavioural recovery with time, demonstrating that even non-specific effects were reversible to some extent. These findings argue against general toxicity or cell death as an explanation for the mechanism of action of Golf oligonucleotides in these

studies. Moreover, the possibility of specific binding of control missense oligonucleotides to as yet undetermined gene sequences cannot be ruled out. Further modification of the experimental protocol may help to resolve some of these issues. In any case, these results support the possibility that a narrow dose-response window where Golf antisense oligonucleotides are effective in specifically reducing functional and behavioural measures of stimulatory G protein activity may exist.

Ultimately, the role of stimulatory G proteins, and Golf specifically, in mediating dopamine receptor supersensitivity remains unproven. Stimulatory G protein levels are clearly modulated in the striatum following dopaminergic denervation in animal models. Adenylyl cyclase activity, which is under positive stimulatory G protein control, is also clearly altered in these animals (Mishra et al., 1974, Pifl et al., 1992). However, direct functional measures of receptor-G protein coupling in biological membranes have proven generally unreliable, especially for the stimulatory G protein pathway. Targeted disruption of G protein gene expression, using for example antisense agents, holds great promise in unraveling the interactions of these signaling components. However, at present the mechanism of action of antisense oligonucleotides in this model is still unclear, and requires further examination.

In addition to the changes in G protein levels discussed here, other components of the signal transduction cascade may also show long-term adaptations to denervation. In particular, the various types of adenylyl cyclases have been suggested to represent critical sites of signal integration (Anholt, 1994). At least eight isoforms of adenylyl cyclase have been described that are differentially regulated by

G proteins. In addition, adenylyl cyclase isoforms have been shown to respond to a wide range of factors, including various second messengers (Lustig et al., 1993, Mons and Cooper, 1995). One particular adenylyl cyclase isoform, type V, is enriched in the striatum and the olfactory tubercle. This pattern of expression is reminiscent of the expression of Golfα, which is localized in the same brain areas (Hervé et al., 1993, Jones and Reed, 1989). Also of interest, γ7, a G protein γ subunit isoform, shares the same specific pattern of expression (Watson et al., 1994). The significance of these findings is unclear, but the restricted co-localization of three specific signal transduction components warrants further investigation. The use of antisense oligonucleotides to specifically inhibit the expression of adenylyl cyclase type V and the γ7 G protein subunit in the striatum is one possible method of elucidating their role, if any, in dopaminergic signal transduction.

Further elucidation of the role of G proteins and other signal transduction elements in dopamine receptor supersensitivity may have significant implications for the study of receptor signaling in general. Ultimately, this knowledge may lead to a greater understanding of the mechanisms involved in mediating compensatory changes to altered neurotransmitter states. The previous narrow focus on alterations in receptor levels and function is clearly no longer sufficient. The development of novel techniques, such as in vivo antisense administration or transgenic gene disruption, offer the potential of separating and elucidating the specific contributions of individual proteins on a scale never previously available. However, it is important to continue to critically assess these techniques using the same rigorous standards applied to other experimental tools.

CHAPTER VI

APPENDICES

APPENDIX A. SEQUENCES OF OLIGONUCLEOTIDES

Golfα antisense (AS), sense (S) and missense (MS) oligonucleotides were synthesized as 20-mer full phosphorothioate oligonucleotides corresponding to the initiation codon of the rat Golfα mRNA as described in Section IV.1.1. Each of the following sequences were tested for lack of homology with known protein sequences by BLAST NIH GenBank search.

Golf S 5' - GCC AGC AGG CAT GGG GTG TT - 3'

Golf AS 5' - AAC ACC CCA TGC CTG CTG GC - 3'

Golf MS-1 5' - CAG CTA GCA CCC CTG TCG CA - 3'

Golf MS-2 5' - CAC TCG CAT CGA GCC ATG CC - 3'

APPENDIX B. DOSING SCHEDULES OF OLIGONUCLEOTIDES

Dosing schedules for oligonucleotide studies described in this thesis are presented below. All oligonucleotides were dissolved in 0.9% sterile saline and infused directly into the striatum in a volume of 2 µl over a 4 min period, as described in Section IV.1.1. Animals were tested for rotational behaviour 20 hours after the last infusion, and sacrificed 4 hours later (24 hours after last infusion).

Very Low Dose	2.5 nmoles / day for 2 days
Low Dose	2.5 nmoles / day for 4 days
High Dose	4.0 nmoles / day for 4 days
Combined Dose	2.5 nmoles / day for 4 days + 4.0 nmoles / day for 3 days

APPENDIX C. MANUSCRIPTS ARISING FROM THIS RESEARCH PROJECT

Marcotte, E.R. and Mishra, R.K., 1998, Stimulatory G proteins and dopamine receptor supersensitivity: the use of in vivo antisense oligonucleotides, In Preparation.

Marcotte, E.R., Chugh, A., Barlas, C. and Mishra, R.K., 1998, Differential regulation of G protein levels following administration of the dopaminergic neurotoxin MPTP in C57 BL/6 mice, In Preparation.

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