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IDENTIFICATION OF THE EFFECTS OF PRO-LEU-GLY-NH<sub>2</sub> (PLG) ON D<sub>2</sub>-  
DOPAMINE RECEPTOR FUNCTION AND GENE EXPRESSION IN THE RAT  
BRAIN

By  
WILLARD JAMES COSTAIN  
B.Sc., M.Sc.

A Thesis  
Submitted to the School of Graduate Studies  
in Partial Fulfilment of the Requirements  
for the Degree  
Doctor of Philosophy

McMaster University

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## PLG REGULATED GENE EXPRESSION AND D<sub>2</sub> RECEPTOR FUNCTION

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Receptor Function And Gene Expression In The Rat Brain**

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## ABSTRACT

Central dopaminergic systems have been implicated in CNS disorders such as schizophrenia and Parkinson's disease. The characteristics of dopamine (DA) receptors has been well studied using a variety of pharmacological and biochemical techniques. DA receptor function is known to be modulated by the endogenous tripeptide pro-leu-gly-NH<sub>2</sub> (PLG). A combination of novel pharmacological (guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate; [<sup>35</sup>S]GTPγS binding) and molecular biological (differential display mRNA by RT-PCR; ddPCR) techniques were used to examine the function of endogenously expressed D2 DA-receptors in the striatum. Studies were undertaken to increase our understanding of the mechanism of action of PLG and its role in D2 receptor regulation and signal transduction. The [<sup>35</sup>S]-GTPγS binding assay was used to study the coupling of D2 receptors to G proteins within the striatum. Assay conditions were optimised for studying D2 receptors in bovine striatum. D2 receptor agonists significantly increased [<sup>35</sup>S]-GTPγS binding with the following rank order of potency: NPA >> bromocryptine > apomorphine > dopamine. No significant differences in agonist efficacy were detected. Pertussis toxin treatment was found to diminish NPA stimulated increases in [<sup>35</sup>S]-GTPγS binding; indicating that this effect is due to activation of the Gi G-protein. Inhibition of NPA stimulated [<sup>35</sup>S]-GTPγS binding by D2 receptor antagonists provided the following rank order of potency: spiperone > haloperidol > butaclamol > sulpiride > chlorpromazine > clozapine. Observed agonist pEC<sub>50</sub> and antagonist pIC<sub>50</sub> values correlate well with values previously reported for transfected D<sub>2short</sub> receptors (Gardner *et al.* 1997; Gardner *et al.* 1996) and D2 receptors in rat brain (Rinken *et al.* 1999). NPA mediated increases in [<sup>35</sup>S]-GTPγS binding were eliminated by pertussis toxin treatment, indicating that the effect is mediated by Gi. Measurement of antagonist effects in the absence of D2 receptor stimulation revealed that basal [<sup>35</sup>S]-GTPγS binding was significantly decreased by haloperidol, butaclamol and chlorpromazine but not clozapine, sulpiride and spiperone. Thus haloperidol, butaclamol and chlorpromazine acted as

negative antagonists; while clozapine, sulpiride and spiperone acted as silent antagonists.

The role of PLG in D2 receptor stimulation of Gi G-proteins was examined using the [<sup>35</sup>S]-GTPγS binding technique. The possible effect of PLG on NPA-stimulated [<sup>35</sup>S]-GTPγS binding was assessed at both maximal (1 μM NPA) and submaximal (0.1 and 0.03 μM NPA) levels of D2 receptor stimulation. It was found that PLG did not significantly alter [<sup>35</sup>S]-GTPγS binding in bovine striatum either in the absence or presence of D2 receptor stimulation; indicating that PLG does not alter the rate of GDP:GTP exchange in the Giα G-protein subunit.

Haloperidol and clozapine have distinct pharmacological profiles and have differential effects on many systems in the brain. This likely accounts for the tendency toward the development of extra pyramidal side effects (EPS) with the use of haloperidol but not clozapine. [<sup>35</sup>S]-GTPγS binding was measured following haloperidol and clozapine treatment to examine functional coupling of D2 dopamine receptors to G-proteins in the rat striatum. Both neuroleptics increased basal [<sup>35</sup>S]-GTPγS binding by ≈ 12% and significantly increased overall binding ( $p < 0.001$ ). Haloperidol, but not clozapine, significantly increased NPA EC<sub>50</sub> values from  $8.55 \pm 0.21$  (in control tissue) to  $9.49 \pm 0.18$  ( $p < 0.04$ ). Thus, “typical” and “atypical” neuroleptics utilise differential effects on D2 receptor coupling to G-proteins in the striatum to produce a similar net effect on signal transduction.

In an attempt to elucidate the mechanism of action of PLG, ddPCR was utilised to discover genes that are regulated by protracted treatment with PLG (20 mg/kg, i.p. for 28 days). Approximately 2400 genes were screened using ddPCR (RNAImage<sup>®</sup> kit1, GenHunter Corp.) and 3 bands were identified that were down-regulated in the PLG treated samples. The down regulated bands were excised from the 6% polyacrylamide sequencing gels, reamplified by PCR and cloned into the pCR-Trap<sup>®</sup> cloning system (GenHunter Corp.). Sequencing of the ddPCR cDNAs (dideoxy chain-termination method, Mobix facility, McMaster University) revealed a total of 6 unique cDNA species



that were termed *PRG1 - PRG6* (PLG Regulated Gene). The cDNA sequence information was searched for homologies with GenBank and it was found that *PRG1* and *PRG3* displayed a high degree of homology to Homo sapiens SAP155 mRNA (AF054284) and Mus musculus protein L mRNA (AB009392) respectively. The other ddPCR cDNAs were not sufficiently homologous to genes in GenBank for positive identification. Elongation of the 5'- and 3'- ends of the cDNAs for *PRG1*, *PRG3* and *PRG4* was performed using RACE-PCR. The 5'- and 3'-RACE-PCR products were cloned into the AdvanTAge cloning system (Clontech), and sequenced at Mobix. Analysis of the sequence information confirmed the homology of *PRG1* and *PRG3* with previously identified genes in GenBank. Analysis of the 5'-RACE-PCR sequence for *PRG4* revealed a significant degree of homology with the human G-protein ( $\gamma_{11}$ ) mRNA (GNG11). Open reading frame analysis of the extended cDNAs for *PRG1*, *PRG3* and *PRG4* and amino acid sequences were obtained. GenBank analysis of the amino acid sequences further confirmed the identity of the extended ddPCR fragment sequences. The RACE-PCR experiments produced partial cDNAs for *PRG1* and *PRG3*, while a complete cDNA sequence was obtained for *PRG4*. RNA expression levels in PLG (20 mg/kg, i.p., 28 days) and Saline (1 ml/kg i.p., 28 days) treated rat striatum were assessed by northern hybridisation for *PRG1 - PRG6*. Blots were stripped and reprobed with  $\beta$ -Actin for accurate determination of expression levels. No signal was detected in striatal poly A<sup>+</sup> when using *PRG2*, *PRG5* or *PRG6* as probes. The *PRG1* probe detected a mRNA species of 4.7 kb that was increased by 54% in the PLG treated tissue. Northern hybridisation analysis of *PRG3* revealed a mRNA species of 2.3 kb that was decreased by 65% in the PLG treated tissue. Analysis of protein L expression by western blot revealed that PLG significantly decreased protein L expression in the striatum and pre-frontal cortex (but not the nucleus accumbens) by 71% and 61% respectively. Northern hybridisation analysis of *PRG4* revealed a mRNA species of 1.1 kb that was not altered as a result of PLG treatment. Analysis of G <sub>$\gamma_{11}$</sub>  protein expression by western blot confirmed that the protein

levels were not altered in either the striatum or pre-frontal cortex. Interestingly,  $G_{\gamma 11}$  expression in the nucleus accumbens was significantly decreased by 39%.

In the present study, I have attempted to further the understanding of D2 receptor coupling to G-proteins in the striatum with respect to agonist efficacy and negative versus silent antagonism. I have also determined that PLG does not modulate D2 receptor signal transduction by altering the rate of GDP:GTP exchange in  $G_i$ . Furthermore, I have used the [ $^{35}$ S]-GTP $\gamma$ S binding technique to compare the effects of typical and atypical neuroleptic treatment on D2 receptor coupling to  $G_i$ .

In a separate set of experiments I have used ddPCR to identify a number of genes that are regulated by PLG treatment, and presumably involved in mediating its physiological effects. These include the DNA/RNA binding proteins SAP155 and protein L as well as the G-protein  $\gamma 11$  subunit. Furthermore, RACE-PCR was used to obtain additional sequence information about these cDNA clones. These newly identified genes may provide insight into the mechanism of action of PLG and certainly warrant further investigation.

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## LIST OF ABBREVIATIONS

[<sup>32</sup>P]-dCTP, [ $\alpha$ <sup>32</sup>P]-deoxycytidine 5'-triphosphate;  
[<sup>32</sup>P]-NAD, [*adenylate*-<sup>32</sup>P]-nicotinamide adenine dinucleotide;  
[<sup>35</sup>S]-GTP $\gamma$ S, [<sup>35</sup>S]-guanosine 5'-( $\gamma$ -thio)triphosphate;  
[ $\alpha$ -<sup>35</sup>S]-dATP, [<sup>35</sup>S]-deoxyadenosine 5'( $\alpha$ -thio)triphosphate;  
6-OHDA, 6-hydroxydopamine;  
AP, alkaline phosphatase;  
BSA, bovine serum albumin;  
cDNA, complementary DNA;  
CPP, (3-(2carboxypiperazin-4-yl)-propyl-1-phosphonic acid);  
DA, dopamine;  
ddPCR, differential display PCR;  
DNA, deoxyribonucleic acid;  
EC<sub>50</sub>, drug concentration that produces a 50% response;  
ECL, enhanced chemiluminescence;  
EDTA, ethylenediaminetetraacetic acid;  
Gi/Go, inhibitory guanine nucleotide binding proteins (G-proteins);  
Golf, stimulatory G-protein / olfactory;  
Gs, stimulatory guanine nucleotide binding protein (G-protein);  
HRP, horseradish peroxidase;  
IC<sub>50</sub>, drug concentration that produces a 50% inhibition;  
IPTG, isopropyl- $\beta$ -D-thiogalactoside;  
LB, Luria-Bertani  
L-DOPA, L-3,4-dihydroxyphenylalanine;  
MIF-1, melanocyte stimulating hormone release inhibiting factor;  
MOPS, 3-[N-morpholino]propanesulfonic acid;  
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;

mRNA, messenger RNA;  
NMDA, N-methyl-D-aspartate;  
PAGE, polyacrylamide gel electrophoresis;  
PCR, polymerase chain reaction;  
 $pEC_{50}$ ,  $-\log(M)$  of  $EC_{50}$ ;  
 $pIC_{50}$ ,  $-\log(M)$  of  $IC_{50}$ ;  
 $pK_i$ ,  $-\log(M)$  of  $pIC_{50}$  adjusted using the Cheng-Prusoff equation;  
PLG, L-prolyl-L-leucyl-glycinamide (Pro-Leu-Gly-NH<sub>2</sub>);  
PT, pertussis toxin  
RACE, rapid amplification of cDNA ends;  
RNA, ribonucleic acid;  
SDS, sodium dodecyl sulfate;  
TBS-T, tris buffered saline with Tween;  
Tris, tris(hydroxymethyl)aminomethane;  
X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside;

## **1.0 Introduction**

Signal transduction by G-protein coupled receptors involves a complex cascade of molecular interaction (Hamm, 1999). The tripeptide PLG (L-prolyl-L-leucyl-glycinamide) is thought to modulate dopamine (DA) D2-receptor mediated signal transduction at the level of the G-protein (Costain *et al.* 1997). The mechanism utilised by PLG is poorly understood. In the following thesis, I attempt to explore aspects of D2 receptor coupling to G-proteins in the striatum using a measure of G-protein activity. Furthermore, I will examine what role, if any, PLG plays in mediating D2 receptor activation of G-proteins. The same methodology will also be used to compare the effect of “typical” and “atypical” neuroleptic drugs on D2 receptor/G-protein function in the striatum.

Although PLG’s modulatory effect on D2 receptors has been studied extensively, there is little information on the effects of PLG in the brain per se. Therefore, I will describe experiments that are directed at elucidating the molecular mechanism of PLG’s action. Specifically, I attempted to discover which genes/proteins are involved in mediating the effects of PLG.

### **1.1 Characteristics and Biological Properties of L-Prolyl-L-Leucyl-Glycinamide (PLG)**

The endogenous neuropeptide melanocyte stimulating hormone release inhibiting factor (MIF-1; L-prolyl-L-leucyl-glycinamide) was originally isolated from bovine hypothalamus by Nair *et al* (1971). PLG belongs to the Tyr-PLG (Tyr-Pro-Leu-Gly-NH<sub>2</sub>) family of related neuropeptides that also includes Tyr-W-PLG (Tyr-Pro-Trp-Gly-NH<sub>2</sub>) and Try-K-PLG (Tyr-Pro-Lys-Gly-NH<sub>2</sub>). Tyr-PLG can bind to the mu opiate receptor as well as its own specific nonopiate receptor in the brain (Kastin *et al.* 1995). Tyr-PLG demonstrates a high degree of selectivity toward the mu opiate receptor, compared to the delta and kappa receptors, but possesses a 50 fold greater affinity for its own receptor (Erchegyi *et al.* 1993).

The synthesis of PLG within the brain has been studied and is thought to be formed by the cleavage of the C-terminus of oxytocin (Misha *et al.* 1983) as well as from the enzymatic cleavage of Tyr-PLG (Kastin *et al.* 1995). PLG mediated MSH release inhibition has been observed to regulate the release of leutinizing hormone as well as progesterone (Caballero & Celis, 1993). PLG has also demonstrated the ability to decrease MSH stimulated increases in intraocular pressure and miosis (McCullen *et al.* 1988)

PLG has been observed to cross the blood brain barrier at a very high rate through a partially saturable transport system (Banks and Kastin, 1994). The rate of transport of PLG from the systemic circulation to the brain is 50 - 100 times greater than that for morphine and  $\approx 10$  times greater than that for Tyr-PLG (Banks and Kastin, 1994). Unlike Tyr-PLG, once PLG enters the brain, it remains sequestered there in a manner similar to morphine. This unidirectional transport mechanism for PLG is distinct from the peptide transport system 1 (PTS-1) utilised by Tyr-PLG (Reed *et al.* 1994; Banks and Kastin, 1994). The rate of metabolism for PLG is much slower than that for Tyr-PLG (Kastin *et al.* 1994). Tyr-PLG is metabolised very quickly in rats and humans with a  $t_{1/2}$  of  $\approx 5$  min for both species. In comparison, the  $t_{1/2}$  for PLG in the rat is  $\approx 30$  minutes; while in man, the  $t_{1/2}$  is  $>5$  days (Kastin *et al.* 1994). Despite the structural similarities between these peptides there is ample evidence from pharmacological studies, (Kastin *et al.* 1995; Kostrzewa and Kastin, 1993; Harrison *et al.* 1998; Harrison *et al.* 1998; Zhang *et al.* 1992) radioligand binding studies (Zadina *et al.* 1982), and differential metabolism and blood-brain barrier transport studies (Banks and Kastin, 1994; Kastin *et al.* 1995) which clearly indicate that PLG acts at sites distinct from those of Tyr-PLG.

## **1.2 Dopamine Receptor / G-Protein Mediated Signal Transduction**

Dopamine (DA) receptors are broadly categorised into two classes. D1 and D2. The original classification of DA receptors was based on their coupling to adenylate

cyclase (Spano *et al.* 1978). This classification system has been expanded and elucidated with the advent of molecular cloning. Within the D1 class are the D<sub>1</sub> and D<sub>5</sub> DA receptor subtypes that are linked to adenylate cyclase stimulation (Lovenberg *et al.* 1991; Otmakhova and Lisman, 1998). The D2 class of receptors contain the D<sub>2S</sub>, D<sub>2L</sub>, D<sub>3</sub> and D<sub>4.1</sub> . . . D<sub>4.10</sub> subtypes that are coupled to adenylate cyclase inhibition (Izenwasser and Côté, 1995; Pilon *et al.* 1994; Van Tol, 1998). D1 and D2 receptor modulation of adenylate cyclase activity is mediated through the GTP binding proteins (G-proteins) Gs/Golf and Gi/Go respectively (Lovenberg *et al.* 1991; Lledo *et al.* 1992; Izenwasser and Côté, 1995; Di Marzo *et al.* 1993). D2 DA-receptors are capable of modulating other second messenger systems, including inositol phospholipid metabolism (Izquierdo-Claros *et al.* 1997; Vaillancourt *et al.* 1995; Tang *et al.* 1994; Canonico *et al.* 1983; Enjalbert *et al.* 1986; Enjalbert *et al.* 1990; Simmonds and Strange, 1985), arachidonic acid release (Vial and Piomelli, 1995; Schinelli *et al.* 1994; Di Marzo *et al.* 1993), potassium currents (Wilke *et al.* 1998; Zhu *et al.* 1997; Liu *et al.* 1996; Lin *et al.* 1996; Werner *et al.* 1996; Pedarzani and Storm, 1995) and calcium currents (Lledo *et al.* 1992; Aguayo and Grossie, 1994; Lledo *et al.* 1990a; Lledo *et al.* 1990b; Zhu *et al.* 1997; Formenti *et al.* 1998; Gomora *et al.* 1996). This broad spectrum of responses to D2 DA-receptor activation are all mediated by the Gi/Go family of G-proteins (Izenwasser and Côté, 1995; Di Marzo *et al.* 1993; Lledo *et al.* 1992).

DA-receptors belong to a larger family of G-protein coupled receptors. These cell-surface receptors interact with heterotrimeric G-proteins ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits) within the cell membrane as part of a cascade of events leading to physiological responses to neurotransmitters. Agonist occupancy of these receptors initiates the activation of G-proteins which undergo a regulatory cascade involving the exchange of a GDP molecule, bound to the  $\alpha$  subunit, for a GTP molecule (activated form) followed by the hydrolysis of the GTP into a GDP molecule (inactive form). It is the short-lived active form of the G-protein that interacts with effector enzymes (such as adenylate cyclase and



phospholipase C) thereby transducing the signal of the stimulated receptor (for review see (Clapham, 1996)).

### **1.3 PLG and Diseases of Dopaminergic Neurotransmission**

Subsequent to the discovery of PLG, it was found to exert an important modulatory effect on central dopaminergic systems. Dopaminergic neurotransmission in the basal ganglia plays an important role in psychomotor processes. Disruptions or alterations in the function of certain central dopaminergic pathways (A9 and A10 neurons) contribute to diseases such as Parkinson's and schizophrenia. Furthermore, changes in central dopamine receptor sensitivity have been implicated in such neurological and mental disorders as tardive dyskinesia, Gilles de la Tourette Syndrome, minimal brain dysfunction, and opiate tolerance and physical dependence (Seeman, 1987a).

Numerous studies have shown that PLG modulates dopaminergic neurotransmission within the central nervous system (Mishra *et al.* 1983). Studies have shown that PLG does not modulate dopaminergic neurotransmission by affecting either dopamine synthesis, uptake, or metabolism (Kostrzewa *et al.* 1975; Kostrzewa *et al.* 1976; Kostrzewa *et al.* 1979a; Torre *et al.* 1984). Rather, biochemical and pharmacological studies indicate that this modulation is brought about by a mechanism in which PLG renders the dopamine receptor more responsive to agonists. This hypothesis is supported by *in vitro* radioligand binding studies in which it has been shown that PLG selectively enhances the affinity of dopamine receptor agonists such as 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) (Johnson *et al.* 1986; Johnson *et al.* 1986), apomorphine (Chiu *et al.* 1981a; Mycroft *et al.* 1987; Bhargava, 1983b), and *N*-propylnorapomorphine (NPA) (Das and Bhargava, 1985a; Srivastava *et al.* 1988) to dopamine receptors. PLG does not affect the binding of dopamine receptor antagonists (Chiu *et al.* 1981a; Mycroft *et al.* 1987; Bhargava, 1983a; Srivastava *et al.*

1988). The modulatory effects of PLG are selective for the dopamine receptor, since PLG does not interact with other aminergic receptors such as the  $\alpha$ -adrenergic (Srivastava *et al.* 1988), GABA-ergic (Miller and Kastin, 1990), and serotonergic receptors (Gulati and Bhargava, 1990). The ability of PLG to modulate the function of these pathways indicates a potential therapeutic benefit in the treatment of Parkinson's disease and schizophrenia (Plotnikoff *et al.* 1971; Barbeau, 1975; Barbeau *et al.* 1976; Barbeau *et al.* 1978; Ehrensing, 1974; Ehrensing *et al.* 1977; Kahn and Davis, 1995; Kostrzewa and Kastin, 1993).

Thus, studying the manner in which PLG modulates central dopamine receptors might not only lead to a better understanding of the above disease states, but also might lead to more effective means of treating people suffering from such disorders. In fact, PLG has already been shown to be effective in aiding some patients with Parkinson's disease (van der Velde, 1983; Schneider *et al.* 1978; Mishra *et al.* 1986) and tardive dyskinesia (Ehrensing *et al.* 1977; Mishra *et al.* 1986). PLG and its analogues would also seem to hold great promise in reversing the adverse side effects associated with the chronic use of neuroleptic drugs (Mishra *et al.* 1986). Finally, recent studies have demonstrated that dopamine D2 receptor agonists are neuroprotective against ischemia-induced brain injury (O'Neill *et al.* 1998). Thus, the demonstrated ability of PLG and its peptidomimetics to enhance D2 dopaminergic neurotransmission suggests that these compounds might have neuroprotective effects and therefore, might be of benefit in neurodegenerative disorders.

### **1.3.1 Dopaminergic Systems in Neurodegenerative and Psychiatric Disorders**

Parkinson's disease is a very well characterised movement disorder resulting from the selective degeneration of the A9 group of dopaminergic neurons (nigrostriatal pathway) within the central nervous system (Jaber *et al.* 1996). These neurons arise from the substantia nigra pars compacta and innervate medium spiny neurons within the

striatum. The A9 neurons modulate the excitatory input received by medium spiny neurons from cortical neurons (Parent and Hazrati, 1995). Thus, degeneration of the A9 neurons disrupts the balance of neuronal activity in the medium spiny neuron and thereby results in loss of motor control (Marsden and Obeso, 1994). Parkinson's disease is typically treated with the dopamine precursor L-DOPA as a means of replenishing the dopaminergic tone of the A9 neurons (Jaber *et al.* 1996).

The dopamine theory of schizophrenia contends that the underlying pathology of the disease is a result of hyperactivity in the mesolimbic dopaminergic pathway (A10) in the brain (Jaber *et al.* 1996; Nestler, 1997). While this theory may be an overly simple interpretation of the disorder, there is substantial evidence implicating a significant role for aberrant dopaminergic function in schizophrenia. An important observation is that the ability of neuroleptic drugs to block D2 dopamine receptors correlates well with their ability to alleviate the psychotic symptoms of schizophrenia (Nestler, 1997). Furthermore, post-mortem analysis of brains obtained from drug naive schizophrenia patients have revealed elevated levels of striatal D2-dopamine receptors (Nestler, 1997). Clearly, there is an alteration in the dopaminergic signal transduction within the basal ganglia.

Typical neuroleptic drugs like haloperidol remain the most effective treatment available for psychotic disorders such as schizophrenia and dementia (Ebadi and Srinivasan, 1995; Seeman, 1995; Dilsaver, 1993; Schwartz and Brotman, 1992; Tanner, 1992). Unfortunately, along with the therapeutic benefits, chronic treatment with haloperidol can result in the manifestation of detrimental movement disorders such as neuroleptic-induced Parkinsonism (Hyman and Nestler, 1993; Tanner, 1992) and tardive dyskinesia (Ebadi and Srinivasan, 1995; MacGibbon *et al.* 1994; Casey, 1993; Tanner, 1992). These side effects are collectively termed extrapyramidal side effects (EPSs) [for a review see (Ebadi and Srinivasan, 1995)]. Both the therapeutic effects and EPS related to haloperidol are attributable to its dopamine D2 receptor antagonistic properties (Seeman and Niznik, 1990; Seeman, 1980; Seeman, 1987b).

A substantial amount of research has been conducted examining the effects of long-term neuroleptic treatment on various aspects of central dopaminergic signal transduction. Numerous studies have demonstrated that long-term treatment with haloperidol results in an up-regulation of D2-dopamine receptors. This up-regulation has been consistently observed in both studies of the expressed protein (western blots and radioligand binding) (Burt *et al.* 1977; Florijn *et al.* 1997; Wan *et al.* 1996; O'Dell *et al.* 1990; See *et al.* 1990; Wilmot and Szczepanik, 1989; Rupniak *et al.* 1985) and mRNA (northern blots, RT-PCR) (Fox *et al.* 1994; Srivastava *et al.* 1990; Buckland *et al.* 1992). A number of haloperidol treatment protocols have been employed in these studies, varying in duration and dosage, and increased D2 receptor protein expression in the striatum has been consistently observed from 2 weeks onward (Creese and Sibley, 1981, Srivastava and Mishra, 1994).

The effects of long term treatment with the atypical neuroleptic clozapine on D2 dopamine receptor expression within the striatum are different from that of typical neuroleptics (Ashby and Wang, 1996). A number of studies have concluded that protracted clozapine treatment does not alter the levels of D2 receptors in the striatum (Rupniak *et al.* 1984; Florijn *et al.* 1997; Wan *et al.* 1996; O'Dell *et al.* 1990; See *et al.* 1990; Wilmot and Szczepanik, 1989; Rupniak *et al.* 1985). The differential effects of clozapine and haloperidol on striatal D2 receptor expression is likely due to their differing affinity toward this receptor population. Clozapine is 50-100 times less potent than haloperidol on striatal D2 receptors (Arnauld *et al.* 1993).

### **1.3.2 *In vivo* Studies**

PLG has also demonstrated positive dopaminergic modulatory effects in a number of *in vivo* paradigms. PLG has been shown to potentiate the behavioural effects of dopaminergic drugs such as L-DOPA (L-3,4-dihydroxyphenylalanine; immediate precursor to dopamine) and apomorphine (Huidobro-Toro *et al.* 1974; Plotnikoff *et al.*

1971; Plotnikoff, 1975; Plotnikoff *et al.* 1974b; Plotnikoff *et al.* 1971; Johnson *et al.* 1978; Plotnikoff and Kastin, 1976; Barbeau, 1975; Kostrzewa *et al.* 1989; Hara and Kastin, 1986b; Kostrzewa *et al.* 1978). PLG has been shown to attenuate some, but not all, apomorphine-induced stereotypies in rats receiving neonatal 6-hydroxydopamine treatment (Gong *et al.* 1993; Kostrzewa *et al.* 1989). Similarly, in the semi-Parkinson rat (animals given a unilateral 6-hydroxydopamine lesion of the substantia nigra) PLG has demonstrated the ability to enhance apomorphine-induced turning behaviour (Ott *et al.* 1996; Smith and Morgan, 1982; Kostrzewa *et al.* 1978). A number of peptidomimetics of PLG have also proven to be effective using this model of dopaminergic activity (Ott *et al.* 1996; Evans *et al.* 1998). PLG has also been shown to attenuate oxotremorine-induced tremor (Plotnikoff and Kastin, 1974a; Plotnikoff and Kastin, 1974b; Huidobro-Toro *et al.* 1974; Bjorkman and Sievertsson, 1977), potentiate CPP (3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; NMDA receptor antagonist) induced locomotion and darting (Savelli *et al.* 1995) as well as catalepsy induced by both morphine and neuroleptics (Chiu *et al.* 1981a; Chiu and Mishra, 1979; Costain *et al.* 1999; Hara and Kastin, 1986a; Kostrzewa and Kastin, 1993). Finally, some studies have indicated that PLG is capable of protecting nigrostriatal dopaminergic neurons from the damaging effects of MPTP (*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; dopaminergic neurotoxin) (Sheng *et al.* 1987; Marcotte *et al.* 1998).

One of the most important observations made to date, regarding the biological effects of PLG, is that it is capable of counteracting the effect of protracted typical neuroleptic treatment on the expression of dopamine D2 receptors (Rajakumar *et al.* 1987b; Chiu *et al.* 1985; Chiu *et al.* 1981b; Saleh and Kostrzewa, 1989; Bhargava, 1984a; Bhargava, 1984b). The increase in dopamine D2 receptor density in the striatum following typical, but not atypical, neuroleptic treatment is thought to contribute to the extra-pyramidal side effects caused by these drugs (Rupniak *et al.* 1984; Ashby Jr. and Wang, 1996). Thus, PLG may be beneficial in preventing the development of neuroleptic

induced movement disorders.

Pharmacological studies *in vivo* also support the concept that PLG makes the dopamine receptor more responsive to agonists. For example, PLG and its active analogues potentiate the behavioural effects of L-DOPA (Plotnikoff *et al.* 1971; Huidobro-Toro *et al.* 1974) and apomorphine (Plotnikoff and Kastin, 1974b; Hara and Kastin, 1986b). PLG also enhances both amphetamine- and apomorphine-dependent rotational behaviour in either 6-hydroxydopamine lesioned rats (Kostrzewa *et al.* 1978; Smith and Morgan, 1982) or rats with electrolytic lesions of the lateral hypothalamic regions (Pereira *et al.* 1990). In another example, PLG has also been shown to antagonize neuroleptic drug induced catalepsy (Costain *et al.* 1999; Hara and Kastin, 1986a).

The ability of PLG to modulate dopamine receptors is also seen in its prevention of drug-induced supersensitivity of post-synaptic dopamine receptors (Chiu *et al.* 1981b). This supersensitivity is manifested at the molecular level by an increase in the population of dopamine receptors. It has been shown that administration of PLG either prior to or following prolonged haloperidol administration can effectively prevent and reverse, respectively, the elevation in the number of specific [<sup>3</sup>H]-spiroperidol binding sites produced by the long-term administration of neuroleptic drugs (Chiu *et al.* 1981b; Chiu *et al.* 1985; Rajakumar *et al.* 1987b). Behavioural studies have corroborated these findings (Bhargava, 1984a; Bhargava, 1984b; Bhargava, 1981b). PLG is also able to down-regulate dopamine receptors in several other paradigms where supersensitivity of dopamine receptors is observed. For example, PLG reverses the up-regulation of dopamine receptors produced after chronic administration of  $\beta$ -endorphin (Bhargava, 1981a), morphine (Das and Bhargava, 1985a; Bhargava, 1983a), and 17 $\beta$ -estradiol (Rajakumar *et al.* 1987a). In another example, PLG, along with two of its analogues, were shown to down-regulate the up-regulated dopamine receptor in spontaneous hypertensive rats, and in the process prevent the development of hypertension in these animals (Das and Bhargava, 1985b; Chiu *et al.* 1982a; Rajakumar *et al.* 1986). Recently, PLG and a

peptidomimetic analogue have been shown to attenuate neuroleptic drug induced immediate early gene expression (such as *c-fos*) in the striatum (Ott *et al.* 1997, Ott *et al.* 2000).

### 1.3.3 *In vitro* Studies

Although the precise mechanism of action for PLG has yet to be elucidated, there is a large body of evidence supporting the contention that PLG enhances dopaminergic neurotransmission mediated by the dopamine D2 receptor family (including the D<sub>2S</sub>, D<sub>2L</sub> and D<sub>4</sub> receptor subtypes). *In vitro* radioligand binding studies have shown that PLG and its peptidomimetic analogues increase agonist binding to dopamine D2 receptors (Chiu *et al.* 1981a; Srivastava *et al.* 1988; Yu *et al.* 1988; Subasinghe *et al.* 1993; Johnson *et al.* 1986; Johnson *et al.* 1986; Johnson *et al.* 1990; Genin *et al.* 1993; Sreenivasan *et al.* 1993; Evans *et al.* 1998; Thomas *et al.* 1998). Furthermore, these studies showed that the effects of PLG are manifest in a bell shaped dose response curve. Further examination of the effect of PLG on dopamine D2 receptor binding revealed that in the presence of PLG, dopamine D2 receptors were maintained in a high affinity state and that PLG counteracted the effects of GTP (or its analogues) on D2 agonist binding (Baures *et al.* 1994; Srivastava *et al.* 1988; Mishra *et al.* 1990). These studies suggest that PLG may exert its effects by altering the dynamics of interaction between the dopamine D2 receptor and G-proteins and that PLG is active in only a subset of dopamine receptors which are coupled to the Gi G-protein.

### 1.3.4 Human Studies

In studies with humans, PLG has been found to potentiate L-DOPA induced behavioural arousal in Parkinsonian patients (Plotnikoff *et al.* 1971; Barbeau, 1975; Barbeau *et al.* 1978), and has also demonstrated an ability to act as an antidepressant (Reed *et al.* 1994; Ehrensing and Kastin, 1978; Ehrensing *et al.* 1994; Pignatiello *et al.*

1989). While not all studies have shown positive results, it is believed that the negative results were due to the use of inappropriate doses of PLG (Reed *et al.* 1994). Although the mechanism involved in PLG's antidepressant effect has not been established, one theory suggests a dopaminergic effect or modification of the hypothalamic-pituitary-adrenal cortical axis (Reed *et al.* 1994). What is clear is that PLG shows promise as a treatment for depression with few or no side effects (Reed *et al.* 1994).

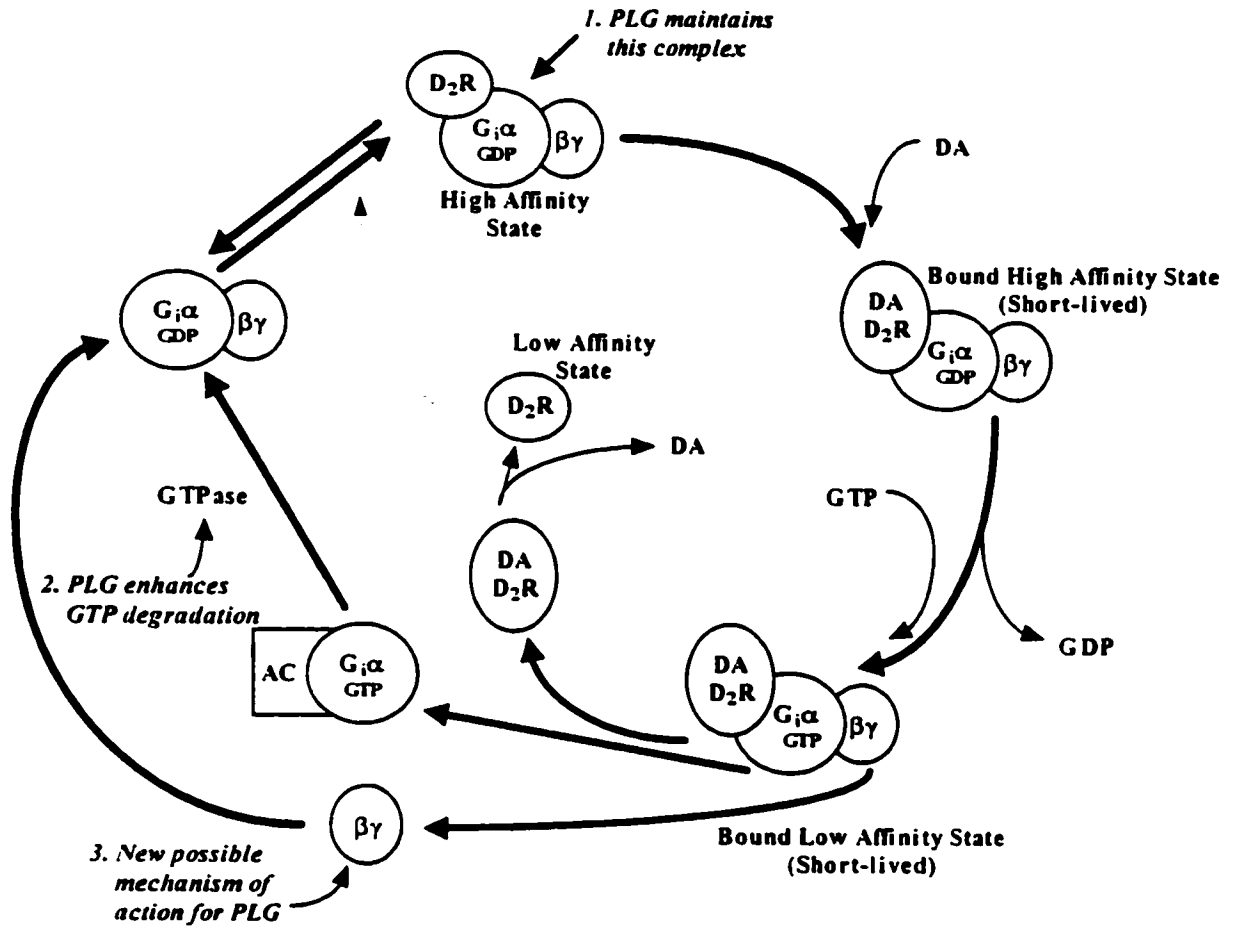
#### **1.4 Theories of PLG's Mechanism of Action**

There have been numerous pharmacological studies that have examined the effects of PLG on the D2-DA receptor / Gi G-protein signal transduction cascade. This research has resulted in the development of putative mechanisms of action for PLG. These "sites of action" are depicted in Scheme 1. The following sections will review the research contributing to the development of these hypotheses.

##### **1.4.1 Receptor**

The biochemical and pharmacological studies that have been conducted with PLG and its analogues clearly demonstrate that PLG modulates the dopamine D2 receptor subtype. The evidence that PLG is able to modulate the D<sub>1</sub> dopamine receptor is less clear. However it has been reported that PLG attenuates SCH 23390-induced ontogenic impairment of rat striatal D<sub>1</sub> dopamine receptors (Kostrzewa and Saleh. 1989). Furthermore, in another study, when a PLG peptidomimetic was given by either intrastriatal or intraperitoneal administration it potentiated the rotational behaviour elicited by the D<sub>1</sub> receptor agonist SK&F 38393 in 6-hydroxydopamine lesioned animals (Mishra *et al.* 1997). However, this compound was found to be considerably more potent in potentiating the rotational behaviour elicited by the D2 dopamine receptor agonist quinpirole than that elicited by SK&F 38393.





**Scheme 1.** Schematic representation of the possible mechanism of action of PLG in regulating the coupling of D<sub>2</sub>-dopamine receptors (D<sub>2</sub>R) to Gi-proteins (G<sub>i</sub>α and βγ) in the striatum. As shown, the agonist dopamine (DA) induces GDP-GTP exchange (step 1) that activates the G-protein, enabling G<sub>i</sub>α to interact with the enzyme adenylyl cyclase (AC). The G<sub>i</sub>α subunit undergoes spontaneous inactivation (step 2) mediated by its inherent GTPase activity. Upon inactivation of the G-protein, the receptor and G-protein subunits reform a complex, thereby completing the cycle. PLG is known to maintain the D<sub>2</sub>R in the high affinity state, and it is thought that this may be accomplished by acting at either step 1 or step 2.

The precise mechanism by which PLG modulates dopamine receptors is not known. It is known that PLG does not interact directly with the same sites that either dopamine receptor agonists or antagonists interact (Chiu *et al.* 1981a; Kostrzewa *et al.* 1979b). Preliminary evidence for the existence of a putative PLG receptor has been demonstrated using a radioreceptor binding assay using [2,3,4,5-<sup>3</sup>H]Pro-Leu-Gly-NH<sub>2</sub> as the radioligand (Chiu *et al.* 1982b; Chiu *et al.* 1983). However, attempts at isolating and purifying this receptor have not yet been successful due to the lack of a highly specific radioligand. Thus, it is not known whether the PLG binding site is a site on the dopamine D2 receptor or whether it is a separate macromolecular receptor that is coupled to the dopamine D2 receptor.

#### **1.4.2 G-protein**

The mechanism of PLG's modulation of dopamine receptors may involve the guanine nucleotide regulatory proteins (G-proteins) (Srivastava *et al.* 1988; Mishra *et al.* 1990; Baures *et al.* 1994; Baures *et al.* 1997). In these studies, PLG was found to selectively enhance agonist binding to striatal D2 dopamine receptors without having an effect on the binding of antagonists. Here PLG decreased the dissociation constant of the high affinity state of the dopamine receptor for agonists, thereby increasing the affinity of the D2 receptor for agonists. Furthermore, the percentage of D2 receptors that existed in the high affinity state were increased. Similarly, PLG has been shown to antagonize the 5'-guanylylimidodiphosphate [Gpp(NH)p]-induced inhibition of high affinity agonist binding and prevent the [Gpp(NH)p]-induced conversion of D2 receptors from a high affinity state to a low affinity state. Certain other peptides, e.g. mastoparan, substance P, bradykinin, have also been shown to interact directly with GTP-binding proteins (G-proteins) (Aridor *et al.* 1990; Higashijima *et al.* 1990; Bueb *et al.* 1990). It may very well be that PLG and its analogues interact with G-proteins directly or indirectly through the putative PLG receptor to modulate affinity states of the D<sub>2S</sub>, D<sub>2L</sub> and D<sub>4</sub> dopamine

receptors. Moreover, this modulation can affect the steady state levels of guanine nucleotide regulatory proteins and/or dopamine receptor levels that could account for the observed behavioural effects in acute as well as chronic studies.

## **1.5 Methods for Discovery of Regulated Gene Expression**

### **1.5.1 Subtractive Hybridisation**

Subtractive hybridisation is a method for identifying differentially expressed genes in two samples. This technique involves the generation of a high quality cDNA library from one of the samples under investigation to which the mRNA from another sample is hybridised. Following hybridisation, only the cDNA molecules that are not present in the mRNA sample remain in the single stranded form. The single stranded cDNA molecules are then separated from the double stranded (cDNA:mRNA) molecules by elution through a hydroxylapatite column. One of the major limitations of this technique is that it only allows one-way comparisons at any given time. That is, only the genes that are present in excess in the cDNA sample, relative to the mRNA sample, will be identified. Thus, any cDNAs that are decreased in abundance will not be identified. Another limitation of this technique is that a very large excess (typically 30 fold greater) of mRNA is required to perform the hybridisation step as a first order reaction. This effectively means that the gene must be present in at least a 30 fold excess in the cDNA sample in order to be identified (Sargent, 1987).

### **1.5.2 DNA Microarray and Gene Chips**

DNA microarrays and gene chips are becoming more popular as a method of detecting differences in gene expression in two different samples. As the number of known genes (and our knowledge of their function) increases, the versatility of this technology will increase. The advantage of this technology is that the level of expression

of a large number of genes can be assessed simultaneously. However, this technology is limited to our knowledge of the genome. That is, this technology currently does not allow one to discover new genes. Furthermore, DNA microarrays and particularly Gene Chips are very expensive, often requiring very expensive computer hardware and software for analysis of the results, compared to more traditional methods of gene discovery. While this technology currently has its limitations, as it becomes more advanced and pervasive it will undoubtedly become an integral component of basic research programs.

### **1.5.3 Differential Display PCR**

The technique of ddPCR lends itself well to the discovery of genes involved in mediating the effects of relatively novel drugs such as PLG. ddPCR has been used successfully to identify genes involved in a number of disorders including bipolar affective disorder (Wang and Young, 1996) and colon cancer (Yeatman and Mao, 1995). This technique has several advantages over other methods such as subtractive hybridisation and differential screening of cDNA libraries. Two major strengths of ddPCR are simplicity and ease of use, utilising PCR and DNA sequencing gel electrophoresis. ddPCR is also an extraordinarily sensitive method; using only 5 µg total RNA when compared to the 100-150 µg total RNA required to construct a cDNA library (Livesey and Hunt, 1996). ddPCR is also highly reproducible, where 90-95% of the bands are reproduced from run to run. ddPCR is very fast and versatile, allowing for high throughput of a large number of comparisons and rapid isolation of fragments of genes of interest. ddPCR also allows for detection of genes that are either up- or down-regulated with high sensitivity.

Several important limitations of ddPCR need to be considered when utilising the technique. The most important factor to consider is the generation of false positives during the PCR step. Two strategies can be employed to control for false positives. Firstly, performing any given PCR combination in duplicate, or preferably in triplicate,

will help identify false positives. The second and surest way of identifying false positives is to use northern blotting to check for the presence of the cDNA species in the total RNA. A second limitation of ddPCR is that it is biased toward the 3'-ends of the mRNA, and the resultant cDNA may lie within an untranslated region. In this case, it is necessary to screen a cDNA library to definitively identify the gene of interest (Livesey and Hunt, 1996). Another concern about ddPCR is that it may be biased toward cloning of low abundance genes (Livesey and Hunt, 1996). While this could be a potential problem in certain situations, in the present study, we expect the genes of interest to be expressed in low levels (Chiu *et al.* 1983).

## **2.0 Hypotheses**

The experiments described herein were conducted in an effort to explore aspects of D2 receptor/G-protein coupling and the role of PLG on this system. Further experiments were conducted attempting to identify genes/proteins integral to the mechanism of action of PLG in the rat brain.

### **2.1 D2 Dopamine Receptor Function Can be Assessed in Brain Tissue Using [<sup>35</sup>S]-GTPγS Binding Assay**

Measurements of [<sup>35</sup>S]-GTPγS binding to G-proteins in response to D2 agonists enables us to assess the functional coupling of these two proteins. Previous studies of dopaminergic receptor signal transduction have been done primarily in homogeneous populations of dopamine receptors expressed in cell lines. Application of the [<sup>35</sup>S]-GTPγS binding technique to natively expressed D2 dopamine receptors will provide pharmacological data that is representative of the pharmacological conditions of the brain *in vivo*. Although this technique only examines initial events of signal transduction, it provides more information about the function of a system than do other techniques that focus on a single component of the signal transduction cascade.

### **2.2 Typical and Atypical Neuroleptic Treatment has Differential Effects on D2 Receptor Coupling to Gi**

Although a large amount of research has examined the effects of neuroleptic treatment on D2 receptor levels and G-protein levels, no attempt has been made at assessing the effect of neuroleptic treatment on the coupling of D<sub>2</sub> receptors to G-proteins. Alterations in the levels of expression of receptors and G-proteins are likely to result in functional alterations. However, in a multi-component system, such as the signal transduction cascade, the effect of altering the levels of a single component on the overall function of the system may be difficult to predict. Comparison of the effects of

typical and atypical neuroleptic treatment on D2 dopamine receptor function in the striatum using the [<sup>35</sup>S]-GTP $\gamma$ S binding technique will provide physiologically important information about the differential effects of typical and atypical neuroleptics on the striatal dopaminergic system.

### **2.3 PLG Modulates D2 DA Receptor Activation of Gi**

The modulatory effects of PLG on D2 DA receptor function in the striatum is well established. The well characterised effects of PLG on D2 receptor agonists has led to the development of a theory on its mechanism of action (Scheme 1). According to this theory, PLG is likely to affect either the activation (GDP:GTP exchange rate) or inactivation (GTPase activity) of the Gi G-protein. Examining the effects of PLG on D2 agonist stimulated [<sup>35</sup>S]-GTP $\gamma$ S binding in the striatum will clarify whether or not PLG affects D2 receptor coupled G-protein activation.

### **2.4 Protracted PLG Treatment Modifies the Expression of Genes Involved PLG Signal Transduction**

PLG is an endogenous neuropeptide with known effects on numerous neurological processes (Kastin *et al.* 1995; Ehrensing *et al.* 1994; Ehrensing, 1974). Prolonged PLG treatment has known effects on central dopaminergic neurotransmission. It is reasonable to expect that a long-term elevation of PLG levels will alter the expression of proteins involved in mediating its effects (particularly in the striatum) as happens in other neurotransmitter systems (Burt *et al.* 1977). Furthermore, observations in our lab indicate that PLG has the ability to modulate the expression of immediate early genes (Ott *et al.* 2000). ddPCR is a technique that enables the detection of numerous genes that are differentially expressed in two or more tissues. Therefore, ddPCR can be used to identify genes that are differentially regulated by prolonged PLG treatment. Genes found to be regulated by PLG will likely be important modulators of its effects and may

provide insight into the mechanism of action of PLG.

The hypothesis I will test is that a protracted treatment with PLG will alter the expression of genes directly involved in PLG signal transduction. The technique of ddPCR will be used to identify a number of genes whose expression is altered as a result of protracted PLG treatment; thereby increasing our understanding of the mechanism of action for PLG.



### 3. Materials and Methods

#### 3.1 Materials

[<sup>35</sup>S]-GTP<sub>γ</sub>S ([<sup>35</sup>S]-guanosine 5'-(γ-thio)triphosphate, 1250 Ci/mmol), [α-<sup>35</sup>S]-dATP ([<sup>35</sup>S]-deoxyadenosine 5'(α-thio)triphosphate, 1250 Ci/mmol), [<sup>32</sup>P]-NAD ([adenylate-<sup>32</sup>P]-nicotinamide adenine dinucleotide, di(triethylammonium) salt, 30 Ci/mmol) and [<sup>32</sup>P]-dCTP ([α<sup>32</sup>P]-deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt, 3000 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA); thymidine was purchased from Calbiochem (San Diego, CA); gel blot paper was purchased from Schleicher & Schuell (Keene, NH). Kits for plasmid isolation (QIAprep Spin Miniprep Kit), DNA gel extraction (QIAEX II Gel Extraction Kit) and poly A<sup>+</sup> mRNA (Oligotex mRNA Mini Kit) isolation were purchased from QIAGEN (Mississauga, ON). The RNImage kit #1 ddPCR kit and pCR-TRAP PCR cloning kit (GenHunter, Nashville, TN) were used for differential display and subsequent cloning. Restriction enzymes (Hind III and Eco RI) were purchased from MBI Fermentas (Hamilton, ON). The Marathon-Ready<sup>™</sup> cDNA (rat brain), Advantage<sup>®</sup> cDNA polymerase mix, Advantage<sup>®</sup> UltraPure PCR Deoxynucleotide (dNTP) Mix, and the AdvanTAge<sup>™</sup> PCR Cloning Kit were purchased from Clontech (Palo Alto, CA). DNase I and Trizol reagent were purchased from GibcoBRL (Life Technologies, Burlington, ON). AmpliTaq was purchased from Perkin-Elmer (PE Biosystems, Foster City, CA) and Bio-Rad reagent was obtained from Bio-Rad Laboratories (Mississauga, ON). Random Primed DNA labelling kit and G-50 Sephadex Quick Spin<sup>™</sup> Columns were purchased from Bohringer Mannheim (Laval, PQ). RNA molecular weight markers, Herring Sperm DNA, goat anti-mouse IgG (H+L) HRP conjugate, goat anti-mouse IgG (H + L) AP conjugate and goat anti-rabbit IgG (Fc) AP conjugate were purchased Promega (Fisher Scientific, Nepean, ON). The donkey anti-rabbit IgG HRP conjugate was purchased from Amersham Pharmacia Biotech, Inc. (Baie d'Urfé, PQ). Actin (Ab-1) monoclonal antibody

kit was purchased from Oncogene Research Products, Cambridge, MA. SEE-BLUE protein molecular weight markers were purchased from Novex (Helixx Technologies, Mississauga, ON). All other chemicals used were reagent grade and were obtained from either Caledon Labs. Ltd. (Georgetown, ON) or BDH Inc. (Toronto, ON).

### **3.2 Animal Dissection and Tissue Handling/Storage**

The animals (male Sprague Dawley rats) used in this study were housed and handled according to the CCAC guidelines for animal handling. The animals were purchased through and housed at the Central Animal Facility at McMaster University. At the end of the treatment and wash out periods for the animals, the animals were anaesthetised with methoxyfluorane and sacrificed by decapitation. Following the sacrifice of the animals, the brains were quickly removed and placed on ice where the various brain regions (striatum, pre-frontal cortex and nucleus accumbens) were dissected out and frozen and stored at -80°C.

#### **3.2.1 Animal Treatment**

The rats were treated in one of two ways. In one study, 18 Male Sprague Dawley rats (350-450g) were divided into three groups receiving either no treatment, haloperidol or clozapine for 14 days. The animals in the control group received daily intraperitoneal injections of sterile saline. The animals in the haloperidol group received daily i.p. injections of 3 mg/kg haloperidol dissolved in sterile water. The animals in the clozapine group received daily doses of 20 mg/kg clozapine orally. In another study, the animals were treated for 28 days with 20 mg/kg PLG i.p. (dissolved in sterile saline) or with an equivalent volume of saline (1 ml/kg).

### **3.3 Membrane Preparation**

### **3.3.1 Crude Striatal Synaptosomal Membrane Preparation (Bovine Striatum)**

Bovine brains, obtained from a local abattoir, were kept on ice until the striata were dissected out and frozen at  $-80^{\circ}\text{C}$ . A purified plasma membrane sample was prepared in the following manner. Frozen striata were thawed and homogenised in 10 vol/wt of an ice cold homogenisation buffer containing 0.25 M sucrose, 50 mM Tris, 1 mM EDTA and 0.1 mM PMSF (pH 7.4). Tissues were homogenized using a motor driven dounce homogeniser with a Teflon pestle. Homogenized tissue was centrifuged at  $1100 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was saved while the pellet was resuspended in 5 vol/wt homogenisation buffer and centrifuged as before. The supernatant from the second spin was collected and pooled with the supernatant from the first spin while the remaining pellet was discarded. The pooled supernatants were then centrifuged at  $35000 g$  for 20 min at  $4^{\circ}\text{C}$ . The resultant pellet was resuspended in 2 vol/wt of a buffer containing 50 mM Tris, 1 mM EDTA, 5 mM  $\text{MgCl}_2$  and 0.1% ascorbic acid (pH 7.4). The final protein concentration was determined by the Bio-Rad method.

### **3.3.2 Crude Striatal Homogenate (Rat Striatum)**

The composition of the homogenisation buffer differed depending on the type of experiment the tissue was to be used in. For [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding studies the homogenisation buffer consisted of: 50 mM Tris, 1 mM EDTA and 0.1 mM PMSF (pH 7.4); while for western blots, the homogenisation buffer consisted of: 50 mM Tris, 1 mM EDTA (pH 7.4). For all experiments, the frozen striata were removed from storage and placed in 1 ml of homogenisation buffer and homogenized using a hand-held glass dounce homogeniser. The crude homogenate was stored at  $-80^{\circ}\text{C}$  following determination of the amount of protein in each sample using the BioRad assay.

### **3.4 Pertussis Toxin Treatment of Bovine Striatum**

The method used was adapted from Hilf *et al.* (1989). Immediately prior to use,

the pertussis toxin (PT) was activated by incubating at 30°C for 30 min in an activation buffer of 20 mM DTT and 0.125% SDS. Purified bovine striatal membranes (250 µg) were incubated for 60 min at 30°C in a buffer containing 100 mM Tris (pH 8.0), 1 mM EDTA, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 3 mM DTT, 10 mM NAD and 15 µg PT in a final volume of 100 µl in a 1.5 ml eppendorf tube. A corresponding control reaction (SHAM) was set up identical to the foregoing except for the exclusion of PT. At the end of the reaction, the reaction was divided into a 40 µl and 60 µl aliquot (aliquots A and B respectively). Aliquot A was placed in a second 1.5 ml eppendorf tube and the reaction was terminated (in both aliquots) by the addition of 1.0 ml ice-cold 10 mM triethanolamine (pH 7.5) and the protein was subsequently pelleted by centrifuging at 16000 x g for 20 min at 4°C. The protein pellets were resuspended in 1.0 ml triethanolamine and centrifuged again. The protein pellet from aliquot B was used for [<sup>35</sup>S]-GTPγS binding. The protein pellets from aliquot A (PT and control reactions) were used in a second PT reaction. For this reaction, both samples were treated the same. The protein pellet (100 µg) was incubated for 60 min at 30°C in a buffer containing 100 mM Tris (pH 8.0), 1 mM EDTA, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 3 mM DTT, 10 µM NAD, 2 µCi [<sup>32</sup>P]-NAD and 5 µg PT in a final volume of 100 µl in a 1.5 ml eppendorf tube. The reaction was terminated as described above, and the resultant protein pellet was resuspended in 50 µl SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2-β-mercaptoethanol; 0.00125% bromophenol blue).

#### **3.4.1 Autoradiography**

The amount of <sup>32</sup>P incorporation was assessed autoradiographically by exposing the dried gel slabs to Kodak X-OMAT AR film at -80°C for 24-72 hours. The calibrated autoradiographic images were analysed using NIH-Image (v1.61).

#### **3.5 [<sup>35</sup>S]-GTPγS Binding Assay**

The method used was adapted from Wieland & Jakobs (1994). Briefly, purified bovine striatal membranes were incubated in a buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM DTT, 0.5% BSA, 3 μM GDP and 50,000 cpm [<sup>35</sup>S]-GTPγS (0.3 - 0.8 nM). The total volume of the assay was 100 μl and it was conducted at 25°C for 45 min. The assay was started by the addition of the striatal membranes and was terminated by the addition of 4 ml ice-cold filtration buffer (containing 50 mM Tris, 5 mM MgCl<sub>2</sub>) followed by rapid filtration over Whatman GF-B filter strips using a Brandell M-24 filtration apparatus. Non-specific binding was defined in the presence of 10 μM cold GTPγS. The filter disks were placed in plastic scintillation vials containing 4 ml scintillation fluid. The radioactivity was then determined as cpm using a Beckman LS5000 scintillation counter. The assay was carried out in the presence and absence of D2 dopamine receptor agonists.

To determine the optimal efficiency of the assay, it was first necessary to examine NPA stimulated [<sup>35</sup>S]-GTPγS binding in the presence of varying concentrations of GDP. From this it was determined that the optimal conditions (greatest magnitude of response and good signal to noise ratio) for the assay were at a GDP concentration of 3 μM. Next, it was necessary to confirm that the time point chosen for the end of the assay (45 minutes) was in a non-limiting range for the reaction. With appropriate assay conditions chosen, the effects of a variety of dopaminergic agonists and antagonists were assayed. The antagonists were assayed by their ability to inhibit NPA (1 μM) stimulated increases in [<sup>35</sup>S]-GTPγS binding.

Specific binding was determined as the difference between total cpm bound and cpm bound in the presence of non-radioactive GTPγS (non-specific binding). Basal levels of binding were determined in the absence of an agonist. The cpm data were converted to fmol [<sup>35</sup>S]-GTPγS bound per milligram protein. To control for inter-experiment variation in basal levels of [<sup>35</sup>S]-GTPγS binding, the data were expressed as a percentage of basal levels of binding. Dose response data were fitted to sigmoidal dose response curves using

least squares regression analysis with the Prism program from GraphPad. Statistical analyses were conducted using a two-way factorial ANOVA with Fisher's PLSD post-hoc test with the program Statview 4.2.

### **3.5.1 Reaction conditions**

### **3.5.2 Data analysis**

Experiments were conducted in a paired manner whereby in each experiment the effects of NPA were measured on one tissue from each treatment group. Specific [<sup>35</sup>S]-GTP $\gamma$ S binding was obtained by subtracting non-specific binding (< 5%) from total binding. The data for all groups were converted to a percentage of the basal [<sup>35</sup>S]-GTP $\gamma$ S binding (in the absence of NPA) observed in the control tissue for each experiment. This was done to control for inter-experiment variation as well as maintain observed differences between groups. Data were fitted to sigmoidal dose response curves by least squares regression analysis using the Prism software package from GraphPad. A two-way factorial ANOVA (treatment and dose) with a Dunnett's post-hoc t-test was used to analyse the dose response data. Comparisons of EC<sub>50</sub> values were made using an unpaired two-tailed Student's t-test. All statistical analyses were performed with an alpha of 0.05 using release 10 of Minitab and Statview 4.02. Six experiments per group were conducted with each experiment being performed in triplicate.

## **3.6 SDS-PAGE For Electrophoresis of Protein Samples**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their size (Laemmli, 1970). Briefly, the protein samples were centrifuged at max speed on a microcentrifuge for 2 minutes, the pellet resuspended in 30  $\mu$ l SDS-PAGE gel loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2- $\beta$ -mercaptoethanol; 0.00125% bromophenol blue) and boiled for 4 minutes

prior to loading on the gel. A discontinuous SDS-PAGE gel (Bio-Rad Mini-PROTEAN II apparatus) was used with a 12% separating gel (12% acrylamide/bis acrylamide, 0.375 M Tris HCL, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate, 0.05% temed) and a 4% stacking gel (4% acrylamide/bis acrylamide, 0.125 M Tris HCL, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, 0.1% temed). Electrophoresis was conducted for  $\approx$  1 hour at a constant voltage of 175 Volts ( $\approx$  110 mAmps). Following electrophoresis, the gels were placed on gel blot paper, covered with Saran Wrap and dried using a Bio-Rad gel slab drier.

Following SDS-PAGE, the protein was transferred to nitrocellulose using a Novex X-Cell II apparatus. The transfer buffer consisted of 12 mM Tris-HCl, 96 mM glycine and 10% methanol. Prior to assembly of the transfer apparatus, the stacking gel is removed, and the nitrocellulose and gel are pre-wet in transfer buffer. The transfer is run at a constant voltage of 30 V for a duration of 2 hours. Following the transfer, the apparatus is disassembled and the nitrocellulose is rinsed briefly with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween<sup>®</sup> 20) buffer and is incubated in a solution of TBS-T containing 5% skim milk powder for 1 hour. After this, the nitrocellulose is rinsed with TBS-T, wrapped in saran wrap and stored at 4°C.

### 3.7 Western Blot

Western blots were performed to assess the levels of expression of two proteins, the heteronuclear protein L and the G-protein subunit  $G_{\gamma 11}$ . The antibody for protein L and  $G_{\gamma 11}$  were generous gifts provided by Dr. G. Dreyfus (University of Pennsylvania, Philadelphia PA; gdreyfuss@hhmi.upenn.edu) (Piñol-Roma *et al.* 1989) and Dr. T. Asano (Institute for Developmental Research, Aichi Japan, toasano@inst-hsc.pref.aichi.jp) (Morishita *et al.* 1998) respectively. These antibodies were used in final dilutions of 1:10000 and 1:1667 for protein L and  $G_{\gamma 11}$  respectively. The protein L antibody is mouse anti-human and the  $G_{\gamma 11}$  antibody is rabbit anti-rat. The protein L antibody was generated

by immunising a BALB/c mouse with the purified protein, while the G<sub>γ11</sub> antibody was generated by immunising a rabbit with a peptide consisting of amino acids Met<sup>1</sup>-Lys<sup>14</sup> of the G<sub>γ11</sub> protein appended with a cysteine (MPALHIEDLPEKEKC) conjugated to keyhole limpet hemocyanin. The blots were also probed for the expression of the housekeeping protein Actin. The primary antibody for Actin was obtained from Oncogene and is reactive with the α-, β- and γ-isoforms of Actin. The antibody is mouse monoclonal anti-Actin IgM (generated by immunising BALB/c mice with cytoskeletal proteins from chicken gizzards and fusion of spleen cells with NS-1 cells), is reactive to a variety of species including rat, and was used in a final dilution of 1:7500.

The nitrocellulose membranes (see section 3.6) were placed in sealed plastic bags and incubated with the primary antibody at 4°C overnight on an orbital shaker. Following labelling with the primary antibody, the nitrocellulose membranes were washed three times with TBS-T (15, 5 and 5 minutes) at room temperature. The nitrocellulose membranes were then placed in new bags and incubated with the secondary antibody for 1 hour at room temperature on an orbital shaker. Following labelling with the secondary antibody, the nitrocellulose membranes were washed three times with TBS-T (15, 5 and 5 minutes) at room temperature.

Two detection methods were employed, NBT/BCIP (Promega) and Enhanced Chemiluminescence (ECL, Amersham). The NBT/BCIP method utilises a secondary antibody conjugated with alkaline phosphatase (AP) that catalyses a colour reaction resulting in the reactive areas of the blot turning purple. This method was used to initially identify the immunoreactive bands and to confirm their molecular mass. Because this method stains the nitrocellulose itself, it is preferable for accurate determination of molecular mass. Briefly, the nitrocellulose blot is incubated in 10 ml of a solution containing 66 µl NBT and 33 µl BCIP for every 10 ml of buffer (100 mM Tris-HCL, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>) for 1-15 minutes, or until the desired band intensity is obtained, then the blot is washed in deionised water.



The ECL method utilises a secondary antibody conjugated with horseradish peroxidase (HRP) that catalyses a chemiluminescent reaction resulting in the reactive areas of the blot emitting light. A permanent record of the reactive areas of the nitrocellulose blots are obtained by exposure to x-ray film. Blots analysed in this manner can be stripped and re-probed for different protein. Thus, this method is useful for quantitative analysis of the expression levels of more than one protein in the same blot. Briefly, the method involves incubating the blot in the ECL detection solution (10 ml total per blot; 5 ml ECL solution 1 + 5 ml ECL solution 2) for exactly 1 minute at room temperature, draining the excess detection solution, wrapping the blot in saran wrap and exposing the blot to x-ray film in a dark room for 15 sec to 5 minutes (depending on the strength of the signal).

Detection of anti-protein L was accomplished by incubating the blots with a 1:7500 dilution of goat anti-mouse IgG (H + L) AP conjugate (Promega) and subsequently visualised using the NBT/BCIP method. Detection of anti-G<sub>γ11</sub> was accomplished by incubating the blots with a 1:7500 dilution of anti-rabbit IgG (HRP conjugate, Amersham Pharmacia) and subsequently visualised using the ECL method. Detection of anti-Actin was accomplished by incubating the blots with a 1:2000 dilution of goat anti-mouse IgM (HRP conjugate, Oncogene) and subsequently visualised using the ECL method.

### **3.8 RNA Preparation, Quantitation and Analysis**

Total RNA was prepared using the Trizol method according to the protocol supplied by the manufacturer. Briefly, the brain tissue (striatum, pre-frontal cortex or nucleus accumbens) from one rat is homogenized in 1.0 ml of Trizol on ice using a glass dounce homogeniser. The homogenate is transferred to a 1.5 ml eppendorf micro centrifuge tube and incubated at room temperature for 5 minutes. To this, 0.2 ml chloroform is added and the sample is shaken vigorously for 15 sec. The sample is then

centrifuged at 12000 x g for 15 min at 4°C in a micro centrifuge. The upper aqueous phase, containing the RNA, is transferred to a new 1.5 ml eppendorf tube and 0.5 ml isopropanol is added and mixed by inverting the tube several times. The sample is incubated at room temperature for 10 min (to precipitate the RNA), and centrifuged at 12000 x g for 10 min at 4°C. The supernatant is discarded, 1 ml 75% ethanol is added and the RNA pellet is washed by vortexing. The sample is centrifuged at 7500 x g for 5 min at 4°C. The supernatant is discarded and the pellet is allowed to air dry at room temperature for 15 min. The pellet is then resuspended in 50 µl DEPC dH<sub>2</sub>O and incubated at 60°C for 15 min prior to storage at -80°C. The amount of mRNA obtained will be determined by measuring the OD at 260 nm and the purity by the ratio of 260/280 nm. The integrity of the RNA (18S and 28S bands) was assessed by running 3 µg on a denaturing 1.2% agarose gel. Prior to use in RT-PCR, mRNA will be purified using a DNase I (Gibco BRL Life Technologies) treatment to remove any contaminating chromosomal DNA. DNase treatment will be done immediately prior to RT-PCR to ensure that the diluted mRNA is not degraded during storage.

### **3.8.1 Poly A<sup>+</sup> mRNA Preparation**

Poly A<sup>+</sup> mRNA was isolated from total RNA for use in northern hybridisation experiments. The Oligotex mRNA Mini Kit (Qiagen) was used to isolate mRNA from total RNA. This kit utilises oligo(dT) anchored latex beads to bind the poly A<sup>+</sup> mRNA and thereby enable its purification from total RNA. The protocol supplied with the kit was used with a few modifications; twice as much Oligotex resin was used and the mRNA hybridisation incubation period was extended to 10 minutes at room temperature. Recovery mRNA ranged from 3.89% to 5.11%.

### **3.9 Agarose Gel Electrophoresis (RNA)**

Denaturing agarose gel electrophoresis was performed using a BioRad Sub GT gel

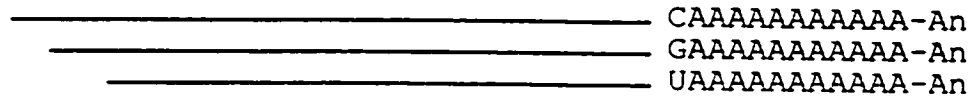
apparatus. Gels prepared for qualitative analysis of RNA integrity were made using 1.2% agarose, while gels used for transfers to nitrocellulose were prepared using 1.0% agarose. The agarose gels were prepared by melting 0.72 - 0.60 g agarose in 60 ml of MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA) containing 6.6% formaldehyde. The melted agarose was then poured into a casting tray and allowed to solidify for at least 30 minutes at room temperature in a fume hood. The gel is placed in the apparatus and the buffer reservoir is filled with MOPS buffer. The samples were prepared by adding 3.545 volumes of sample premix (12.9% 5X MOPS, 8.35% formaldehyde, 64.5% deionised formamide) to the RNA sample and heating to 55°C for 15 minutes. Gel loading buffer (0.1 volumes; 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) is then added to the samples prior to loading on the gel. The gel is run at a constant voltage of 60 V for 1.5 - 2 hours.

### **3.10 Differential Display PCR**

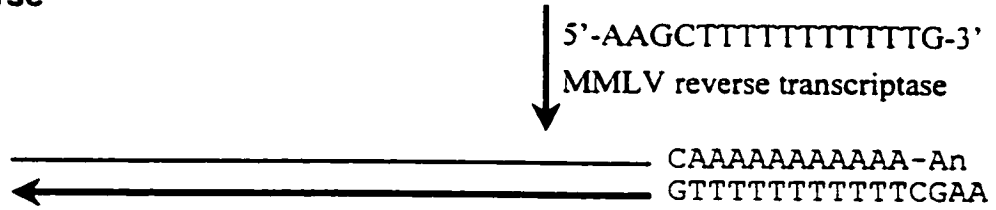
Differential display RT-PCR (ddPCR) was performed using the GenHunter ddPCR RNAimage kit #1 (Scheme 2). Briefly, 1 µg of total RNA (isolated from rat striatum, section 3.8) undergoes a DNase three reverse transcription reactions with three different oligo-dT primers (H-T<sub>11</sub>G, H-T<sub>11</sub>C, H-T<sub>11</sub>A). The products of each RT reaction are then used in 8 different PCR reactions (MJ Research MiniCycler). Each PCR reaction uses the same oligo-dT primer used in RT and one of eight arbitrary primers. Hence, each mRNA sample undergoes a total of 24 PCR reactions. Approximately 100 cDNAs are generated by each PCR reaction, therefore 2400 cDNAs in total are generated by this method.

Initially, 1 µg of total RNA was treated with 1 unit of DNase I (in a total volume of 10 µl with 1X DNase I reaction buffer), for 15 minutes at room temperature. The reaction was stopped by the addition of 1 µl of 25 mM EDTA and incubated at 65°C for 10 minutes. A 2 µl aliquot of the DNase treated RNA was used for reverse transcription.

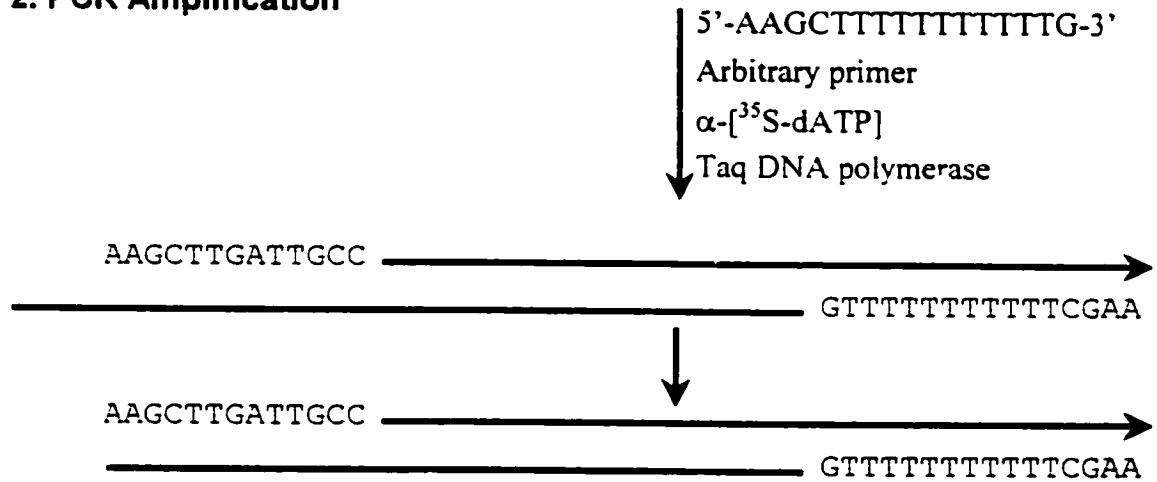
**mRNA Population**



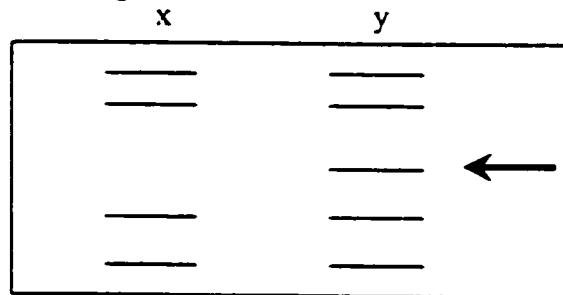
**1. Reverse**



**2. PCR Amplification**



**3. Denaturing Polyacrylamide gel**



**Scheme 2.** Schematic representation of ddPCR method.

The reverse transcription reaction was conducted in a total volume of 20  $\mu\text{l}$  with a 2  $\mu\text{l}$  aliquot of the DNase I treated RNA (0.09  $\mu\text{g}$ ), 20  $\mu\text{M}$  dNTPs, 4  $\mu\text{l}$  5X RT reaction buffer (125 mM Tris-HCl, pH8.3, 188 mM KCl, 7.5 mM  $\text{MgCl}_2$ , 25 mM DTT), 0.2  $\mu\text{M}$  of one of three oligo dT anchored primers (H-T<sub>11</sub>N). The reaction was then incubated at 37°C for 10 minutes and 1  $\mu\text{l}$  of MMLV reverse transcriptase (100 units) was added to the reaction. The reaction was allowed to continue for 50 minutes at 37°C, then for 5 minutes at 75°C and then the samples were stored at -20°C.

PCR reactions were then conducted on the cDNAs created in the reverse transcription reaction. Eight different arbitrary primers (H-AP<sub>N</sub>) were used in combination with the oligo dT anchored primers (H-T<sub>11</sub>N) used in the reverse transcription reaction. Each PCR reaction (total volume of 20.0  $\mu\text{l}$ ) contained an aliquot of 2  $\mu\text{l}$  of the cDNA created in the reverse transcription reaction, 2  $\mu\text{M}$  dNTPs, 0.2  $\mu\text{M}$  H-T<sub>11</sub>N, 0.2  $\mu\text{M}$  H-AP<sub>N</sub>, 1 Unit AmpliTaq and 2  $\mu\text{l}$  10X PCR buffer (100 mM Tris-HCl, pH 8.4, 500 mM KCl, 15 mM  $\text{MgCl}_2$ , 0.01% gelatin).

Following the completion of the ddPCR reactions, the ddPCR products were visualised on a 6% denaturing polyacrylamide sequencing gel (apparatus BioRad Sequi-Gen<sup>®</sup> GT Nucleic Acid Electrophoresis Cell). A 6% acrylamide solution (19:1 acrylamide/bis-acrylamide) is prepared in TBE buffer (89.1 mM Tris, pH 8.3, 88.9 mM boric acid, 2.5 mM EDTA) containing 7 M urea. Polymerisation is initiated with the addition of 0.08% TEMED and 0.02% ammonium persulfate. The electrode buffer (TBE) is heated to 55°C prior to use, and a pre-run is performed for 30 - 60 minutes (until gel temperature is  $\approx$  55°C) at a 70 watts (constant). The samples are loaded onto the gel, and the gel is run for 2 hours at 60 watts. When completed, the gel is placed on gel blotting paper, covered with saran wrap and dried using a BioRad gel slab dryer (model 583). When dry, the gel is exposed to Kodak XAR film for 3 days at room temperature. The ddPCR products are visible on the developed film.

For comparisons between control and PLG treated samples, the ddPCR process is

conducted side by side on equal amounts of mRNA for both samples. Following electrophoresis, the gel is removed from the gel apparatus and dried using a BioRad gel slab drier. The dried gel is then exposed to Kodak BioMax MR film for 3 days at room temperature. PCR products from similar reactions for the control and PLG samples are run in adjacent lanes for easy comparison of amplified bands. This allows for easy visualisation of up- or down-regulated PCR products.

### **3.10.1 PCR Fragment Recovery And Reamplification**

Those bands identified as being regulated by PLG were excised from the gel and solubilized in 100  $\mu$ l dH<sub>2</sub>O, precipitated and finally resuspended in 10  $\mu$ l dH<sub>2</sub>O. The recovered cDNA (PCR fragments) were reamplified using the same primers by which they were generated. The resultant PCR products were run on a 1.7% agarose gel and their size was checked with what was expected from the acrylamide gel.

### **3.10.2 Agarose Gel Electrophoresis (DNA)**

Non-denaturing agarose gel electrophoresis was performed using a BioRad Sub GT gel apparatus. Gels prepared for analysis of DNA were prepared using between 1.2% and 2.0% agarose. The agarose gels were prepared by melting 0.72 - 1.2 g agarose in 60 ml of TAE buffer (40 mM Tris-HCl, 0.1142% glacial acetic acid, 1 mM EDTA) containing and 0.5  $\mu$ g/ml ethidium bromide. The melted agarose was then poured into a casting tray and allowed to solidify for at least 30 minutes at room temperature in a fume hood. The samples were prepared for loading onto the gel by adding 0.1 volumes of a 10 X gel loading buffer (40% sucrose, 0.25% bromophenol blue, 400 mM Tris-HCl, 1.142% glacial acetic acid, 10 mM EDTA). The buffer reservoir is filled with TAE buffer and the gel is run at 80 V for 1 - 1.5 hours (depending on the amount of agarose used). The DNA bands are then visualised under UV light, and photographed in black and white with a POLARIOD instant camera.

### 3.11 Cloning of and Analysis of ddPCR Products

The reamplified cDNA was extracted from the agarose gel using the QIAEX II gel extraction kit. The recovered cDNA was cloned into the pCR-TRAP cloning system (GenHunter) using blunt end ligation and expressed in competent cells. Transformed cells were plated on LB plates containing 20 µg/ml tetracycline and incubated overnight at 37°C. The presence of cDNA inserts were screened for by using Tetracycline resistance (Tet<sup>R</sup>) and checked by colony-PCR according to the pCR-TRAP protocol. The use of a phage Lambda repressor gene by this cloning system allows only those plasmids that have a cDNA insert to acquire Tet<sup>R</sup>. To ensure that the Tet<sup>R</sup> colonies contain cDNA inserts, a colony-PCR was performed on DNA recovered from the Tet<sup>R</sup> colonies using a specific set of PCR primers that flank the cloning site. The presence of a cDNA insert will result in larger PCR products that are visualised on a 1.7% agarose gel. A number of insert positive colonies were grown overnight in liquid culture (LB medium + 20 µg/ml tetracycline) and the plasmid DNA harvested the next day using QIAGEN Miniprep system. Plasmid DNA were sequenced at the MOBIX facility at McMaster. Sequence information will be searched on GenBank to see if the genes had previously identified. The cDNA inserts were excised by Hind III restriction enzyme digestion and separated on a 1.7% agarose gel. The cDNA was recovered from the gel using the QIAEX II method and labelled using a random primed labelling kit (Boehringer Mannheim) and subsequently used as a probe in northern hybridisation. The northern blot was done for two reasons; 1/ to eliminate the possibility that the band is non-specific (due to a PCR error) and 2/ to confirm the effect of the treatment on the levels of expression of the mRNA. PCR fragments which produce positive northern blots and do not correspond to known genes will make good candidates for full length cloning.

The pCR-TRAP vector, supplied by GenHunter, was chosen for use in cloning because it is designed to be used with ddPCR fragments generated by the GenHunter

RNAimage kits. The pCR-TRAP vector is constructed in such a way that insertion of a foreign cDNA sequence into the cloning site disrupts the expression of a gene that represses the expression of the plasmid's tetracycline resistance gene. Thus, when competent cells (transformed with the pCR-TRAP vector) are grown on agar plates containing tetracycline, all of the colonies should contain a cDNA insert. This vector is also designed with a *HindIII* restriction consensus sequence at the cloning site that corresponds to the *HindIII* sequence integrated into the ddPCR primers of the RNAimage kits. Thus, when the plasmid is digested with the *HindIII* restriction enzyme, the excised cDNA insert possesses none of the flanking plasmid sequence.

The reamplified ddPCR fragments obtained in the previous step were cloned directly into the pCR-TRAP vector. Following the ligation reaction, the vector was used to transform competent cells (GH cells, provided by GenHunter with the pCR-TRAP kit) which were subsequently plated onto agar plates containing 20 µg/ml tetracycline. The agar plates were incubated overnight at 37°C and subsequently placed at 4°C for storage. Ten colonies from each cloning were chosen for plasmid isolation. Overnight cultures (5 ml LB medium + 10 µg/mg tetracycline) were made from each colony chosen and 50% glycerol stocks were prepared and stored at -70°C. Plasmid DNA was isolated by Miniprep on 4 - 6 replicates, and was subsequently sent for sequencing at MOBIX.

### **3.12 RACE-PCR**

Elongated clones of the novel cDNAs identified by ddPCR were obtained by the rapid amplification of cDNA ends (RACE) method for obtaining full-length cDNA clones. In doing this, I utilised the Marathon™ cDNA amplification kit (Clontech, Palo Alto, CA) which employs a combination of long-distance PCR and RACE to produce full-length clones from partial cDNAs. This method does not require the construction or screening of a cDNA library. The only requirement is that a 23-28 nucleotide sequence is known for the cDNA being amplified. This requirement is easily met, since our method



selects ddPCR fragments that are in the range of 100-400 base pairs in size. The cDNA is synthesized by Clontech and involves first strand cDNA synthesis from poly A<sup>+</sup> RNA using MMLV reverse transcriptase primed with a lock-docking oligo(dT) cDNA synthesis primer. Second strand synthesis is carried out with an enzyme cocktail containing RNase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase. Treatment of the resultant double stranded DNA with T4 DNA polymerase produces blunt-ends suitable for ligation (T4 DNA ligase) with a double stranded cDNA adapter. The adapter ligated cDNA, supplied by Clontech, is then used in 5' and 3' RACE reactions. The long-distance PCR reactions were carried out using Clontech's Advantage KlenTaq™ polymerase mix which includes a minor amount of proof-reading polymerase to provide 3' → 5' proof-reading. Gene specific primers (based on the known sequence of the original cDNA) were synthesised at McMaster's MOBIX facility and used along with a primer specific for the adapter. The primers were to be designed so that they were 20-38 base pairs in length with 50-70% G/C content and a  $T_m \approx 70$  °C. Thus, with appropriate design of gene specific primers, the cDNA can be extended in both the 5' and 3' directions. Since the ddPCR products we have obtained are located at the 3' end of the mRNA transcript by design, 3' RACE reaction were not expected to elongate the cDNA, whereas the 5' RACE reaction was potentially capable of yielding the full length cDNA without further effort. The size of the 5' RACE products were compared to the expected size of the mRNA (obtained from northern blot) to determine if the full-length cDNA has been obtained. The RACE products obtained were characterised to ensure that the desired product has been amplified. This was done by cloning and sequencing of RACE product.

### 3.12.1 Primer design

Primers for RACE-PCR were designed according to the recommendations in the Marathon cDNA kit. Briefly, primers were designed so that they would ideally possess a melting temperature ( $T_m$ ) between 68-72°C, were specific for the gene of interest and

were devoid of primer dimers or hairpin loops. In some instances, the short length or sequence peculiarities of the ddPCR fragments limited the design of the primers. For control purposes, the primers used for 5' RACE were positioned closer to the 3' end of the ddPCR fragment while the primers used for 3' RACE were positioned closer to the 5' end of the ddPCR fragment. By doing this, it was possible to confirm the amplification of the correct gene by comparing the overlapping regions of the ddPCR fragment and the RACE-PCR products.

### 3.12.2 Cloning of and Analysis of RACE-PCR Products

RACE-PCR products were cloned using the AdvanTage™ PCR Cloning Kit (Clontech). This kit is based on TA cloning and utilises the *lacZ* operon for blue/white screening of positive colonies. Disruption of the expression of the *lacZ* gene results in white colonies. Briefly, the PCR products (less than 1 day old) were ligated to 50 ng of the pT-Adv vector in a 10 µl reaction containing 4 units of T4 DNA ligase, 6 mM Tris-HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 5 mM NaCl, 0.1 mg/ml BSA, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine, and 20 fmol of the RACE-PCR product. The ligation reaction was carried out for 14 hours at 14°C. A mixture containing 2 µl of the ligated DNA, 2 µl 0.5 M β-mercaptoethanol and 50 µl of the TOP10F' *E. coli* competent cells were incubated on ice for 30 min, heat shocked for 30 sec at 42°C, and then placed on ice for 2 min. A 250 µl aliquot of room temperature SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose) was added to the transformation mixture and placed in a rotary shaking incubator at 37°C for 1 hour at 225 rpm. The transformed cells were kept on ice prior to plating (50 and 200 µl aliquots on two separate plates) on LB/X-Gal/IPTG (40 µl each of 40 mg/ml X-Gal in dimethylformamide and 100 mM IPTG) plates containing 50 µg/ml ampicillin. After the liquid had absorbed, the plates were incubated at 37°C for 18 hours, then stored at 4°C.

### 3.13 Northern Hybridisation

Total RNA and poly A<sup>+</sup> mRNA samples were prepared as described in sections 3.8 and 3.81. Denaturing agarose gel electrophoresis was conducted as described in section 3.9. For northern hybridisation, 1.0 µg mRNA was loaded onto the agarose gels and electrophoresed for 2 hours. The gels were then prepared for transfer to nitrocellulose by soaking in DEPC dH<sub>2</sub>O (3 times 10 min) followed by 20X SSC (3 M NaCl, 75 mM sodium citrate, pH 7.2) for 45 min. The mRNA was then transferred overnight to nitrocellulose, by capillary action. Following the transfer, the lanes were marked on the blot in pencil, and the blot rinsed in 5X SSC for 5 min and air dried on a piece of Whatman filter paper. The RNA was fixed to the blot by baking at 80°C for 2 hours. The blots were then stored at room temperature in dry conditions. Prior to hybridisation, the blot was pre-wet with 2X SSPE (3 M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 20 mM EDTA) and sealed into a plastic bag containing 5 ml pre-hybridisation solution (5X SSPE, 50% formamide, 5X Denhard's reagent (100 X, 1% Ficoll<sup>®</sup> type 400, 1% polyvinylpyrrolidone, 1% BSA), 0.5% SDS, and 100 µg/ml denatured herring sperm DNA). The blot was pre-hybridised at 42°C for 2 hours in a Hybaid hybridisation chamber. After pre-hybridisation, the <sup>32</sup>P labelled probe was denatured, and added to the bag (1 e<sup>6</sup> cpm/ml). Hybridisation was conducted at 42°C with rotation overnight. Hybridisation was terminated by conducting a low stringency wash of the blot for 30 minutes at 42°C in a 50 ml volume of a solution containing 2X SSPE and 0.1% SDS. The probes used for northern hybridisation were obtained by agarose gel purification (QIAEX II method) of the cloned ddPCR fragments (pCR-TRAP system, GenHunter) following *Hind*III restriction enzyme digestion. Alternatively, the larger cDNAs produced by RACE-PCR were used as probes for *PRG1*, *PRG2* and *PRG4*. These cDNAs were obtained by agarose gel purification of the cloned RACE-PCR products (AdvanTAge system, Clontech) following *Eco*RI restriction enzyme digestion.

### 3.13.1 Probe Labelling

The Random Primed DNA labelling kit (Bohringer Mannheim ) was used to label the probes obtained by ddPCR or RACE-PCR. Briefly, 25 ng of DNA were denatured by heating to 95°C for 5 min, and added to a 50 µl reaction containing 10 nM dATP, 10 nM dGTP, 10 nM dTTP, random primers buffer mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl<sub>2</sub>, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD<sub>260</sub> units/ml oligodeoxyribonucleotide primers (hexamers), pH 6.8), ≈ 50 µCi [ $\alpha^{32}$ P]-dCTP, and 3 Units Klenow fragment (large fragment of DNA polymerase I). The reaction was carried out at 25°C for 1 hour, and then 5 µl of a stop buffer (0.5 M EDTA, pH 8.0) was added. The unincorporated radionucleotides were removed from the sample by elution through a Sephadex G-50 column (G-50 Sephadex Quick Spin™ Columns, Bohringer Mannheim). The number of cpm in a 2 µl aliquot of the labelled DNA sample were determined using a liquid scintillation counter. The specific activity of the probes was then calculated and those probes with a minimum of 1e<sup>8</sup> cpm/µg were used for northern hybridisation.

### 3.13.3 Blot Stripping

Northern blots were stripped by incubating the nitrocellulose in a solution containing 5 mM Tris-HCl (pH 8.0), 2 mM EDTA and 0.1 X Denhardt's solution for 2 hours at 65°C in a hybridisation tube with rotation. Immediately thereafter, the blots were transferred to a 2X SSPE solution at room temperature and subsequently sealed in a plastic bag for storage.

### 3.13.4 Autoradiographic Densitometry

Image analysis (autoradiography, chemiluminescence and AP staining) was performed using the computer software package NIH Image (v 1.61; NIH, Bethesda Maryland; <http://rsb.info.nih.gov/nih-image/Default.html>). Northern and western blots

were quantitated using the line scan method with the uncalibrated optical density setting.

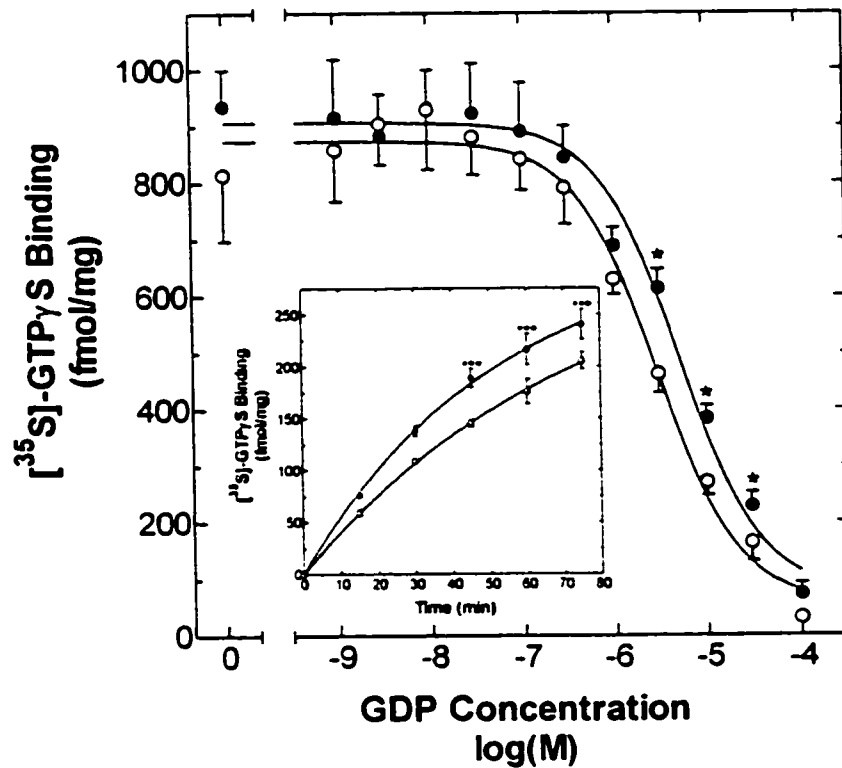
## **4.0 Results**

### **4.1 Characterisation of the Role of PLG in D2 Receptor Activation of G-Proteins in the Striatum**

#### **4.1.1 Establishment of [<sup>35</sup>S]-GTP $\gamma$ S Binding Assay**

Preliminary measurements of [<sup>35</sup>S]-GTP $\gamma$ S binding in purified bovine striatal membrane preparations revealed very high levels of constitutive binding ( $\approx$  900 fmol/mg protein in the absence of GDP, Figure 1). The high levels of constitutive [<sup>35</sup>S]-GTP $\gamma$ S binding precluded the detection of a significant increase in [<sup>35</sup>S]-GTP $\gamma$ S binding resulting from the addition of D2 agonists. Therefore, it was necessary to perform the assay in the presence of a competing guanine nucleotide (GDP) so that the signal to noise ratio of the assay was sufficiently reduced to enable the detection of agonist stimulated increases in [<sup>35</sup>S]-GTP $\gamma$ S binding. This made it possible to consistently detect significant increases in binding in the presence of a D2 receptor agonist. Figure 1 shows a GDP dose response experiment conducted in the absence and presence of 1  $\mu$ M NPA. The addition of NPA significantly increased [<sup>35</sup>S]-GTP $\gamma$ S binding when GDP concentration were between 3 and 30  $\mu$ M ( $p < 0.05$ , ANOVA). Although the signal to noise ratio was greatest at 10  $\mu$ M GDP, a concentration of 3  $\mu$ M GDP was used throughout because this concentration provided the greatest magnitude of response to D2 agonist. This concentration of GDP is consistent with that used in other studies. The dose response effect ( $EC_{50}$  values) of NPA was the same at 3 and 10  $\mu$ M GDP (data not shown), indicating that the concentration of GDP did not have an effect on the D2 receptor function.

The inset of Figure 1 shows that [<sup>35</sup>S]-GTP $\gamma$ S binding increases with time beyond the chosen experimental endpoint of 45 minutes, indicating no substrate limitations for the assay. The data was best described by a one-phase exponential association with correlation values of 0.984 and 0.973 for - NPA and + NPA respectively. The presence of



**Figure 1.** Effect of GDP concentration on [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in bovine striatum. A GDP titration is performed to determine the optimal signal to noise ratio for the assay. The assay is performed with a 45 minute incubation time in the absence (O) and presence (●) of 1  $\mu\text{M}$  NPA, with the greatest signal to noise ratio found at a concentration of 3  $\mu\text{M}$  GDP. Inset shows that the binding of [ $^{35}\text{S}$ ]-GTP $\gamma$ S, in the presence of 3  $\mu\text{M}$  GDP, increases with time and is best described by a one-phase exponential association curve fit. Importantly, [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding increases well beyond the 45 minute time point, thereby ensuring that there is not a substrate limitation at the endpoint chosen for subsequent experiments. Data are given as mean of  $n = 4 \pm \text{S.E.M.}$  (\*  $p < 0.05$ , \*\*\*,  $p < 0.0001$ , one-way ANOVA).

1  $\mu$ M NPA increased the amount of [ $^{35}$ S]-GTP $\gamma$ S binding at all time points and significant differences were observed at time points > 45 minutes ( $p < 0.0001$ , ANOVA).

#### 4.1.2 Characterisation of D2 DA-Receptor Agonist Pharmacology

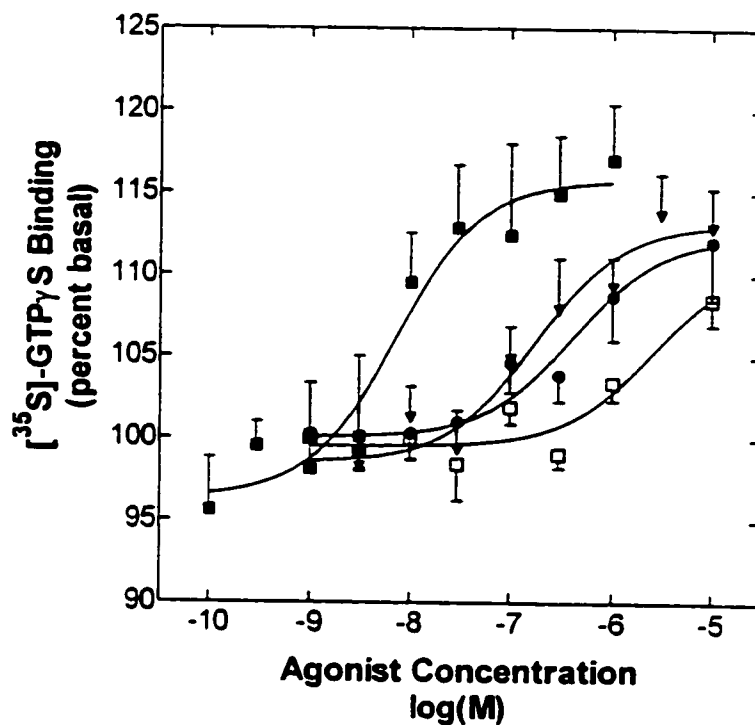
Dose response measurements for a variety of D2 receptor agonists were performed in purified bovine striatal membranes. Figure 2 shows the dose response curves for the four agonists tested. The rank order of potency of the agonists is as follows: NPA >> bromocryptine > apomorphine > dopamine. The  $pEC_{50}$  values obtained from the analysis of the data in Figure 2 are presented in Table 1. The  $pEC_{50}$  values from the present study are consistently between the  $K_D$  values reported for the high and low affinity binding states of the drugs studied (Gardner *et al.* 1997; Seeman, 1995; Mishra *et al.* 1990). The rank order of potency observed here, with the exception of bromocryptine, corresponds well with the rank order of potency observed in radioligand binding studies (Gardner *et al.* 1997; Seeman, 1995; Mishra *et al.* 1990). It should be noted that receptor binding studies using bromocryptine detect only a single affinity state which is not affected by the presence of guanine nucleotides in the assay (Gardner *et al.* 1997; Seeman, 1995). Furthermore, the  $pEC_{50}$  values presented here correlate very well with the values obtained by Gardner *et al.* (1997) for the  $D_{2S}$  receptor expressed in CHO cells.

Comparison of the efficacy of the agonists tested revealed a certain degree of variability. Data for the efficacy (maximal stimulation of [ $^{35}$ S]-GTP $\gamma$ S binding) of the agonists tested is provided in Table 1. The data in Figure 2 and Table 1 are expressed as a percentage of the [ $^{35}$ S]-GTP $\gamma$ S binding observed in the absence of dopaminergic ligands. Previous reports have used the [ $^{35}$ S]-GTP $\gamma$ S binding assay as a means of detecting partial agonists (Gardner *et al.* 1997; Malmberg *et al.* 1998; Newman-Tancredi *et al.* 1997; Chabert *et al.* 1994). The data presented here (Table 1) indicates that there were no significant differences in the maximal levels of stimulation. Thus, all of the agonists tested behaved as full agonists in the bovine striatum.



**Table 1.** Summary of D2 dopamine receptor agonist stimulation of [<sup>35</sup>S]-GTP $\gamma$ S binding in bovine striatum.

<b>AGONIST</b>	<b>pEC<sub>50</sub> (EC<sub>50</sub>, nM)</b>	<b>Maximal effect (% of basal)</b>	<b>Number of Observations</b>
NPA	8.15 ± 0.17 (7.1)	115.8 ± 1.19	6
Bromocryptine	6.76 ± 0.17 (175)	113.2 ± 1.04	4
Apomorphine	6.39 ± 0.18 (411)	112.3 ± 1.31	4
Dopamine	5.62 ± 0.36 (2400)	110.7 ± 2.79	6



**Figure 2.** Effect of D2 dopamine receptor agonists on [ $^{35}$ S]-GTP $\gamma$ S binding in bovine striatum. The graph shows the effect of NPA (■), bromocryptine (▼), apomorphine (●) and dopamine (□) on [ $^{35}$ S]-GTP $\gamma$ S binding. The results are expressed as a percentage of the amount of binding observed in the absence of drugs (basal binding = 100%). Each of the agonists significantly increased [ $^{35}$ S]-GTP $\gamma$ S binding ( $p < 0.05$ , one-way ANOVA). The data are presented as mean  $\pm$  S.E.M. of 6, 4, 4 and 6 experiments for NPA, apomorphine, bromocryptine and dopamine respectively.

#### 4.1.3 Characterisation of D2 DA-Receptor Antagonist Pharmacology

A series of D2 receptor antagonists were tested for their ability to inhibit NPA stimulated [ $^{35}$ S]-GTP $\gamma$ S binding in purified bovine striatum. The dose response data for the antagonists tested is displayed in Figure 3. The data were best described by one-site inhibition curves and the pIC<sub>50</sub> values were adjusted to pK<sub>i</sub> values using the Cheng-Prusoff equation to account for the competition with NPA. The pK<sub>i</sub> values are given in Table 2 with a rank order of potency as follows: spiperone > haloperidol > butaclamol > sulpiride > chlorpromazine > clozapine. The rank order of potency observed here correlates very well with previously reported data using receptor binding assays (Gardner *et al.* 1996; Seeman and Van Tol, 1994).

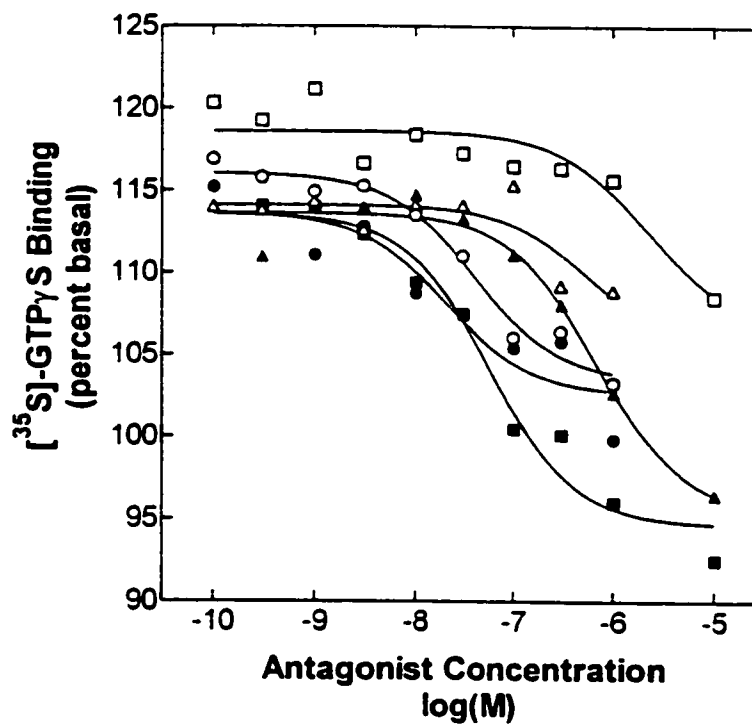
It was observed that the D2 antagonists haloperidol, butaclamol, clozapine and chlorpromazine reduced [ $^{35}$ S]-GTP $\gamma$ S binding in the absence of D2 receptor stimulation. However, this effect was not observed for all of the antagonists tested. Figure 4 shows the effect of the antagonists in the absence of D2 receptor stimulation on [ $^{35}$ S]-GTP $\gamma$ S binding. One-way ANOVA revealed that only haloperidol ( $p = 0.0009$ ), butaclamol ( $p < 0.0001$ ) and chlorpromazine ( $p = 0.0004$ ) significantly reduced [ $^{35}$ S]-GTP $\gamma$ S binding. The data for the antagonists causing significantly reduced [ $^{35}$ S]-GTP $\gamma$ S binding were fit to one-site inhibition curves. Table 2 gives the pIC<sub>50</sub> values as well as the maximal inhibition of basal binding for those antagonists which caused significantly decreased basal [ $^{35}$ S]-GTP $\gamma$ S binding. The pIC<sub>50</sub> values obtained in the absence of D2 receptor stimulation correlate well with pIC<sub>50</sub> values obtained in the presence of D2 receptor stimulation, providing a similar rank order of potency (haloperidol > butaclamol > chlorpromazine).

#### 4.1.4 Activation of D2 DA-Receptors Increases [ $^{35}$ S]-GTP $\gamma$ S Binding in Gi G-Proteins

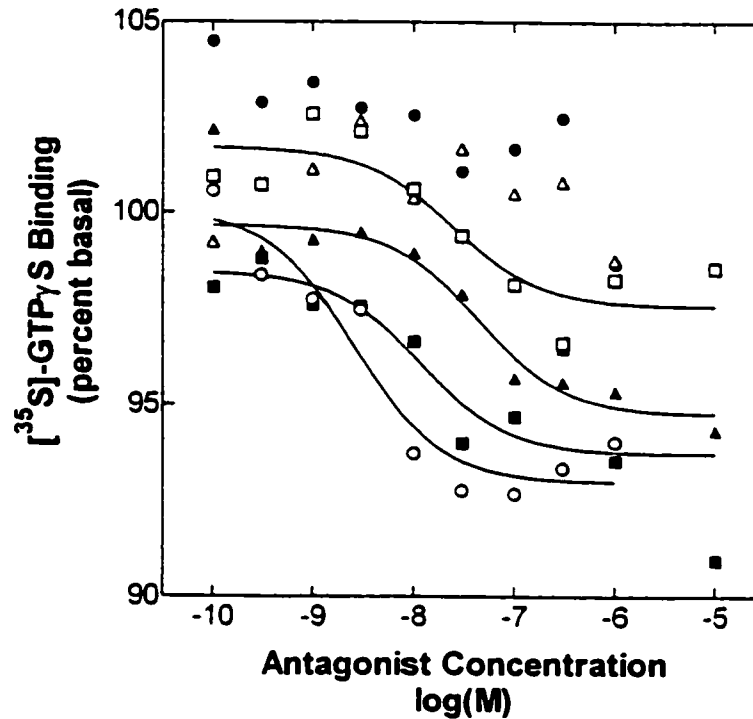
The ability of NPA to stimulate [ $^{35}$ S]-GTP $\gamma$ S binding in bovine striatal

**Table 2.** Summary of inhibition of [<sup>35</sup>S]-GTP $\gamma$ S binding in bovine striatum by D2 dopamine receptor antagonists in the presence and absence of D2 receptor stimulation with 1  $\mu$ M NPA. <sup>†</sup> Adjusted according to the Cheng-Prusoff equation. \* p = 0.0009; \*\* p < 0.0001; \*\*\* p = 0.0004 one-way ANOVA.

ANTAGONIST	NPA antagonism	Basal inhibition	Max inhibition	# of obs
	pK <sub>i</sub> <sup>†</sup> (K <sub>i</sub> , nM)	pIC <sub>50</sub> (IC <sub>50</sub> , nM)	of basal (% of basal)	
Siperone	9.85 ± 0.57 (0.142)	ND		3
Haloperidol	9.55 ± 0.24 (0.282)	8.58 ± 0.39 (2.6)	7.02 ± 0.92*	6
Butaclamol	9.39 ± 0.17 (0.406)	7.94 ± 0.40 (11.6)	6.28 ± 0.64**	4
Sulpiride	8.36 ± 1.10 (4.40)	ND		3
Chlorpromazine	8.31 ± 0.16 (4.89)	7.36 ± 0.32 (44.0)	5.26 ± 0.68***	4
Clozapine	7.82 ± 0.41 (15.3)	ND		3



**Figure 3.** Effect of D2 dopamine receptor antagonists on NPA (1  $\mu$ M) stimulated [ $^{35}$ S]-GTP $\gamma$ S binding in bovine striatum. The graph shows the ability of haloperidol (O), clozapine (□), sulpiride ( $\Delta$ ), spiperone ( $\bullet$ ), butaclamol ( $\blacksquare$ ) and chlorpromazine ( $\blacktriangle$ ) to inhibit NPA stimulated [ $^{35}$ S]-GTP $\gamma$ S binding. The results are expressed as a percentage of the amount of binding observed in the absence of drugs (basal binding = 100%). Each of the antagonists significantly reduced NPA stimulated [ $^{35}$ S]-GTP $\gamma$ S binding ( $p < 0.01$ , one-way ANOVA). The data are presented as the mean of 6, 3, 3, 3, 3 and 3 experiments for haloperidol, clozapine, sulpiride, spiperone, butaclamol and chlorpromazine respectively.



**Figure 4.** Effect of D2 dopamine receptor antagonists on basal [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in bovine striatum. The graph shows the ability of haloperidol (○), clozapine (□), sulpiride (Δ), spiperone (●), butaclamol (■) and chlorpromazine (▲) to inhibit [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in the absence of a D2 receptor agonist. The results are expressed as a percentage of the amount of binding observed in the absence of drugs (basal binding = 100%). One-way ANOVA revealed that only haloperidol, butaclamol and chlorpromazine significantly reduced [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding ( $p = 0.0009$ ,  $0.0001$  and  $0.0004$  respectively). The data are presented as the mean of 6, 3, 3, 3, 3 and 3 experiments for haloperidol, clozapine, sulpiride, spiperone, butaclamol and chlorpromazine respectively.

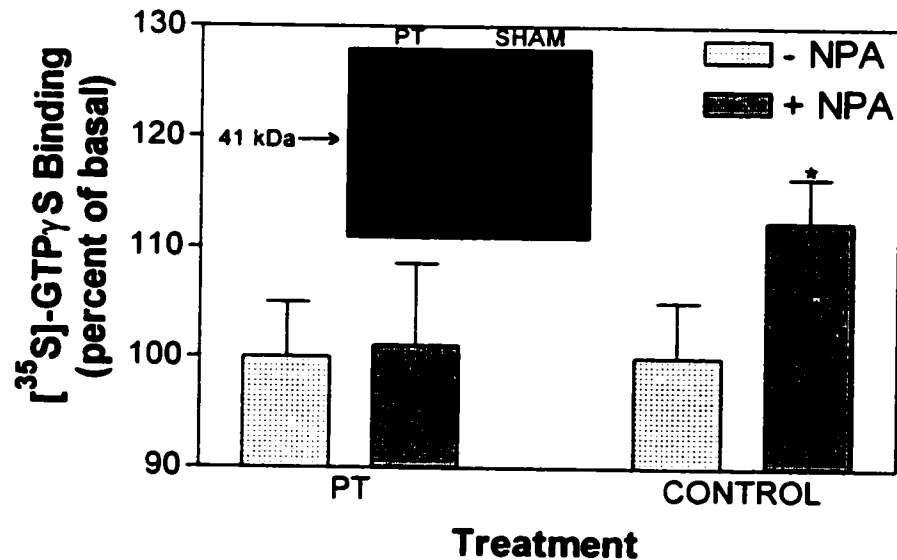
membranes treated with PT was assessed in order to identify the species of G-protein being activated. Figure 5 shows that NPA stimulated [<sup>35</sup>S]-GTP $\gamma$ S binding was diminished in tissue treated with PT; indicating that the Gi G-protein is involved in mediating the effects of NPA. The inset of Figure 5 is an autoradiogram showing that the amount of PT mediated ADP-ribosylation was decreased  $\approx$  60% in the tissue previously treated with PT. This data is consistent with previous research indicating that the D2-DA receptor is coupled to adenylate cyclase via the Gi G-protein (Lovenberg *et al.* 1991; Lledo *et al.* 1992; Di Marzo *et al.* 1993; Izenwasser and Côté, 1995).

#### **4.1.5 Effect of PLG and PLG analogues on D2 receptor stimulation of G-proteins**

The possibility that PLG modulates D2 receptor mediated signal transduction by affecting the function of Gi at the level of GDP:GTP exchange was assessed using the [<sup>35</sup>S]-GTP $\gamma$ S binding method. It is a general property of G-protein coupled receptors, particularly those receptors coupled to Gi, that the affinity of an agonist is decreased in the presence of GTP in *in vitro* binding assays. Since PLG is known to increase the affinity of D2 receptor agonists, it is possible that it is doing so by affecting the rate of GDP:GTP exchange. Figure 6 shows that PLG did not affect the levels of [<sup>35</sup>S]-GTP $\gamma$ S binding either in the absence or presence of NPA. This data indicates that PLG, or its potent analogue, do not affect the rate of GDP:GTP exchange.

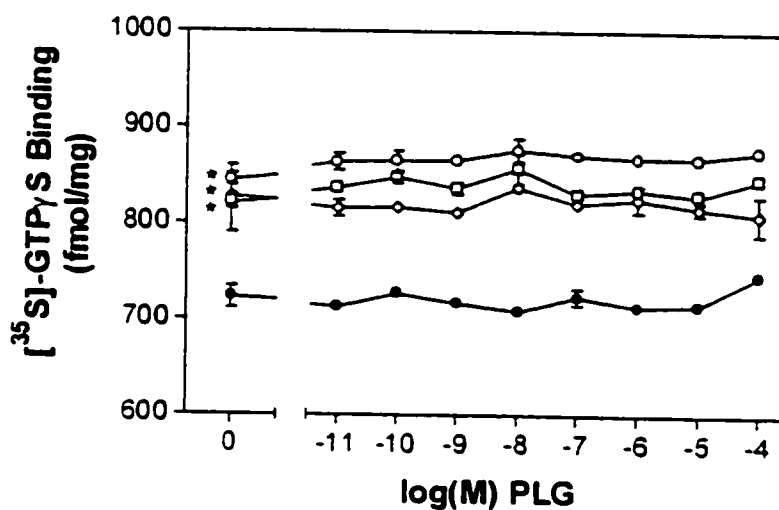
## **4.2 Characterisation of Effects of Typical and Atypical Neuroleptic Treatment on D2 Receptor Activation of [<sup>35</sup>S]-GTP $\gamma$ S Binding in the Rat Striatum**

### **4.2.1 Effect of Haloperidol and Clozapine Treatment on Levels of [<sup>35</sup>S]-GTP $\gamma$ S Binding**



**Figure 5.** Effect of pertussis toxin on NPA stimulated [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in bovine striatum. The graph shows that NPA (1  $\mu\text{M}$ ) significantly (\*,  $p < 0.05$ , t-test) increased [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in tissue receiving the sham treatment, while no such increase was observed in the PT treated tissue. The data are represented as mean of  $n = 3 \pm \text{S.E.M.}$ . The inset of the Figure is an autoradiogram that shows the efficacy of the PT treatment. Here an aliquot of the sham and PT treated tissues were subjected to a second PT treatment using. Incorporation of  $^{32}\text{P}$ -NAD was determined to be reduced by  $\approx 60\%$  in the PT treated tissue.





**Figure 6.** Effect of PLG on [ $^{35}$ S]-GTP $\gamma$ S binding in bovine striatum in the absence (●) and presence of 1  $\mu$ M (○), 0.1  $\mu$ M (□) and 0.03  $\mu$ M (◇) NPA. In the presence of NPA, [ $^{35}$ S]-GTP $\gamma$ S binding was significantly (\*,  $p < 0.05$ , t-test) increased. The figure also shows that PLG did not affect (either positively or negatively) the levels [ $^{35}$ S]-GTP $\gamma$ S binding in either the absence or presence of D2 receptor stimulation. The data are represented as mean of  $n = 3 \pm$  S.E.M..

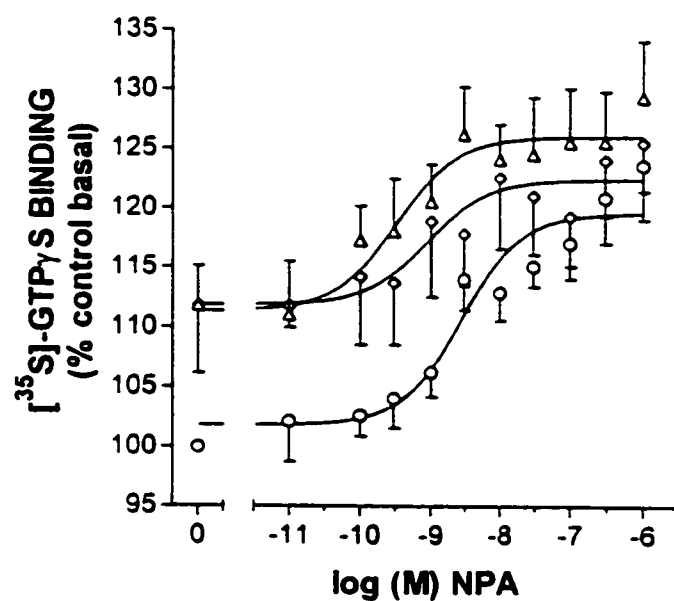
Previous studies have demonstrated that the neuroleptic treatments used in this study lead to decreased and increased expression of the  $G_i$  family of G-proteins in the rat striatum for haloperidol and clozapine respectively (Gupta and Mishra, 1993; Shin *et al.* 1995). In the present study, it was observed that both haloperidol (3 mg/kg, i.p., 14 days) and clozapine (20 mg/kg, oral, 14 days) treatment (section 3.2.1) increased the unstimulated (basal) levels of [ $^{35}$ S]-GTP $\gamma$ S binding in the rat striatum (by  $11.8 \pm 3.3\%$  and  $11.8 \pm 5.6\%$  respectively) as well as the maximal levels of NPA stimulated binding (Figure 7). Two-way ANOVA confirmed that [ $^{35}$ S]-GTP $\gamma$ S binding (group means) was significantly increased compared to control levels as a result of treatment with either haloperidol or clozapine ( $p < 0.001$ ). These results indicate that neuroleptic treatment increases the constitutive activity of  $G_i$  G-proteins in the striatum and that the D2 receptor mediated signal transduction pathway is potentiated.

#### **4.2.2 Effect of Haloperidol and Clozapine Treatment on NPA Stimulation of G-Protein GTP Binding**

The dose-response effect of NPA on [ $^{35}$ S]-GTP $\gamma$ S binding in control and neuroleptic treated rat striatum is displayed in Figure 7. NPA produced similar dose response effects in each of the treatment groups. The NPA  $EC_{50}$  values are given in Table 3. Comparisons of  $EC_{50}$  values using Student's t-test revealed that haloperidol caused a significant decrease in NPA  $EC_{50}$  (increased affinity) compared to control ( $p < 0.04$ ) while clozapine did not ( $p > 0.38$ ). This differential effect between haloperidol and clozapine is consistent with the differential effects of these drugs on D2 receptor density in the striatum (Creese and Sibley, 1981; Ashby Jr. and Wang, 1996; See *et al.* 1990).

### **4.3 Identification of Genes Regulated by Protracted PLG Treatment Using ddPCR**

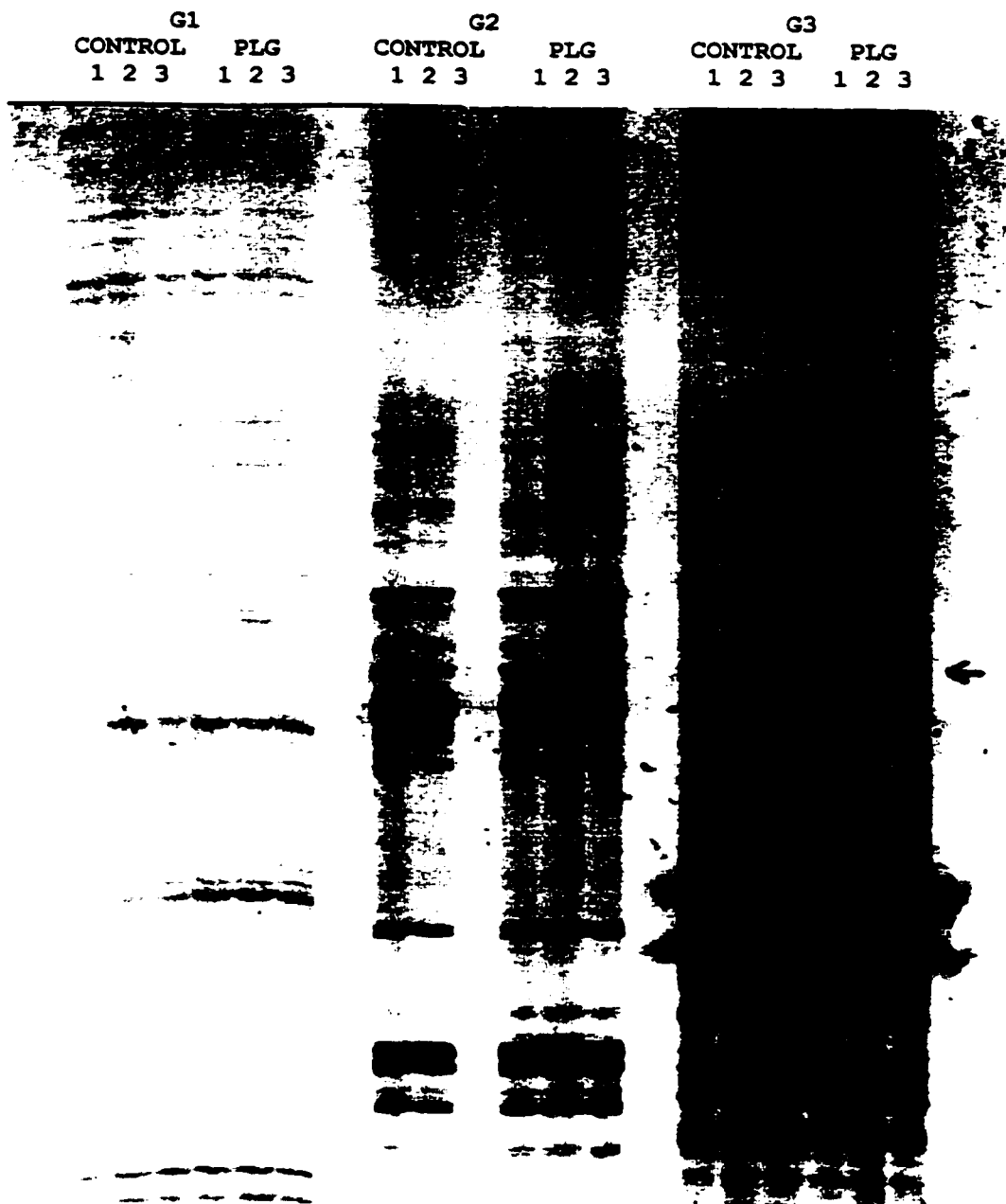
#### **4.3.1 ddPCR Screening of 2400 Genes**



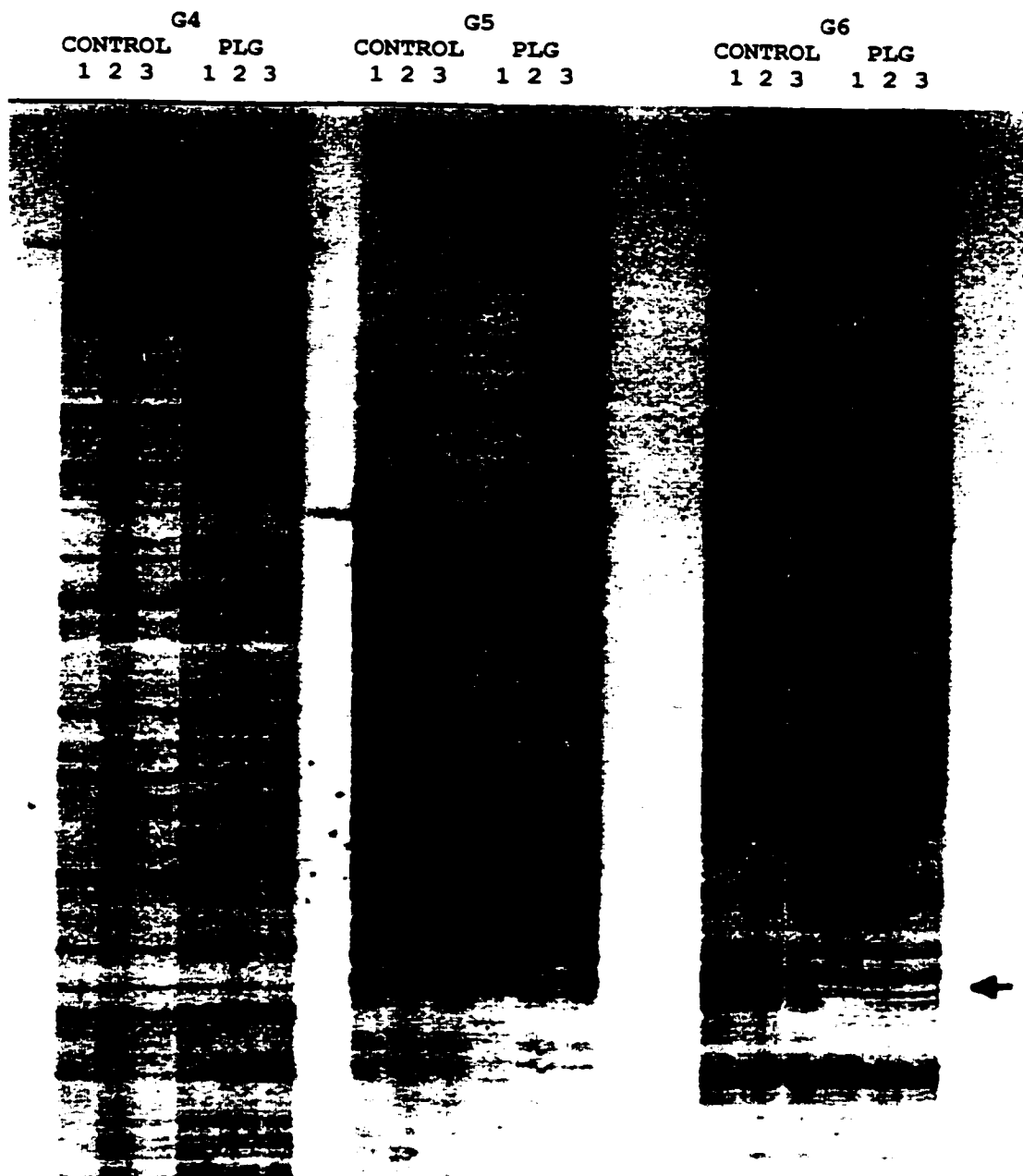
**Figure 7.** NPA stimulated [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in striatum from control (O), haloperidol ( $\Delta$ ) and clozapine ( $\diamond$ ) treated rats. The data are expressed as a percentage of the basal (absence of NPA) levels of [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in the control tissue for each experiment. Experiments were paired so that each experiment measured [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding in one tissue from each group. The data were analysed using a two-way factorial ANOVA with Dunnett's post-hoc t-test. [ $^{35}\text{S}$ ]-GTP $\gamma$ S binding was significantly increased in both the haloperidol and clozapine treated groups ( $p < 0.001$ ). Data are presented as mean  $\pm$  s.e.m. ( $n = 6$ ).

**Table 3.** Table of EC<sub>50</sub> values for NPA stimulation of [<sup>35</sup>S]-GTPγS binding in control and neuroleptic treated rat striatum. Comparisons of EC<sub>50</sub> values were made using a two-tailed unpaired t-test. Haloperidol treatment caused a significant decrease in the NPA EC<sub>50</sub> compared to control (\* p < 0.04) whereas no differences were detected between control and clozapine (p > 0.38). Data are given as mean ± s.e.m. (n = 6).

<b>Animal Treatment</b>	<b>NPA Affinity pEC<sub>50</sub> (EC<sub>50</sub>, nM)</b>
CONTROL	8.55 ± 0.21 (35.5)
HALOPERIDOL	9.49 ± 0.18 * (0.324)
CLOZAPINE	9.05 ± 0.28 (1.12)



**Figure 8.** Representative autoradiogram from a ddPCR gel. In this figure, the PCR products are run in groups of 6; each group consisting of 3 replicates of PCR products of control and PLG treated mRNA. From left to right, the groups are the products of the primer combinations of H-T<sub>11</sub>G and arbitrary primers 1, 2 and 3 respectively.

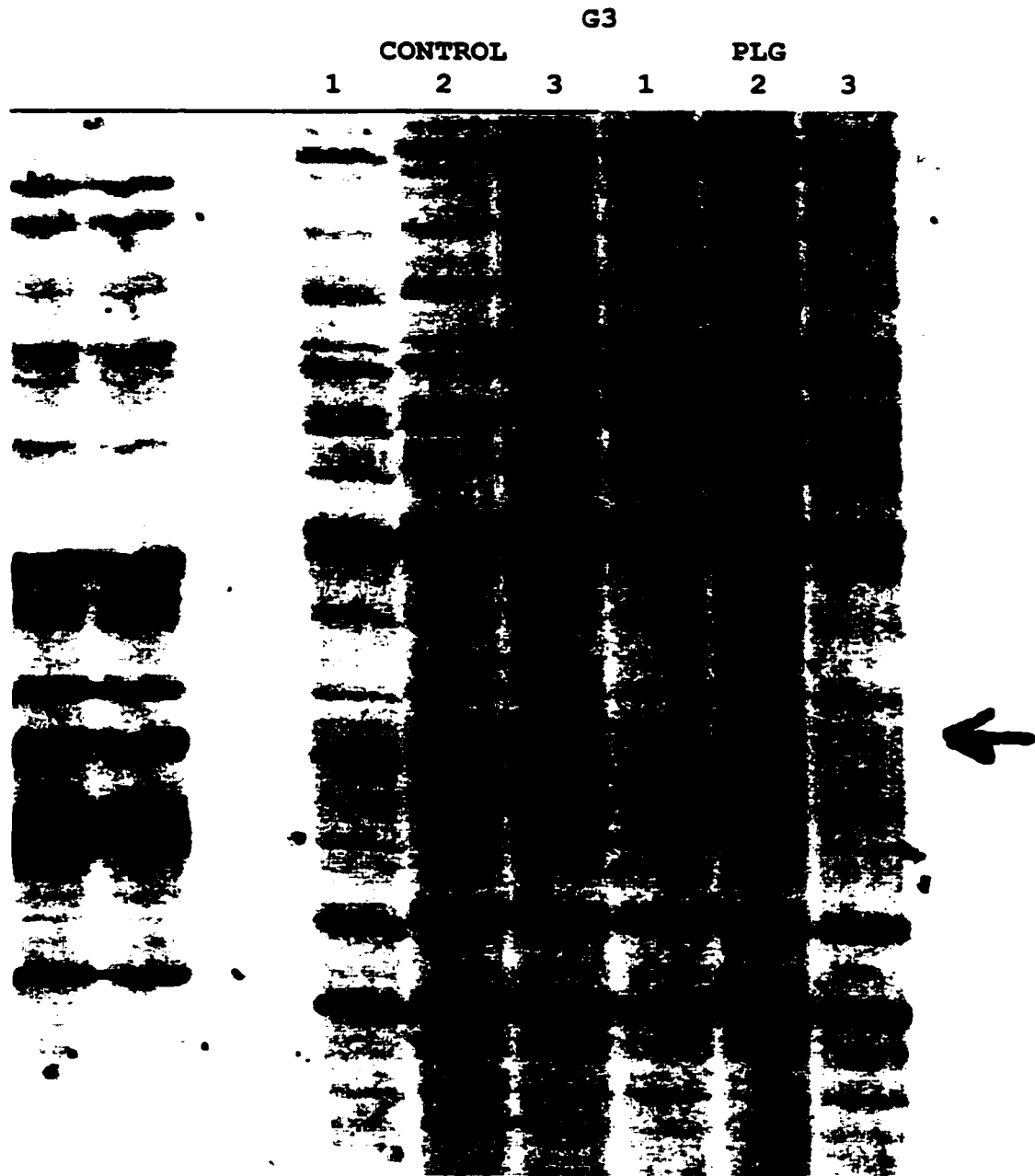


**Figure 9.** Representative autoradiogram from a ddPCR gel. In this figure, the PCR products are run in groups of 6; each group consisting of 3 replicates of PCR products of control and PLG treated mRNA. From left to right, the groups are the products of the primer combinations of H-T<sub>11</sub>G and arbitrary primers 4, 5 and 6 respectively.

Figures 8 and 9 are representative autoradiograms of ddPCR gels. Each Figure shows three groups of six lanes. Each group consists of 3 control lanes and 3 PLG lanes. Each of the replicate lanes were from separate PCR reactions. The ddPCR reactions were conducted on total RNA isolated from control and PLG treated rat striatum. The reproducibility of the cDNA fragments generated during the PCR reactions is evident. Similarly, a certain degree of variability resulting from the PCR reaction can also be observed.

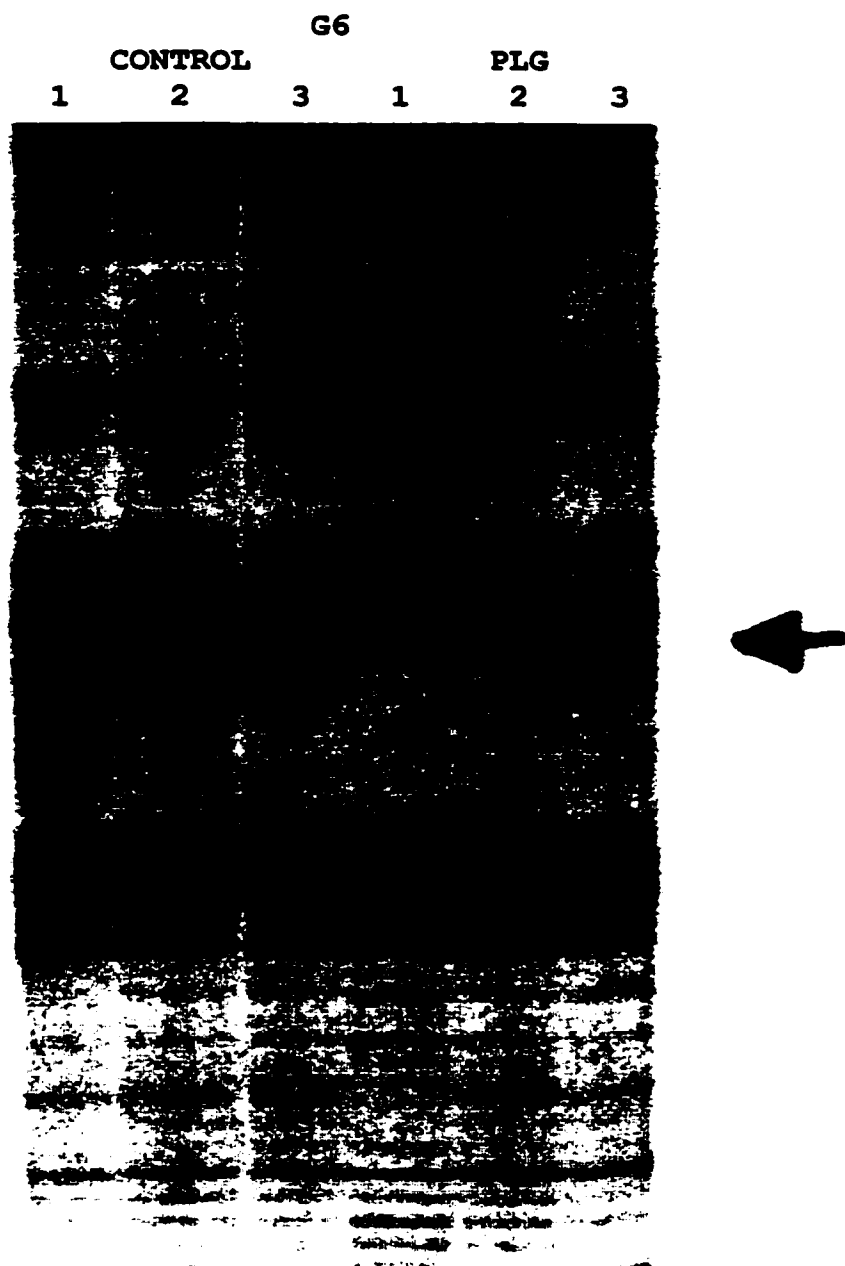
Figures 10 and 11 show a close view of bands that are consistently down regulated in the PLG treated animals. These bands represent good candidates for further investigation. Three candidate bands were identified, resulting from the combination of H-T<sub>11</sub>G primer and arbitrary primers #3 (Figures 8 and 10) #6 (Figures 9 and 11) as well as H-T<sub>11</sub>C and arbitrary primer #1. All of the candidate bands appear to be down-regulated as a result of PLG treatment.

The differentially regulated bands were recovered from acrylamide sequencing gels and reamplified by PCR. The reamplified cDNA fragments were then separated on a 1.7% agarose gel along with a 100 bp molecular weight marker. The approximate sizes of the three bands were determined to be  $\approx$  130, 170 and 210 base pairs (Figure 12). The bands were recovered from the agarose gels using the QIAEX II method and the purified cDNA were then cloned into the pCR-TRAP cloning system. Transformation of competent GH cells (included with pCR-TRAP kit) with the modified vector was then performed. The transformed cells were plated on LB plates containing 20  $\mu$ g/ml tetracycline and allowed to grow overnight at 37°C. Following the overnight culture, several colonies were observed and ten from each plate were chosen for confirmation of cDNA insert by *Hind*III restriction fragment analysis. Figure 13 shows that *Hind*III digestion of the pCR-TRAP vector produces four DNA fragments ( $\approx$  4 kb, 2 kb, 280 bp and 264 bp) plus a fragment corresponding to the inserted DNA (variable length).

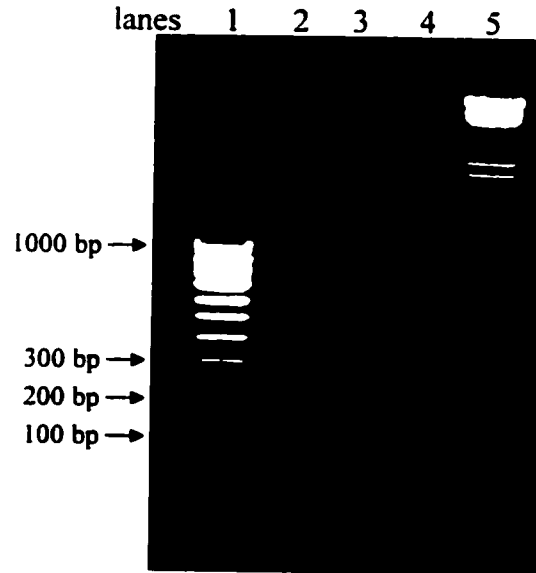


**Figure 10.** Closer view of differentially regulated PCR fragment displayed in Figure 8. It can be seen that the indicated band is absent in the three PLG lanes.

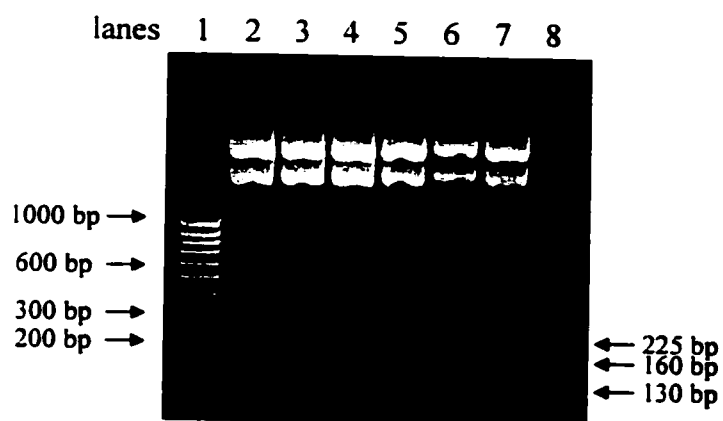




**Figure 11.** Closer view of differentially regulated PCR fragment displayed in Figure 9. It can be seen that the indicated band is absent in the three PLG lanes.



**Figure 12.** Reamplification of PLG down-regulated ddPCR products recovered from sequencing gels. The figure shows molecular weight markers in lanes 1 (100 bp ladder) and 5 ( $\lambda$  DNA/HindIII) as well as PCR products of three down-regulated bands recovered from a sequencing gel. Lanes 2, 3 and 4 correspond to RNAimage primer combinations H-T<sub>11</sub>C & H-AP1, H-T<sub>11</sub>G & H-AP6 and H-T<sub>11</sub>G & H-AP3 respectively.



**Figure 13.** HindIII restriction enzyme digestion of ddPCR fragments cloned into the pCR-TRAP vector. The figure shows that pCR-TRAP vector is cut into 4 fragments and that the inserted ddPCR fragment can be isolated from the gel by recovering the appropriate DNA fragment. Lane 1, 100 bp DNA ladder; Lane 2 & 3, *PRG1*; Lane 4, *PRG5*; Lane 5, *PRG6*; Lane 6 & 7, *PRG4*; lane 8,  $\beta$ -actin (PCR product).

### 4.3.2 Sequence Analysis of ddPCR Products

Sequencing of the cloned ddPCR products was performed at MOBIX. From each cloning, ten colonies were selected for plasmid isolation. From the 10 plasmid samples, a minimum of three were sequenced and the DNA sequences were compared. A total of 6 unique gene sequences were obtained, and the resultant sequences are provided in Table 4. The sequence information was then compared to gene sequences in GenBank using the Blast search program (<http://www.ncbi.nlm.nih.gov/blast/>). The cDNAs obtained did not possess significant homology to any rat gene sequences contained in GenBank. However, *PRG1* and *PRG3* displayed significant homology to non-rat gene sequences (Table 5).

### 4.4 RACE-PCR Analysis of PLG Regulated Genes

Three cDNAs were chosen for full length cloning by RACE-PCR; *PRG1*, *PRG3* and *PRG4*. RACE-PCR was performed on Marathon-Ready rat brain cDNA using the Marathon™ cDNA Amplification kit from Clontech (Scheme 3). Gene specific primers (primary and nested) were synthesised at MOBIX. An initial RACE-PCR was performed on the cDNA library followed by a second nested PCR amplification of an aliquot of the diluted initial PCR product. Typically, the initial RACE-PCR reaction produced a few identifiable bands accompanied by smear. The subsequent nested PCR produced definite bands that were recovered from the agarose gels, reamplified and sequenced at MOBIX. The PCR products were cloned and sequenced at MOBIX.

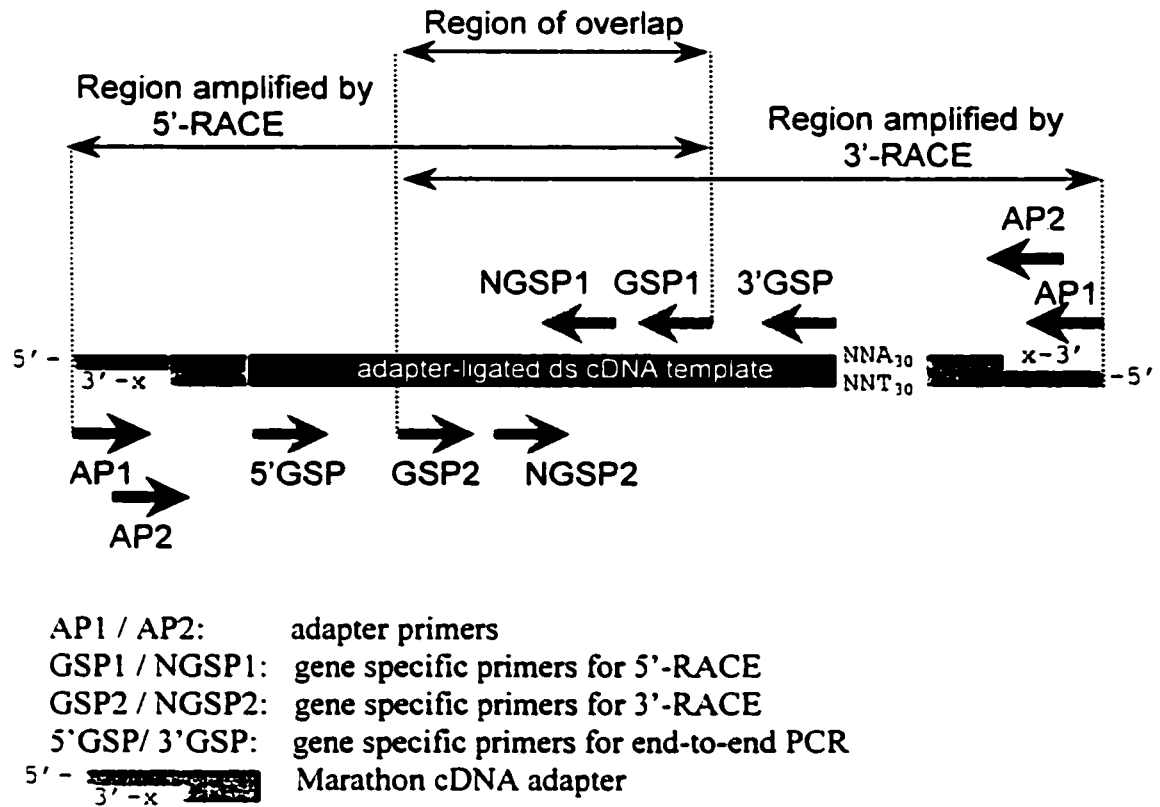
As shown in Table 5, *PRG1* and *PRG3* displayed good homology with SAP155 and Protein L respectively. *PRG4* however, did not possess significant homology with any genes present in GenBank. The objective of the RACE-PCR were as follows: 1/ to confirm the sequence homology of *PRG1* and *PRG3* with SAP155 and Protein L respectively, 2/ to obtain full length clones of the PLG regulated genes, 3/ generate larger sequences for use in northern hybridisation. and 4/ to possibly gain more useful information about the sequence of *PRG4*.

<i>ddPCR Clone</i>	<i>Length (bp)</i>	<i>Sequence (plus strand, 5' → 3')</i>
<b><i>PRG1</i></b>	225	AAGCTTTGGTCAGAAGAAGCCTGGATATCATGCCCTGTGGCAT TGCTTAATGATATACCACAGTCAACAGAACAGTATGATCCATTC GCTGAGCATCGTCCTCCAAAGATTGCAGACCGGGAAGATGAATA TAAAAGCATAGGCGGACCATGATAATTTCCCCAGAGCGTCTTG ATCCTTTTGCAGATGGAGGGAAGACCCCTGATCCCCAAAAA AGCTT
<b><i>PRG2</i></b>	228	AAGCTTTGGTCAGTCCCCAGTAAAATAACAAACAAATAAACTCA TGGAGAGAGGACAGGGCAGGCAATCTCTATGAATTCAGGCCT ACCTTGTCTACCTAATGAGTTCGAGACAGCCAGAGCTACACAG TGAGATCGTATCTCTGGGAGAAAAATAAAAAAAAAATAAAAA ATAAAAAAACATCAACTCCCCAAAAACAAGACTACAAAAA AAAAGCTT
<b><i>PRG3</i></b>	224	AAGCTTTGGTCAGTAGAGGCTTAACCACAACAAATGCTGGAAC CTGGAGGGGGGAGGGGAACTGGTATCTCCCAAGATTAACCTTC ACTTTTAAAATAACTGTATGTGTGATTCTTTTTTCTGTTC TACATTTGTGCTGCCCATGTATCTTGGCACATTTCAATAAAAT GTTTGAAAAATAAACTATTTCGCTGGGGCTCCCCAAAAA GCTT
<b><i>PRG4</i></b>	133	AAGCTTGATTGCCACACACCCTCTCTCAGAGGAGAGCACCTGA GTGCAGTTATTGGATGAAGGCCTGGCTCTCCCTTTCAATATACT GCTTGCTGTCTCCAATAAAGTTTTACCTTGAAAAAAAAAAGCT T
<b><i>PRG5</i></b>	166	AAGCTTGCACCATATAAAGAAACCTAGCACAGTGACACATACTT AGAATCCCAGAATTCAGGATCAGAAGTTTAAGGCCATCCTTGAC TTTATAGCAAACCTCAAGGCTAGCCTGAATTACACAAGACCATGT GTCTTGTCCCCACACCCCCAAAAAAGCTT
<b><i>PRG6</i></b>	166	AAGCTTGCACCATACAGAAAGAAAGTGTAAATCTCCTTGCAAT GAAACCAAAAGACACACAAACATAATTTACCTCTAACAAATGAA CATAACAGGAAGCAACAATCACTATTCCTTAATATCTCTCAACA TCAATGGACTCAATCCCCAAAAAAGCTT

**Table 4.** Sequence information for *PLG* down-regulated cDNAs isolated using ddPCR. Underlined regions indicate polyadenylation signals.

<b>ddPCR Clone</b>	<b>Clone length (bp)</b>	<b>Match length (bp)</b>	<b>Score/Expect</b>	<b>Gene Sequence Match (length bp) (accession #)</b>
<b><i>PRG1</i></b>	225	197 / 205; 96%	335 / $2e^{-90}$	Homo sapiens spliceosomal protein SAP155 mRNA (4259 bp) (AF054284)
<b><i>PRG2</i></b>	228	58 / 68; 85%	56 / $3e^{-6}$	Mus musculus semaF cytoplasmic domain associated protein 1 (Semcap1) (1605 bp) (AF061263)
<b><i>PRG3</i></b>	224	168 / 187; 89% <sup>†</sup>	234 / $6e^{-60}$	Mus musculus mRNA for protein L, partial cds (958 bp) (AB009392)
<b><i>PRG4</i></b>	133	18 / 18; 100%	36.2 / 1.7	Schistosoma mansoni annexin mRNA (1245 bp) (AF065599)
<b><i>PRG5</i></b>	166	27 / 27; 100%	54 / $9e^{-6}$	Mus musculus cosmid MPMGc121L12287 (46872 bp) (AF073797)
<b><i>PRG6</i></b>	166	88 / 97; 90%	121 / $5e^{-26}$	Mus musculus MHC locus class II region a chain (245439 bp) (AF050157)

**Table 5.** Summary of GenBank search (conducted Jun. 7, 1999) information for *PLG* regulated gene sequences (*PRG1* - *PRG6*) obtained using *ddPCR*. All of the identified *ddPCR* fragments were down-regulated as a result of *PLG* treatment. Table provides the length of the *ddPCR* fragments obtained as well as the match information (length and percentage of match, score and expect values) for the best match reported by BLASTN 2.0.8. The best matches are given along with their GenBank accession numbers. <sup>†</sup> indicates match had gaps = 3/187 (1%).



**Scheme 3.** Clontech Marathon RACE-PCR method.

#### 4.4.1 RACE-PCR Analysis of *PRG1*

The ddPCR fragment *PRG1* matched well with a human gene present in GenBank (Homo sapiens spliceosomal protein SAP155 mRNA, 4259 bp; AF054284, (Wang *et al.* 1998)). Initial and nested primers for the 5' and 3' RACE reactions were designed near the 3' and 5' ends of *PRG1* respectively (Table 6). This was done so that the sequence of the RACE PCR product would overlap with the sequence of *PRG1*. Thus, the overlapping sequences could confirm that the RACE PCR product corresponded to the sequence of *PRG1*. The 5'-RACE PCR reaction for *PRG1* provided a product of  $\approx$  500 bp while the initial 3'-RACE product was  $\approx$  1.8 kb (Figure 14). These results suggested that *PRG1* was not located at the 3' end of the cDNA, and were consistent with the region of identity with SAP155 (Figure 15). Figure 15 compares the identity of the combined RACE-PCR nucleotide sequences for *PRG1* to that of the four genes in GenBank that displayed the highest homology with *PRG1*. The overall homology for the genes were 92% (1367/1474 identities), 98% (386/392), 95% (409/428), and 82% (651/792) for SAP155, rat PMSG-induced ovarian mRNA, Homo sapiens full-length insert cDNA, and Xenopus laevis mRNA for 146 kDa nuclear protein respectively.

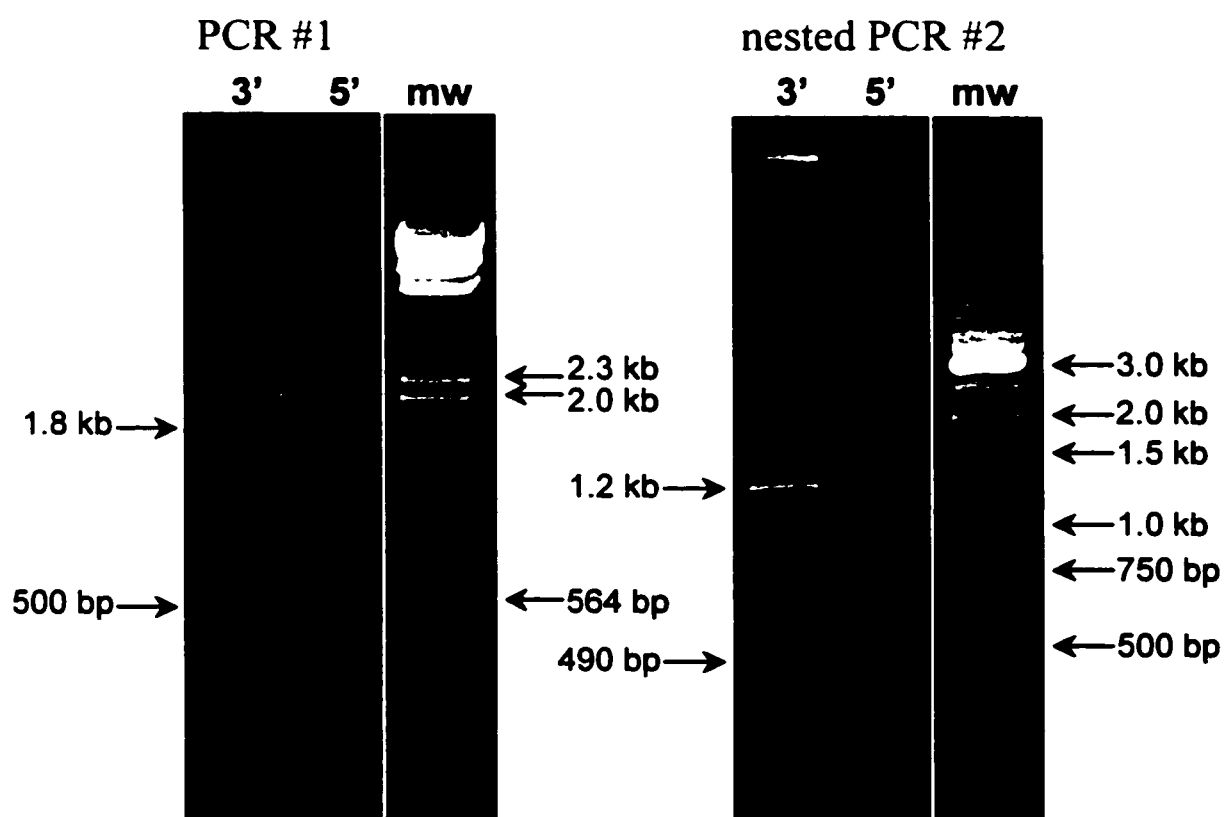
Figure 16 provides an analysis of the open reading frame for *PRG1* (GenBank accession number AF260435). Putative initiation (ATG) and termination codons (TGA) were found at 13 and 985 bp respectively. The amino acid sequence for *PRG1* is also provided in Figure 16. The resultant translation product is 324 aa in length with an expected mass of 35.7 kDa. Figure 17 is an analysis of the two amino acid sequences with the greatest homology to *PRG1* in GenBank, SAP155 and Xenopus laevis 146kDa nuclear protein. SAP155 has an overall homology with *PRG1* of 99% (316/317 identities) and the Xenopus laevis 146kDa nuclear protein has an overall homology of 90% (285/322 identities).

#### 4.4.2 RACE-PCR Analysis of *PRG3*



Gene	Direction	Region / Tm	Sequence
<b><i>PRG1</i></b>	5' GSP	200 – 179 / 66°	5' -GTCTTCCCTCCATCTGCAAA-3'
	5' NGSP	185 – 164 / 66°	5' -GCAAAAGGATCAAGACGCTCTG-3'
	3' GSP	3 - 25 / 68°	5' -GCTTTGGTCAGAAGAACCTGGAT-3'
	3' NGSP	23 – 45 / 70°	5' -GATATCATGCCCTGTGGCATTG-3'
<b><i>PRG3</i></b>	5' GSP	160 – 140 / 66°	5' -GCCAAGATACATGGGCAGCAC-3'
	5' NGSP	90 – 67 / 68°	5' -GTGAAGGTTAATCTTGGGAGATAC-3'
	5' GSP#2	47 – 25 / 66°	5' -CAGAGTTCCAGCATTTGTTGTG-3'
	5' NGSP#2	26 - 3 / 68°	5' -GGTTAAGCCTCTACTGACCAAAGC-3'
	3' GSP	3 - 26 / 68°	5' -GCTTTGGTCAGTAGAGGCTTAACC-3'
	3' NGSP	25 - 47 / 66°	5' -CACAACAAATGCTGGAACCTCG-3'
<b><i>PRG4</i></b>	5' GSP	101 - 80 / 64°	5' -GGAGACAGCAAGCAGTATATTG-3'
	5' NGSP	75 - 56 / 70°	5' -GGAGAGCCAGGCCTTCATCC-3'
	3' GSP	3 - 24 / 70°	5' -GCTTGATTGCCACACACCCTC-3'
	3' NGSP	30 - 50 / 68°	5' -GAGGAGAGCACCTGAGTGCAG-3'

**Table 6.** Table of primers used in RACE-PCR reactions. Gene specific (GSP) and nested gene specific (NGSP) primers were designed for both the 5' and 3' RACE reactions. Primers were designed for three of the genes identified by ddPCR (*PRG1*, *PRG3* and *PRG4*). The Table provides the sequences and melting temperatures (Tm) of the primers, as well as the regions of the PLG regulated genes to which they correspond. Tm was calculated using the following equation:  $T_m = (((A+T)*2) + ((G+C)*4))$ .



**Figure 14.** Agarose gel (1.2%) electrophoresis of *PRGI* RACE-PCR products (5'- and 3'- RACE-PCR reactions). The figure shows that 5'-RACE-PCR reaction resulted in a major band of  $\approx 500$  bp in length, while the 3'-RACE-PCR reaction produced a major band of  $\approx 1.4$  kb. The bands were visible in both the primary and nested PCR reactions.

## Sequences producing significant alignments:

			Score	E
			(bits)	Value
gb AF054284 AF054284	Homo sapiens spliceosomal protein SAP ...	2044	0.0	
dbj D84487 RATN10	Rat PMSG-induced ovarian mRNA, 3' sequence...	728	0.0	
gb AF086296 HUMZD50B08	Homo sapiens full length insert cDNA...	698	0.0	
emb Y08997 XLNUCB2PR	Xenopus laevis mRNA for 146 kDa nuclea...	422	e-115	

<b>PRG1</b>	1	cgagtggacaaaatggcgaagatcgccaagactcacgaagatatcgaagcacagattcga	60
AF054284	1	[REDACTED]	48
AF086296	4	[REDACTED]	63
Y08997	83	[REDACTED]	135
<b>PRG1</b>	61	gaaattcaaggcaagaaggcagctcttgatgaagcccaaggagtgggccttgattccaca	120
AF054284	49	[REDACTED]	108
AF086296	64	[REDACTED]	123
Y08997	136	[REDACTED]	195
<b>PRG1</b>	121	ggttattatgaccaagaaatttatggtggaagtgatagcaggttgctggatattgagaca	180
AF054284	109	[REDACTED]	168
AF086296	124	[REDACTED]	183
Y08997	196	[REDACTED]	255
<b>PRG1</b>	181	tcaattgctgcgactgaacttgaagatgatgacgatgactactcatcatccactagcttg	240
AF054284	169	[REDACTED]	228
AF086296	184	[REDACTED]	243
Y08997	256	[REDACTED]	257
<b>PRG1</b>	241	cttggtcagaagaagcctggatcatgcccctgtggcattgcttaatgatataccacag	300
AF054284	229	[REDACTED]	288
AF086296	244	[REDACTED]	303
Y08997	355	[REDACTED]	369
<b>PRG1</b>	301	tcaacagaacagtatgatccattcgtgagcatcgtcctccaagattgcagaccgggaa	360
AF054284	289	[REDACTED]	348
AF086296	304	[REDACTED]	363
Y08997	370	[REDACTED]	429
<b>PRG1</b>	361	gatgaatataaaaagcataggcggaccatgataatttccccagagcgtcttgatcctttt	420
AF054284	349	[REDACTED]	408
AF086296	364	[REDACTED]	423
Y08997	430	[REDACTED]	489
<b>PRG1</b>	421	gcagatggagggagaagaccctgatccaaaaatgaatgctagaacctacatggatggtatg	480
AF054284	409	[REDACTED]	468
AF086296	424	[REDACTED]	431
Y08997	490	[REDACTED]	549
<b>PRG1</b>	481	cgagaacaacatttgactaaggaagagagagaattaggcaacaactagcagaaaaagct	540
AF054284	469	[REDACTED]	528
Y08997	550	[REDACTED]	609
<b>PRG1</b>	541	aaagctggagaactaaaagtgtcaacggagcagcagcatcccagcctccctcaaaacga	600
AF054284	529	[REDACTED]	588
Y08997	610	[REDACTED]	629
<b>PRG1</b>	601	aaacggcgtgggatcaaactgctgaccagactcctggtgccactccccaaaaagctgtca	660
AF054284	589	[REDACTED]	648



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PRG1      1321 cacctttgggcggtatgactggttttcacatgcaaactgaagacagaactatgaaaagtg 1380
AF054284   1307 ██████████t████████████████████c████████████████████tc████████████████████ 1366
D84487     168 ████████████████████████████████████████████████████████████████████████████ 109
Y08997     1403 ██████a█████a████t████████g████c████t████c████a████g████t████t████c██████████ 1462

PRG1      1381 tcaacgaccagccatctgggaatcttccatttctaaaacctgatgacattcagtactttg 1440
AF054284   1367 ██████t████████████████████a████████████████████t████████████████████a██████████ 1426
D84487     108 ████████████████████████████████████████████████████████████████████████████ 49
Y08997     1463 ██████t█████g████a████t████████a████████████████████c████g████████████████t██████████████ 1522

PRG1      1441 acaaactattggttgatgtagatgagtcaacacttagtccagaagaccaaaaaaaaaaaaaa 1500
AF054284   1427 ██████t████████████████████t████████████████████a██████████████████████████████ 1472
D84487     48 ████████████████████████████████████████████████████████████████████████████ 3
Y08997     1523 ██████t████████████████████t████████████████████a██████████████████████████████ 1568

PRG1      1501 aaaaaaaaaaaaaaagcggcgctgaattctagggtttaaactatTTTTGTGTGTTTTATTTA 1560

PRG1      1561 ataataaaatatcaaccattggtggcagacaaaaatagtatcatttttGTCTATGTGAA 1620

PRG1      1621 aggatgtgaagccattttaaggactcactttccttctagaattcagcggccgctgaattc 1680

PRG1      1681 tag 1683

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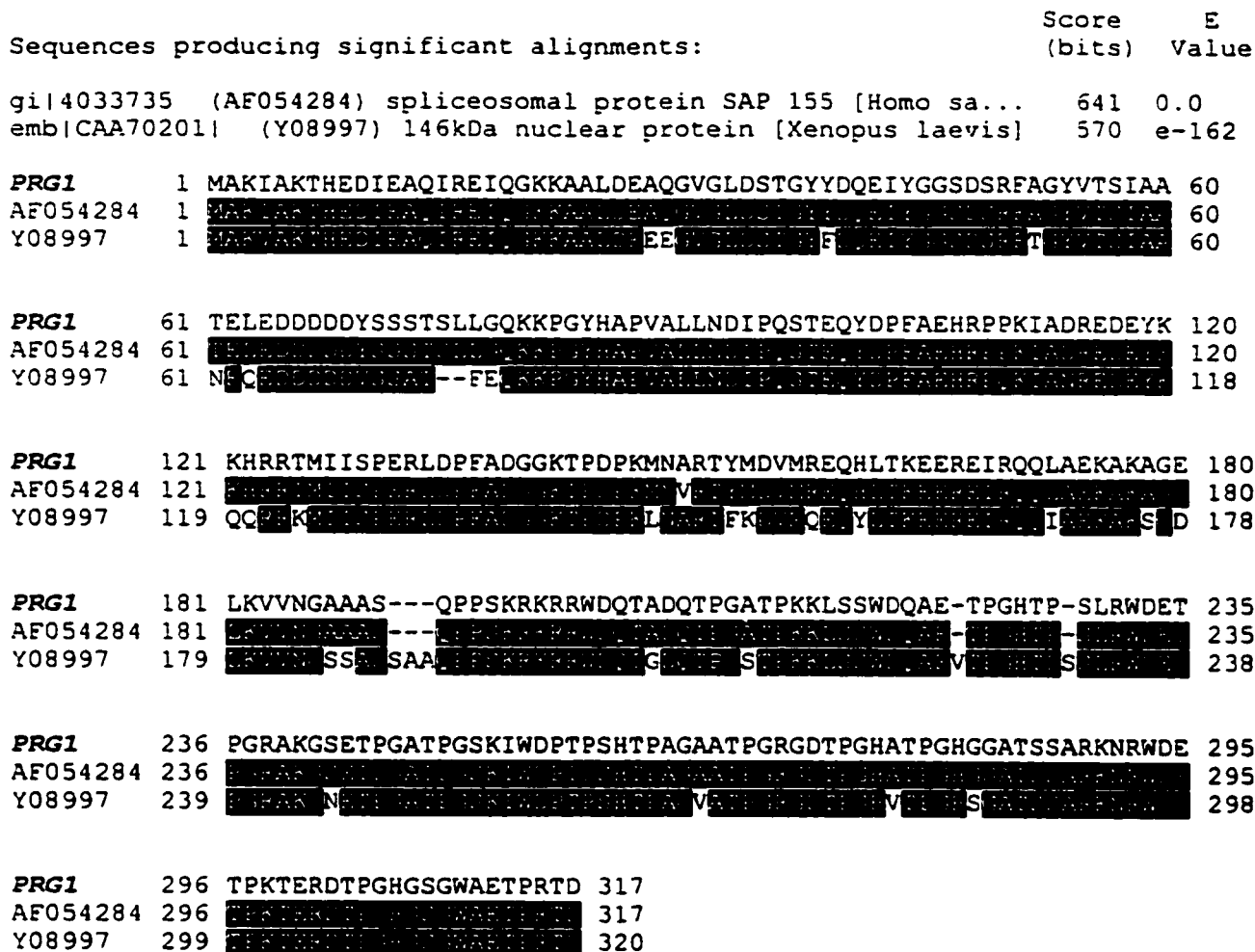
**Figure 15.** Multiple sequence alignment for *PRG1* cDNA with its four nearest matches in GenBank. The sequence identity between *PRG1* and AF054284, D84487, AF086296 and Y08997 are 92%, 98%, 95% and 82% respectively.

```

1 CGAGTGGACA AAATGGCGAA GATCGCCAAG ACTCACGAAG ATATCGAAGC ACAGATTCGA
  M A K I A K T H E D I E A Q I R
61 GAAATTCAAG GCAAGAAGGC AGCTCTTGAT GAAGCCCAAG GAGTGGGCCT TGATTCCACA
  E I Q G K K A A L D E A Q G V G L D S T
121 GGTATTATG ACCAAGAAAT TTATGGTGGG AGTGATAGCA GGTTTGCTGG ATATGTGACA
  G Y Y D Q E I Y G G S D S R F A G Y V T
181 TCAATTGCTG CGACTGAACT TGAAGATGAT GACGATGACT ACTCATCATC CACTAGCTTG
  S I A A T E L E D D D D D Y S S S T S L
241 CTTGGTCAGA AGAAGCCTGG ATATCATGCC CCTGTGGCAT TGCTTAATGA TATACCACAG
  L G Q K K P G Y H A P V A L L N D I P Q
301 TCAACAGAAC AGTATGATCC ATTCGCTGAG CATCGTCCTC CAAAGATTGC AGACCGGGAA
  S T E Q Y D P F A E H R P P K I A D R E
361 GATGAATATA AAAAGCATAG GCGGACCATG ATAATTTCCC CAGAGCGTCT TGATCCTTTT
  D E Y K K H R R T M I I S P E R L D P F
421 GCAGATGGAG GGAAGACCCC TGATCCAAAA ATGAATGCTA GAACCTACAT GGATGTTATG
  A D G G K T P D P K M N A R T Y M D V M
481 CGAGAACAAC ATTTGACTAA GGAAGAGAGA GAAATTAGGC AACAACTAGC AGAAAAAGCT
  R E Q H L T K E E R E I R Q Q L A E K A
541 AAAGCTGGAG AACTAAAAGT TGTC AACCGGA GCAGCAGCAT CCCAGCCTCC CTCAAAACGA
  K A G E L K V V N G A A A S Q P P S K R
601 AAACGGCGCT GGGATCAAAC TGCTGACCAG ACTCCTGGTG CCACTCCCAA AAAGCTGTCA
  K R R W D Q T A D Q T P G A T P K K L S
661 AGTTGGGATC AGGCAGAGAC CCCTGGGCAT ACCCCTTCTT TAAGATGGGA TGAGACACCG
  S W D Q A E T P G H T P S L R W D E T P
721 GGTCGTGCAA AAGGAAGTGA AACACCTGGA GCAACCCAG GCTCAAAAAT ATGGGATCCT
  G R A K G S E T P G A T P G S K I W D P
781 ACACCTAGTC ATACACCTGC GGGAGCTGCT ACTCCTGGAC GAGGAGATAC ACCAGGCCAT
  T P S H T P A G A A T P G R G D T P G H
841 GCAACCCCGG GCCATGGCGG TGCAACTTCC AGTGCCCGTA AAAACAGATG GGATGAGACC
  A T P G H G G A T S S A R K N R W D E T
901 CCCAAAACAG AAAGAGATAC TCCTGGGCAT GGAAGTGGGT GGGCTGAGAC TCCTCGAACA
  P K T E R D T P G H G S G W A E T P R T
961 GACCCGAGGT GGAGACTCTA TTGGTGAGAC ACCAACTTCC TGGAGCAAGT AAAAGAAAAGT
  D P R W R L Y W *
1021 TTTCTGTTGGG ACGAAACACC AGCTAGTCAA ATGGGGGAAG CACTCCTGTT CTGACTCCAG
1081 GAAAAACACC AATTGGCACA CCAGCCATGA ACATGGCCAC CCCCACTCCA GGTCACATAA
1141 TGAGCATGAC TCCTGAGCAG CTTCAGGCTT GGCGGTGGGA GAGAGAGATT GATGAGCCGA
1201 ACCGCCCACT TTTTGATGAG GAATTAGATG CTATGTTCCC AGAAGGATAC AAGGTCCTTC
1261 CCCCTCCAGC TGGCTATGTT CCTATTCGAA CTCCAGCTCG AAAGCTGACA GCAACTCCCA
1321 CACCTTTGGG CGGTATGACT GGTTTTTACA TGCAAACTGA AGACAGAACT ATGAAAAGTG
1381 TCAACGACCA GCCATCTGGG AATCTTCCAT TTCTAAAACC TGATGACATT CAGTACTTGT
1441 ACAAACTATT GGTTGATGTA GATGAGTCAA CACTTAGTCC AGAAGACCAA AAAAAAAAAA
1501 AAAAAAAAAA AAAAGCGGCG CTGAATTCTA GGTTTAAACT ATTTTTGTGT GTTTTATTTA
1561 ATAATATAAT ATCAAACCAT TGGTGGCAGA CAAAAATAGT ATCATTTTTG TCTATGTGAA
1621 AGGATGTGAA GCCATTTTAA GGACTCACTT TCCTTCTAGA ATTCAGCGGC CGCTGAATTC
1681 TAG

```

**Figure 16.** Combined 5' and 3' RACE-PCR sequences for *PRGI* with the corresponding protein sequence displayed below the nucleotide sequence. Sequence identity with *PRGI* is indicated by double underlined (nucleotides 242-451). Nucleotides 1 - 423 were generated by 5' RACE-PCR and nucleotides 424 - 1681 were generated by 3' RACE-PCR. Sequence analysis indicates an open reading frame between nucleotides 13 and 985 (initiation and termination codons are underlined). Polyadenylation signals are indicated as inverse text. GenBank accession number AF260435.

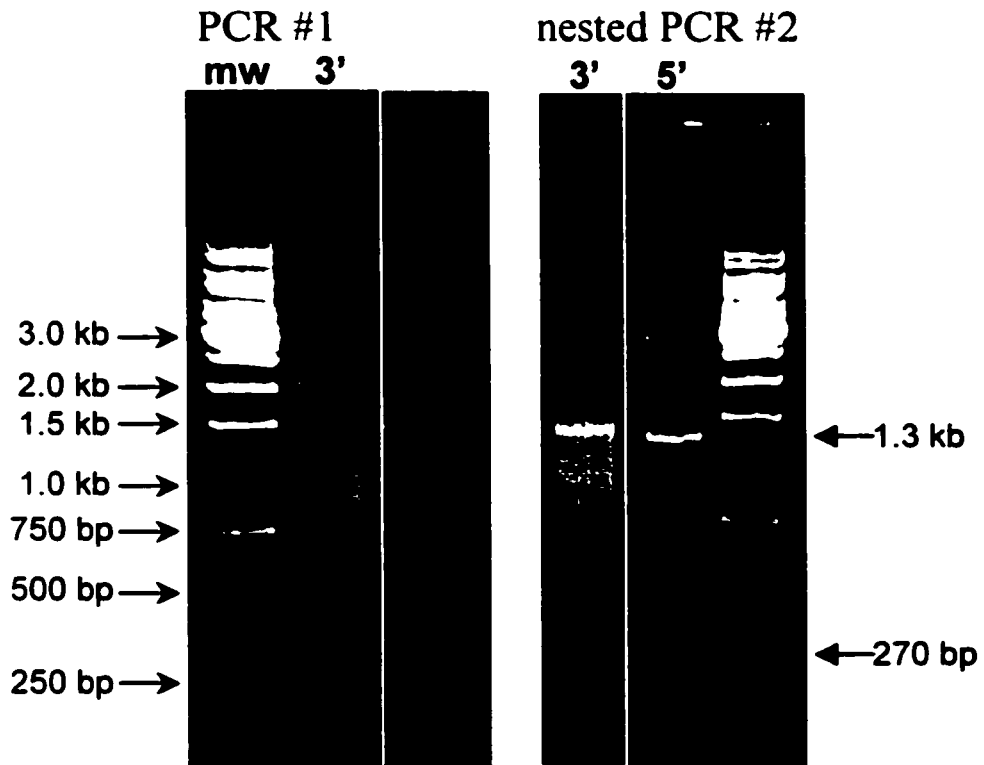


**Figure 17.** Multiple sequence alignment for putative *PRG1* protein. Identities are 99% and 90% for AF054284 and Y08997 respectively.

The ddPCR fragment *PRG3* matched well with a murine gene present in GenBank (Mus musculus mRNA for protein L, partial cds, 958 bp; AB009392, (Piñol-Roma *et al.* 1989)). The next best match is to a 146kDa nuclear protein from *Xenopus laevis*. Initially, 5' and 3' RACE PCR primers were designed in the same manner as described for *PRG1* (Table 6). Using these primers, the 5'-RACE PCR reaction for *PRG3* did not produce a distinct PCR product, despite several attempts at PCR optimization. However, the 3'-RACE PCR reaction produced a product of  $\approx 270$  bp (Figure 18). Because there were no other suitable regions within the cDNA of *PRG3* for designing primers, it was decided that primers corresponding to the same regions used for the 3' RACE reaction would be used for 5' RACE. Using these primers, the 5' RACE reaction produced 6 bands ranging in size from  $\approx 3$  kb to 1.3 kb (Figure 18). These results suggested that *PRG3* was located at the 3' end of the cDNA, and are consistent with the region of identity with Protein L (Figure 19). Figure 19 is an analysis of the nucleotide sequence identity of the combined *PRG3* RACE-PCR product with that of the two genes with the highest homology to *PRG3* in GenBank. The overall homology for the genes were 95% (475/497 identities) and 90% (389/429 identities) for Mus musculus mRNA for protein L, and homo sapiens hnRNP L (protein L) respectively.

Figure 20 provides an analysis of the open reading frame for *PRG3* (GenBank accession number AF260436). A putative termination codon (TAA) was found at 423 bp. Although a possible initiation codon is present at 33 bp, it is not likely to be the beginning of the open reading frame, as the clone of the 5'-RACE-PCR reaction was smaller than the maximal PCR product. Thus, the combined sequence for *PRG3* is not likely to be a full-length clone. The amino acid sequence for *PRG1* is also provided in Figure 20. The resultant translation product is 140 aa in length with an expected mass of  $\approx 15.6$  kDa. Figure 21 is an analysis of the four amino acid sequences with the greatest homology to *PRG1* in GenBank: Mus musculus protein L, Homo sapiens Protein L, Drosophila melanogaster homologous to human hnRNP L, and Caenorhabditis elegans





**Figure 18.** Agarose gel (1.2%) electrophoresis of *PRG3* RACE-PCR products (5'- and 3'- RACE-PCR reactions). The figure shows that 5'-RACE-PCR reaction produced 6 bands ranging in size from  $\approx 3$  kb to 1.3 kb, while the 3'-RACE-PCR reaction produced a major band of  $\approx 270$  bp. The bands were visible in both the primary and nested PCR reactions.

Sequences producing significant alignments:			Score	E
			(bits)	Value
dbj AB009392 AB009392	Mus musculus mRNA for protein L, part...	803	0.0	
ref NM001533.1 HNRPL	Homo sapiens heterogeneous nuclear r...	533	e-149	
<b>PRG3</b>	1	tgaatgtctgtgtctccaagcaaccagccattatgcccgggtcagtcatatgggctagaag	60	
AB009392	177	[REDACTED]	236	
NM001533	1281	[REDACTED]	134	
<b>PRG3</b>	61	acggatcttgcagttacaagacttcagcgagtcagggaacaatcggttctctactccag	120	
AB009392	237	[REDACTED]	296	
NM001533	1341	[REDACTED]	140	
<b>PRG3</b>	121	agcaggcagctaagaaccgcattccagcaccctagcaatgttctgcacttctcaatgctc	180	
AB009392	297	[REDACTED]	356	
NM001533	1401	[REDACTED]	146	
<b>PRG3</b>	181	cctggaggtgactgaggagaacttcttgagatctgcgatgaactgggagtgaagcggc	240	
AB009392	357	[REDACTED]	416	
NM001533	1461	[REDACTED]	152	
<b>PRG3</b>	241	caacctctgtgaaagtatttcaggcaaaagcgagcgtagctcctctgggctgctggagt	300	
AB009392	417	[REDACTED]	476	
NM001533	1521	[REDACTED]	158	
<b>PRG3</b>	301	gggactccaagagtgcagccctggagaccttgggcttctctgaaccattaccagatgaaa	360	
AB009392	477	[REDACTED]	536	
NM001533	1581	[REDACTED]	164	
<b>PRG3</b>	361	acccaatggcccgtatccatacactctgaagttgtgcttctccaccgcacagcagcct	420	
AB009392	537	[REDACTED]	596	
NM001533	1641	[REDACTED]	170	
<b>PRG3</b>	421	cctaattagatgctgggaaggaagactccatccgagcaggaagatgtttcttctctta	480	
AB009392	597	[REDACTED]	655	
NM001533	1701	[REDACTED]	170	
<b>PRG3</b>	481	tgctggtggtttctttggtttgggctttatTTTTTgcagttctTTTTTTccatttaaagc	540	
AB009392	656	[REDACTED]	672	
AB009392	745	[REDACTED]	753	
<b>PRG3</b>	541	taggtcagtagaggcttaaccacaacaaatgctggaactcgcaacaacaaatgctggaact	600	
AB009392	782	[REDACTED]	794	
AB009392	754	[REDACTED]	795	
		\		
NM001533	1330	taggtttgtagaggctta	184	
<b>PRG3</b>	601	ctggagggggggagggggaactggtatctcccaagattaaccttcactttttaaataact	660	
AB009392	795	[REDACTED]	854	
NM001533	1891	[REDACTED]	193	
<b>PRG3</b>	661	gtatgtgtgattctTTTTTctgttcatacatttgtgctgccatgtatcttggcacat	720	
AB009392	955	[REDACTED]	915	
		\		

NM001533 1961 [REDACTED] 200

t  
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i  
c

*PRG3* 721 ttcaataaaaattgtttgaaaaat 743  
 AB009392 916 [REDACTED] 938  
 NM\_001533 2002 [REDACTED] 2024

**Figure 19.** Multiple sequence alignment for *PRG3* cDNA with its two nearest matches in GenBank. The sequence identity between *PRG3* and AB00932 and NM001533 are 95% and 90% respectively.

```

1 TGAATGTCTG TGTCTCCAAG CAACCAGCCA TTATGCCCGG TCAGTCATAT GGGCTAGAAG
   N V C V S K Q P A I M P G Q S Y G L E
61 ACGGATCTTG CAGTTACAAA GACTTCAGCG AGTCAAGGAA CAATCGGTTC TCTACTCCAG
   D G S C S Y K D F S E S R N N R F S T P
121 AGCAGGCAGC TAAGAACCGC ATCCAGCACC CTAGCAATGT TCTGCACTTC TTCAATGCTC
   E Q A A K N R I Q H P S N V L H F F N A
181 CCCTGGAGGT GACTGAGGAG AACTTCTTTG AGATCTGCGA TGAACTGGGA GTGAAGCGGC
   P L E V T E E N F F E I C D E L G V K R
241 CAACCTCTGT GAAAGTATTT TCAGGC AAAA GCGAGCGTAG CTCCTCTGGG CTGCTGGAGT
   P T S V K V F S G K S E R S S S G L L E
301 GGGACTCCAA GAGTGACGCC CTGGAGACCT TGGGCTTCCT GAACCATTAC CAGATGAAAA
   W D S K S D A L E T L G F L N H Y Q M K
361 ACCCAAATGG CCCGTATCCA TACACTCTGA AGTTGTGCTT CTCCACCGCA CAGCAGCCT
   N P N G P Y P Y T L K L C F S T A Q H A
421 CCTAATTAGA TGCTGGGAAG GAAGACTCCA TCCGAGCAGG GAAGATGTTT CTTTCCTTTA
   S
481 TGCTGGTGGT TTCTTTGTTT TGGGCTTTAT TTTTGCAGTT CTTTTTTTCC ATTTAAATGC
541 TAGGTCAGTA GAGGCTTAAC CACAACAAAT GCTGGAACTC GCACAACAAA TGCTGGAACT
601 CTGGAGGGGG GGAGGGGAAC TGGTATCTCC CAAGATTAAC CTTCACTTTT TAAAATAACT
661 GTATGTGTGA TTCTTTTTTT CCTGTTTATA CATTGTGCT GCCCATGTAT CTGGGCACAT
721 TTCA TTCA TTGTTGGAA A TTGTTGGAA AAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
781 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA CAAAAAAAAA AAAAAAAAAA AAAAA

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**Figure 20.** Combined 5' and 3' RACE-PCR sequences for *PRG3* with the corresponding protein sequence below the nucleotide sequence. Sequence identity with *PRG3* is indicated by double underlined (nucleotides 582-747). Nucleotides 1-561 were generated by 5' RACE-PCR and nucleotides 561 - 835 were generated by 3' RACE-PCR. Sequence analysis indicates an open reading frame between nucleotides 1 and 423 (the termination codon is underlined). Polyadenylation signals are indicated as inverse text. GenBank accession number AF260436.

Sequences producing significant alignments:		Score	E
Value		(bits)	
dbj BAA24237	(AB009392) protein L [Mus musculus]	295	9e-80
ref NP_001524.1 PHNRPL	heterogeneous nuclear ribonucleopro...	292	8e-79
emb CAA66282	(X97706) homologous to human hnRNP L [Drosoph...	102	1e-21
gi 861311	(U28928) similar to heterogeneous ribonucleoprote...	98	3e-20
<b>PRG3:</b>	1 NVCVSKQPAIMPQSYGLEDGSCSYKDFESERNRNFSTPEQAAKNRIQHPSNVLHFFNAP	60	
AB009392	60 [REDACTED]	119	
PHNRPL	419 [REDACTED]	478	
X97706	328 [REDACTED] P H P E EYTG K L S A S P K I T	370	
U28928	377 [REDACTED] P P E Y S Q L A A I I F T T W	419	
<b>PRG3:</b>	61 LEVTEENFFEICDELGVKRPTSVKVFSGKSERSSSGLLEWDSKSDALETGLFLNHYQMKN	120	
AB009392	120 [REDACTED]	179	
PHNRPL	479 [REDACTED] S H E P A S K A S D F N N H N	538	
X97706	371 PGL DQLIG FNIKD P A R L P L T I F S N I Q V L A I M K C L P I E G	429	
U28928	420 GTMD QALLDLMA KTEHK VKIDI PSRN A A T A FETVEI N V A L V T P V N S	479	
<b>PRG3:</b>	121 PNGPYPYTLKLCFSTAQHAS	140	
AB009392	180 [REDACTED]	199	
PHNRPL	539 [REDACTED]	558	
X97706	430 KGTKF FIM SSK	446	
U28928	480 Y	482	

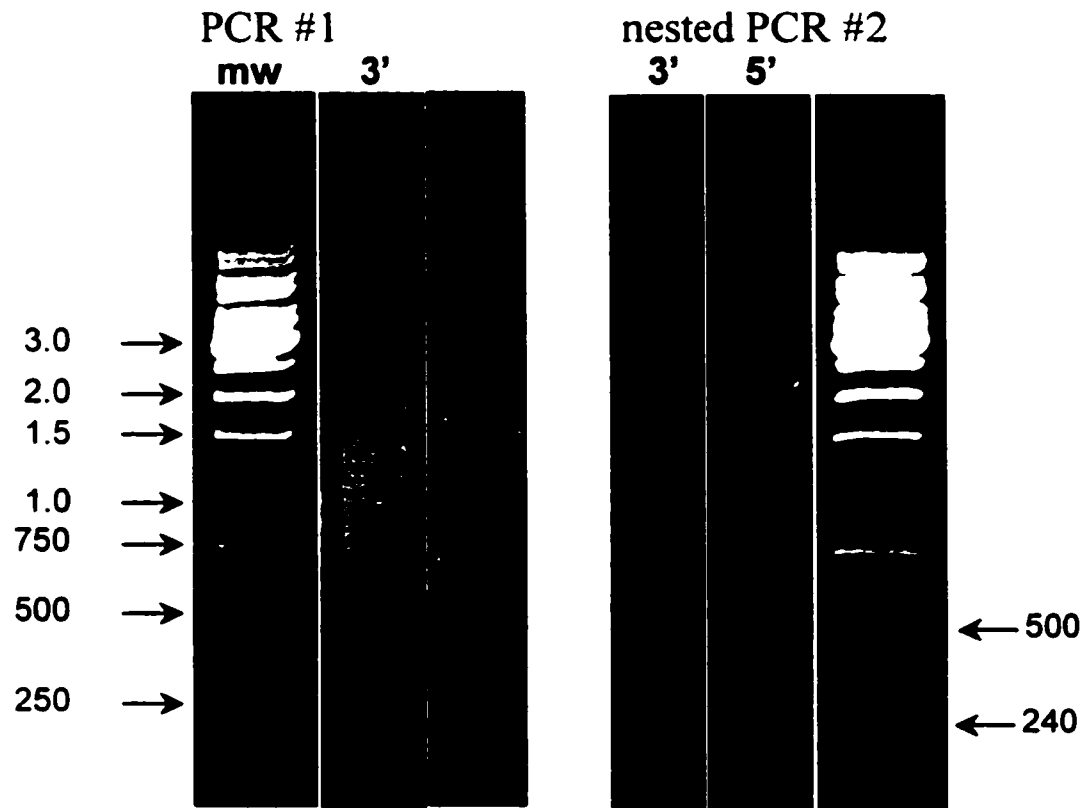
**Figure 21.** Multiple sequence alignment for putative *PRG3* protein translation product. Protein sequence identity between *PRG3* protein and its nearest matches in GenBank; identities are 100%, 98%, 44% and 44% for AB009392, PHNRPL, X97706, U28928 respectively.

similar to heterogeneous ribonucleoprotein L have overall homologies with *PRG3* of 100% (140/140 identities), 98% (138/140 identities), 44% (53/120 identities) and 44% (47/106 identities) respectively.

#### 4.4.3 RACE-PCR Analysis of *PRG4*

The sequence of the ddPCR fragment *PRG4* produced a poor match with the sequences in GenBank (Table 5). This presented the possibility that *PRG4* represented a novel gene. Initial and nested primers for the 5' and 3' RACE reactions were again designed near the 3' and 5' ends of *PRG4* respectively (Table 6); producing RACE PCR products with sequences overlapping that of *PRG4*. The 5'-RACE PCR reaction for *PRG4* provided a product of  $\approx$  550 bp while the 3'-RACE product was  $\approx$  240 bp (Figure 22). These results suggested that *PRG4* was located at the 3' end of the cDNA. Figure 23 compares the identity of the combined RACE-PCR nucleotide sequences for *PRG4* to that of the five genes in GenBank that displayed the highest homology with *PRG4*. The overall homology for the genes were 91% (211/230 identities), 94% (117/124), 89% (57/64), and 89% (57/64) for Human G protein gamma-11 (Ray *et al.* 1995), Human BAC clone GS345D13, human photoreceptor transducin gamma subunit, and human transducin gamma subunit respectively.

Figure 24 provides an analysis of the open reading frame for *PRG4* (GenBank accession number AF257110). Putative initiation (ATG) and termination codons (TAA) were found at 31 and 250 bp respectively. The amino acid sequence for *PRG4* is also provided in Figure 24. The resultant translation product is 73 aa in length with an expected mass of 8.5 kDa. Figure 25 is an analysis of the four amino acid sequences with the greatest homology to *PRG4* in GenBank. Human G protein gamma-11, human G protein gamma-1, Bovine G gamma-1 and PhosducinTransducin G chain. The overall homologies with *PRG1* were 100% (73/73 identities), 75% (53/74), 75% (53/74) and 75% (50/66) for Human G protein gamma-11, human G protein gamma-1, Bovine G



**Figure 22.** Agarose gel (1.2%) electrophoresis of *PRG4* RACE-PCR products (5'- and 3'- RACE-PCR reactions). The figure shows that 5'-RACE-PCR reaction produced a major product of  $\approx 500$  bp, while the 3'-RACE-PCR reaction produced a major product of  $\approx 240$  bp. The bands were visible in both the primary and nested PCR reactions.





```

1 GTGACTTCTG GGCCCAGCTC TGGGGCAAAG ATGCCCGCCC TTCACATTGA GGATCTGCCG
      M P A L H I E D L P
61 GAAAAGGAAA AACTGAAGAT GGAGGTTGAG CAACTTCGCA AAGAAGTGAA GTTGCAGAGA
      E K E K L K M E V E Q L R K E V K L Q R
121 CAACAGGTGT CTAAATGTTT TGAGGAAATA AAGAACTACA TTGAAGAACG TTCTGGAGAG
      Q Q V S K C S E E I K N Y I E E R S G E
181 GATCCTCTGG TAAAGGGAAT TCCAGAAGAC AAGAATCCCT TCAAAGAAAA GGGCAGCTGT
      D P L V K G I P E D K N P F K E K G S C
241 GTCATTTCTT AAGGAACTCT GGAGGGAAAT TGTTTTTCAGT TGGGTCAGAT TGTTCTTTTG
      V I S *
301 TTTAATTTTT CCCCCAAATG AAGCCAAAGT GTGTGTGAAG AATTGAGGAA AAATGAAATC
361 GAGAAAAAGA CTGTCATATA AGCACTCTCC AAGCACTTTG TGCATAGAAC AAACCTGCTT
421 CTCACCCACA CACCCTCTCT CAGAGGAGAG CACCTGAGTG CAGTTATTGG ATGAAGGCCT
481 GGCTCTCCCT TTCAATATAC TGCTTGCTGT CTCCA CTCCA GTTTTACCTC GAAAAAAAAA
541 AAAAAAAAAA AAAAAAAGCG GCCGCTGAAT TCTAGGCCGC TTTTTTTTTT TTTTTTTTTT
601 TTTTTTTTC

```

**Figure 24.** Combined 5' and 3' RACE-PCR sequences for *PRG4* with the corresponding protein sequence below the nucleotide sequence. Sequence identity with *PRG4* is indicated by double underlined (nucleotides 425-542). Nucleotides 1 - 442 were generated by 5' RACE-PCR and nucleotides 443 - 609 were generated by 3' RACE-PCR. Sequence analysis indicates an open reading frame between nucleotides 31 and 250 (initiation and termination codons are underlined). Polyadenylation signal is indicated as inverse text. GenBank accession number AF257110.



gamma-1 and Phosducin/Transducin G chain respectively.

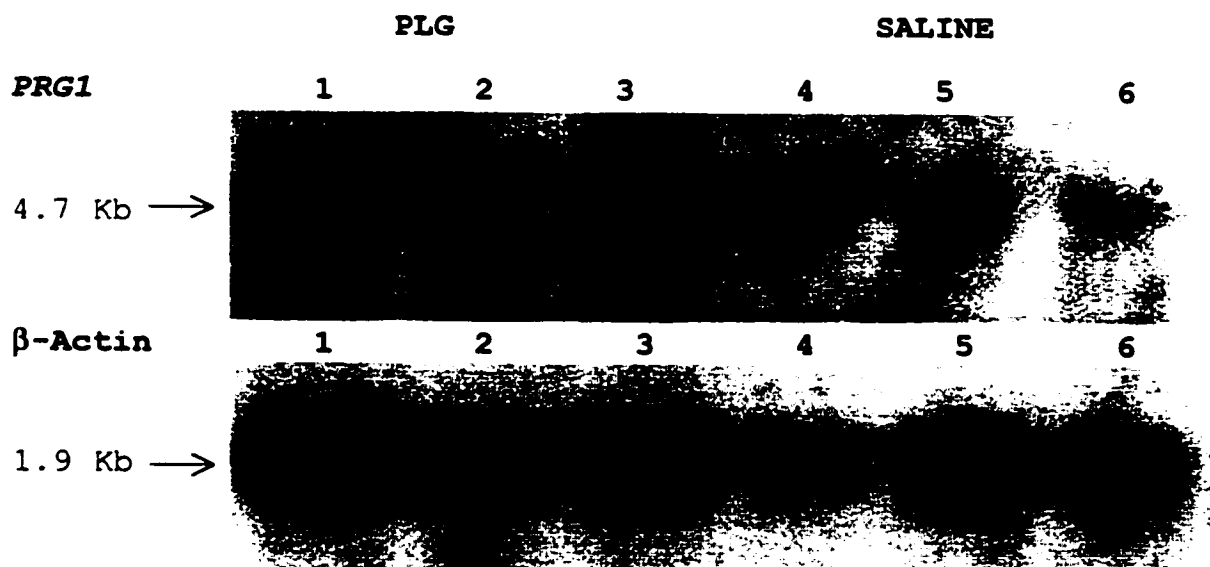
#### **4.5 Northern Hybridisation Analysis of *PRG* Expression**

##### **4.5.1 Analysis of *PRG1* Expression by Northern Hybridisation**

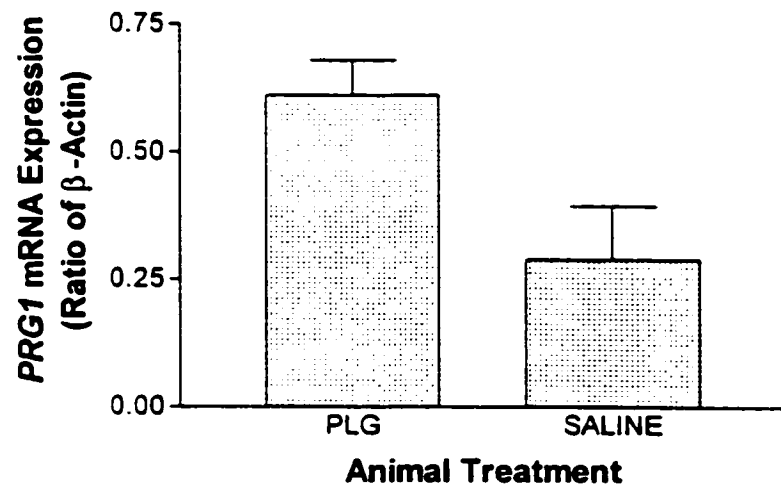
*PRG1* is highly homologous with the nucleotide sequence of the human SAP155 cDNA (Figure 15) and the putative translation product is also highly homologous with that of the SAP155 protein (Figure 18). A 4259 bp cDNA clone of human SAP155 has been identified (Wang *et al.* 1998) with a protein product that displays significant homology to the corresponding protein found in lower organisms (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Caenorhabditis elegans*). The partial rat clone identified here is 1683 bp in length and hybridises with a mRNA species of 4.7 kb (Figure 26). Figure 26 shows that a weak signal was detected using the 3' RACE-PCR product as a probe. The probe corresponded to nucleotides 424-1681 (Figure 16), and contained a substantial part of the coding region, as well as the 3' non-coding region. Analysis of the expression levels, relative to that of  $\beta$ -Actin, revealed that the gene was upregulated by  $\approx$  54% following PLG treatment (Figure 27).

##### **4.5.2 Analysis of *PRG3* Expression by Northern Hybridisation**

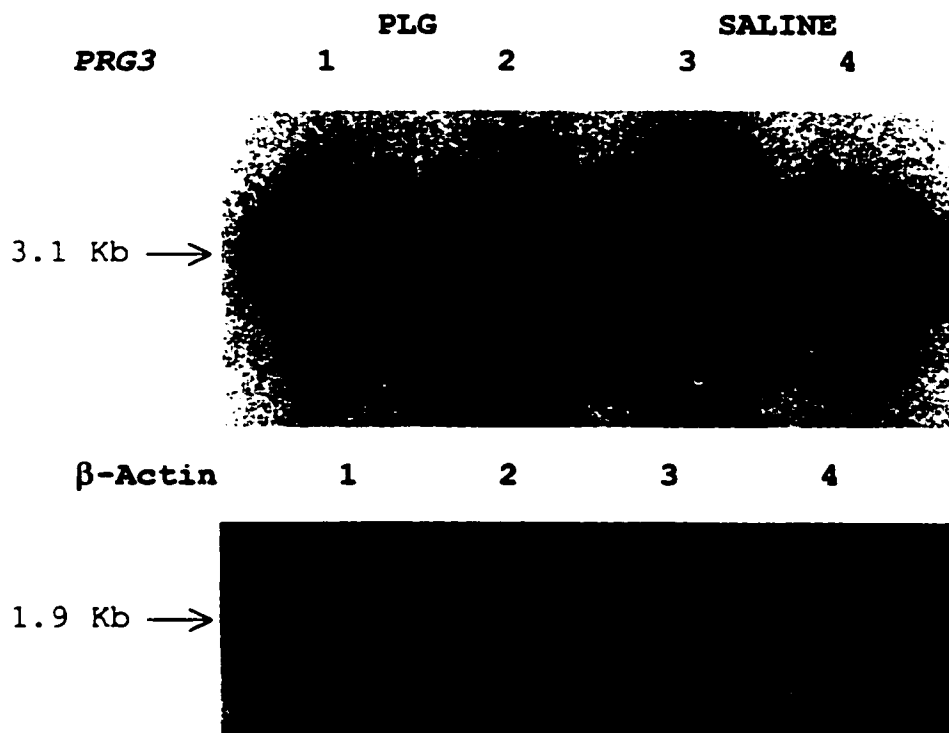
*PRG3* is highly homologous with the nucleotide sequence of the human protein L cDNA (Figure 19), and the putative translation product is 100% and 99% match with that of murine and human protein L respectively (Figure 21). The partial rat protein L cDNA clone is 958 bp in length, while the full length human cDNA clone is 2033 bp. Northern blot analysis of human protein L expression resulted in the detection of a single mRNA species of 2.3 kb (Piñol-Roma *et al.* 1989). The partial rat clone identified here is 835 bp in length and hybridises with a single mRNA species of 3.1 kb (Figure 28). Figure 28 shows that a strong signal was detected using the 5' RACE-PCR product as a probe. The



**Figure 26.** Northern blot of *PRG1* and  $\beta$ -actin in rat striatal total RNA (10  $\mu$ g per lane). Total RNA was isolated from the striata of PLG (28 days with 20 mg/kg, i.p.) and Saline (28 days with 1 ml/kg, i.p.) treated animals.



**Figure 27.** Graph of *PRG1* mRNA expression in the rat striatum following treatment with PLG (20 mg/kg i.p.) or Saline (1 ml/kg, i.p.). Results were determined in triplicate ( $\pm$  S.E.M.) and are expressed as a ratio of  $\beta$ -Actin expression.



**Figure 28.** Northern blot of *PRG3* and  $\beta$ -actin in rat striatal mRNA (1  $\mu$ g per lane). Poly A<sup>+</sup> mRNA was isolated from the striata of PLG (28 days with 20 mg/kg, i.p.) and Saline (28 days with 1 ml/kg, i.p.) treated animals.

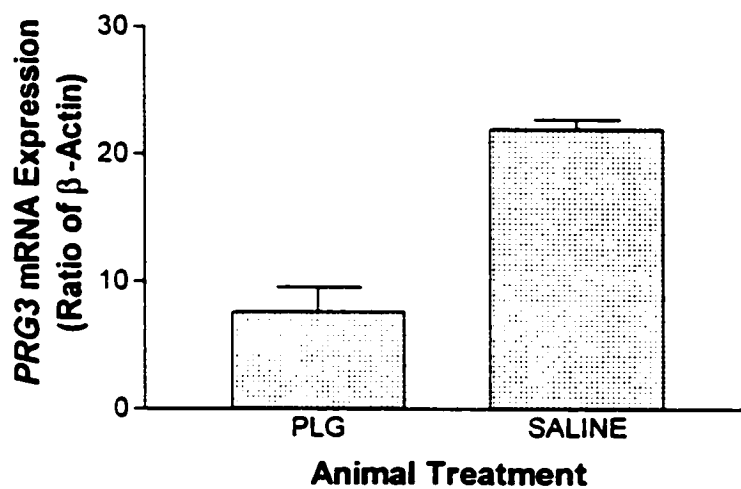
probe corresponded to nucleotides 1-561 (Figure 20), and contained a substantial part of the coding region, as well as a section of the 3' non-coding region. Analysis of the expression levels, relative to that of  $\beta$ -Actin, confirmed that the gene was down regulated by  $\approx 65\%$  following PLG treatment (Figure 29).

#### 4.5.3 Analysis of *PRG4* Expression by Northern Hybridisation

*PRG4* is highly homologous with the nucleotide sequence of the human  $G_{\gamma 11}$  subunit cDNA (Figure 23), and the putative translation product is an exact match with that of human  $G_{\gamma 11}$  (Figure 25). The human  $G_{\gamma 11}$  clone is 654 bp in length and has been shown to hybridise with two mRNA species of 1.0 and 1.2 kb in humans (Ray *et al.* 1995). The rat clone identified here is 609 bp in length and hybridises with a single mRNA species of 1.1 kb (Figure 30). Figure 30 shows that a weak signal was detected using part of the 5' RACE-PCR product as a probe. The probe corresponded to nucleotides 198-488 (Figure 24), and contained a substantial part of the coding region, as well as a section of the 3' non-coding region. Analysis of the expression levels, relative to that of  $\beta$ -Actin, revealed that the gene was not altered following PLG treatment (Figure 31).

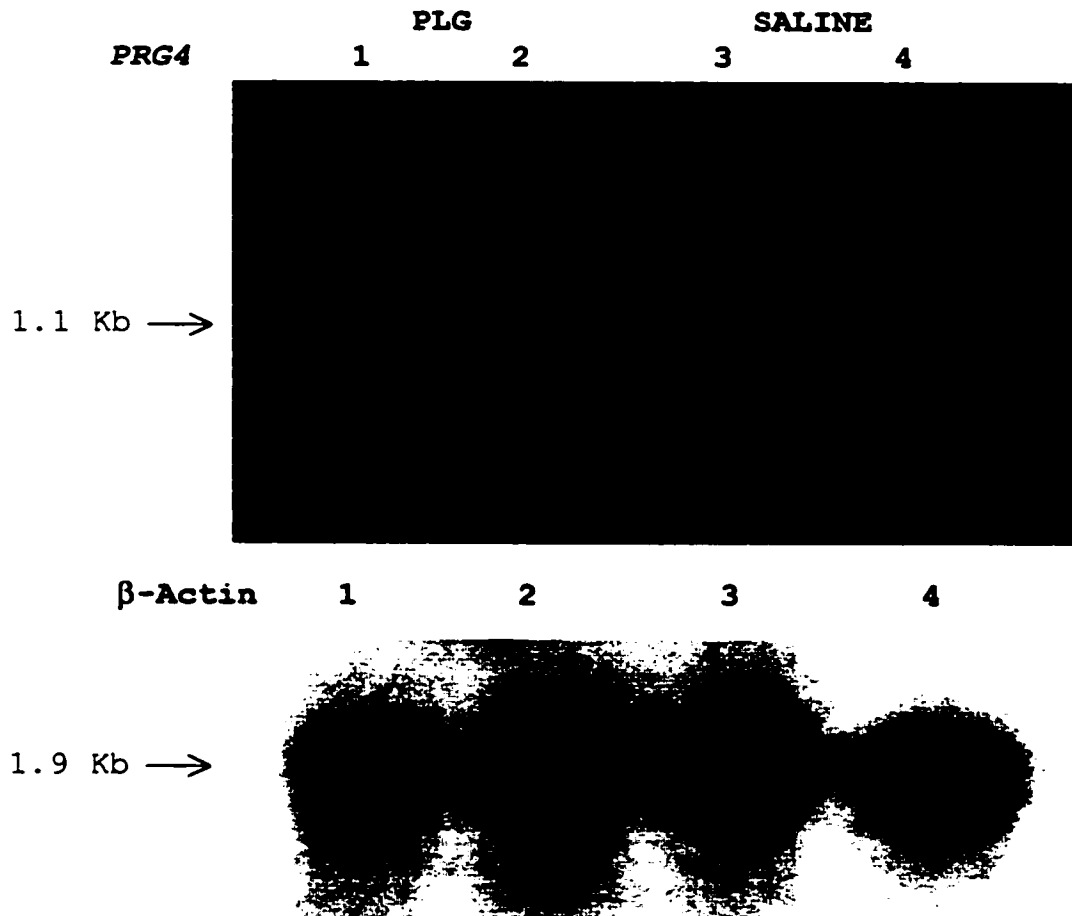
#### 4.5.4 Analysis of *PRG2*, *PRG5* and *PRG6* Expression by Northern Hybridisation

The expression of *PRG2*, *PRG5* and *PRG6* in striatal mRNA was assessed using northern hybridisation. Previous experiments have shown that when the ddPCR products were used as probes against total RNA, non-specific hybridisation to the 28S and 18S RNA was problematic (Figure 32). This necessitated the use of poly A<sup>+</sup> mRNA for assessing the expression of these genes in northern blots. The labelled cDNAs for *PRG2*, *PRG5* and *PRG6* were hybridised against 1  $\mu$ g of mRNA obtained from the striatum of PLG and saline treated rats (section 3.2). Initially, a very low stringency wash (30 min at room temperature with 2X SSPE + 0.1% SDS) was performed followed by a moderate

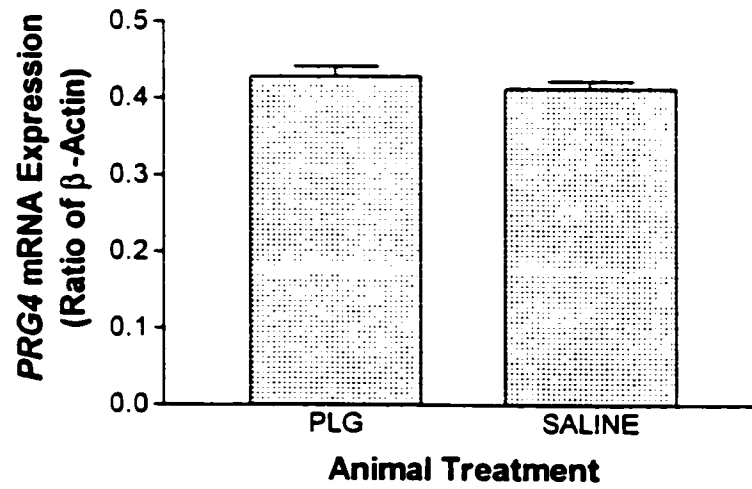


**Figure 29.** Graph of *PRG3* mRNA expression in the rat striatum following treatment with PLG (20 mg/kg i.p.) or Saline (1 ml/kg, i.p.). Results were determined in duplicate ( $\pm$  S.D.) and are expressed as a ratio of  $\beta$ -Actin expression.

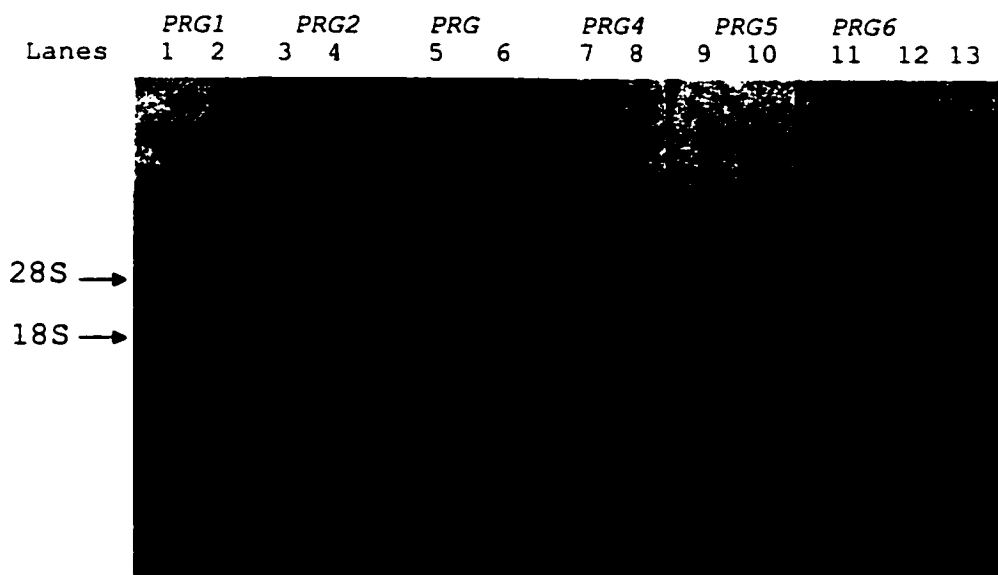




**Figure 30.** Northern blot of *PRG4* and  $\beta$ -actin in rat striatal mRNA (1  $\mu$ g per lane). Poly A<sup>+</sup> mRNA was isolated from the striata of PLG (28 days with 20 mg/kg, i.p.) and Saline (28 days with 1 ml/kg, i.p.) treated animals.



**Figure 31.** Graph of *PRG4* mRNA expression in the rat striatum following treatment with PLG (20 mg/kg i.p.) or Saline (1 ml/kg, i.p.). Results were determined in duplicate ( $\pm$  S.D.) and are expressed as a ratio of  $\beta$ -Actin expression.



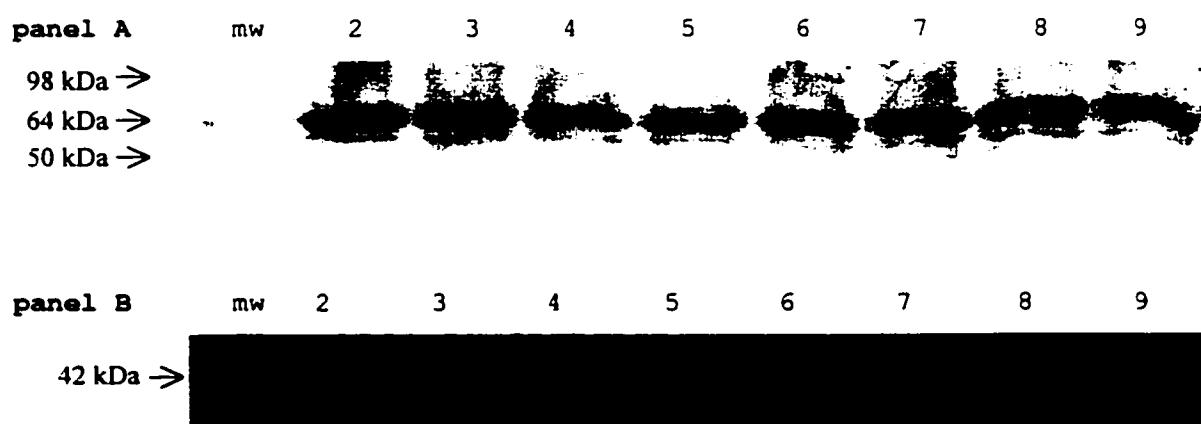
**Figure 32.** Confirmation of expression of ddPCR fragments by northern hybridisation with rat pre-frontal cortex and striatal total RNA. DNA probes, obtained from HindIII digestion of plasmid DNA, were  $^{32}\text{P}$  labelled using random primed labelling kit with specific activity in excess of  $10^8$  cpm/ $\mu\text{g}$  DNA. Position of the 28S and 18S ribosomal RNA is indicated on the blot. Even numbered lanes are pre-frontal cortex (20  $\mu\text{g}$ ); odd numbered lanes are striatum (20  $\mu\text{g}$ ) and lane 13 is a RNA molecular weight marker. The probes used are indicated above the lane markers.

stringency wash (15 min at 42°C with 0.1X SSPE + 0.1% SDS). Figure 33 shows that a non-specific signal (smear) was detected for *PRG2* and *PRG5* following the low stringency wash. Following the moderate stringency wash, no signal was detectable for *PRG1* and *PRG5*. No signal was detectable for *PRG6*, either at low or moderate stringency. In comparison to the signal for  $\beta$ -Actin, it is clear that these probes do not detect a mRNA species.

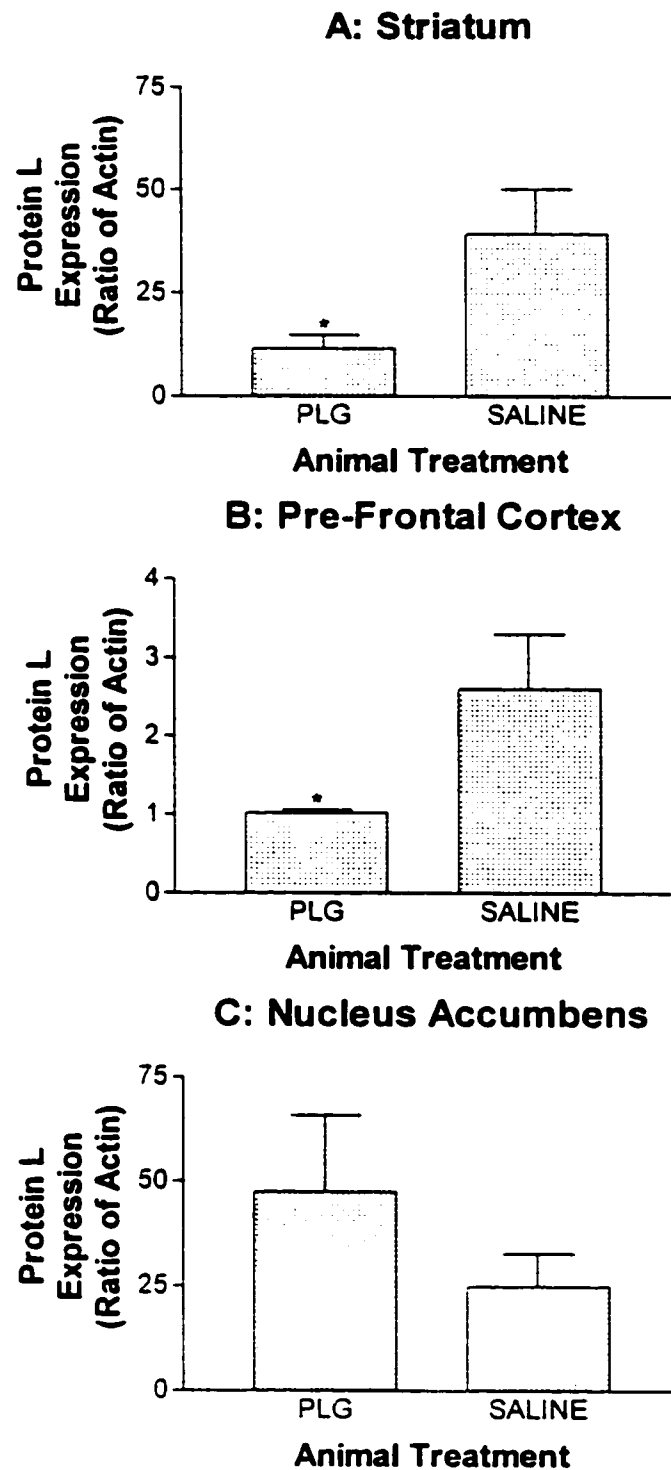
#### **4.6 Effect of PLG Treatment on Protein L Expression in Rat Brain Regions**

The levels of protein L were assessed using western blots in rat brain tissues isolated from animals treated with either PLG or saline (section 3.2). Three brain regions were studied, the prefrontal cortex (PFC), striatum (STR) and nucleus accumbens (NA). The homogenized tissues (5  $\mu$ g) were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed by western blotting. The levels of both protein L and actin (immunoreactive for  $\alpha$ ,  $\beta$  and  $\gamma$  subtypes) were determined in the same blot. Figure 34 is a representative western blot obtained from rat striatal tissue. The primary antibody for protein L is reactive with numerous proteins ranging in size from 16 to 64 kDa with a major band at  $\approx$  64 kDa. The protein L antibody has previously been shown to possess considerable cross reactivity with other members of the hnRNP family of proteins (Piñol-Roma *et al.* 1989) and the major species observed at 64 kDa is consistent with the observed size for purified protein L (Piñol-Roma *et al.* 1989). Figure 34 also shows that the Actin antibody was immunoreactive with a single species of protein of the expected size of  $\approx$  42 kDa.

Figure 35 is a bar graph of the analysis of the levels of expression of protein L in tissues obtained from PLG and saline treated rats. The values for protein L are expressed as ratio of the expression of the control protein Actin. This Figure shows that protein L levels were not significantly different in the PLG treated NA tissue. In the STR and PFC, however, protein L levels were reduced by 70.6% and 60.7%, respectively, in the PLG



**Figure 34.** Western blot in rat striatal tissue (5  $\mu$ g/lane) from PLG (28 days with 20 mg/kg, i.p.; lanes 2-5) and Saline (28 days with 1 ml/kg, i.p.; lanes 6-9) treated animals with anti-protein L (panel A) and anti-actin (panel B).



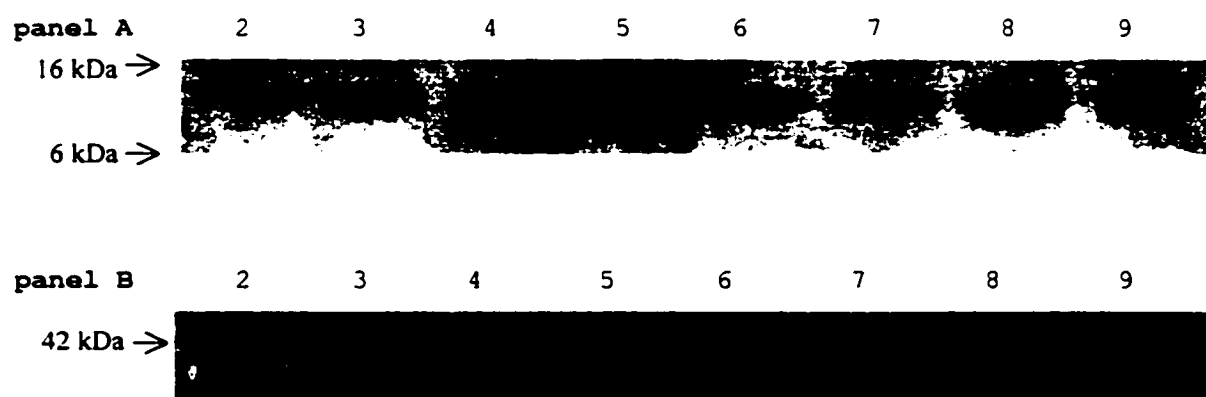
**Figure 35.** Levels of expression of protein L in rat brain tissues (5  $\mu\text{g}/\text{lane}$ ) from PLG (28 days with 20 mg/kg, i.p.) and Saline (28 days with 1 ml/kg, i.p.) treated animals as determined by western blotting. Data are given for striatum (panel A), pre-frontal cortex (panel B) and the nucleus accumbens (panel C). Values for protein L in graph are given as mean  $\pm$  S.E.M. ( $n=4$ ) and are expressed as a ratio of the level of actin (protein L/actin). \*  $p < 0.05$ , unpaired t-test.

treated animals compared to the saline treated animals ( $p < 0.05$ , unpaired t-test). The effect of PLG on the expression of protein L is consistent with the observed decrease ( $\approx 65\%$ ) in protein L mRNA expression (Figure 29).

#### **4.7 Effect of PLG Treatment on $G_{\gamma 11}$ Expression in Rat Brain Regions**

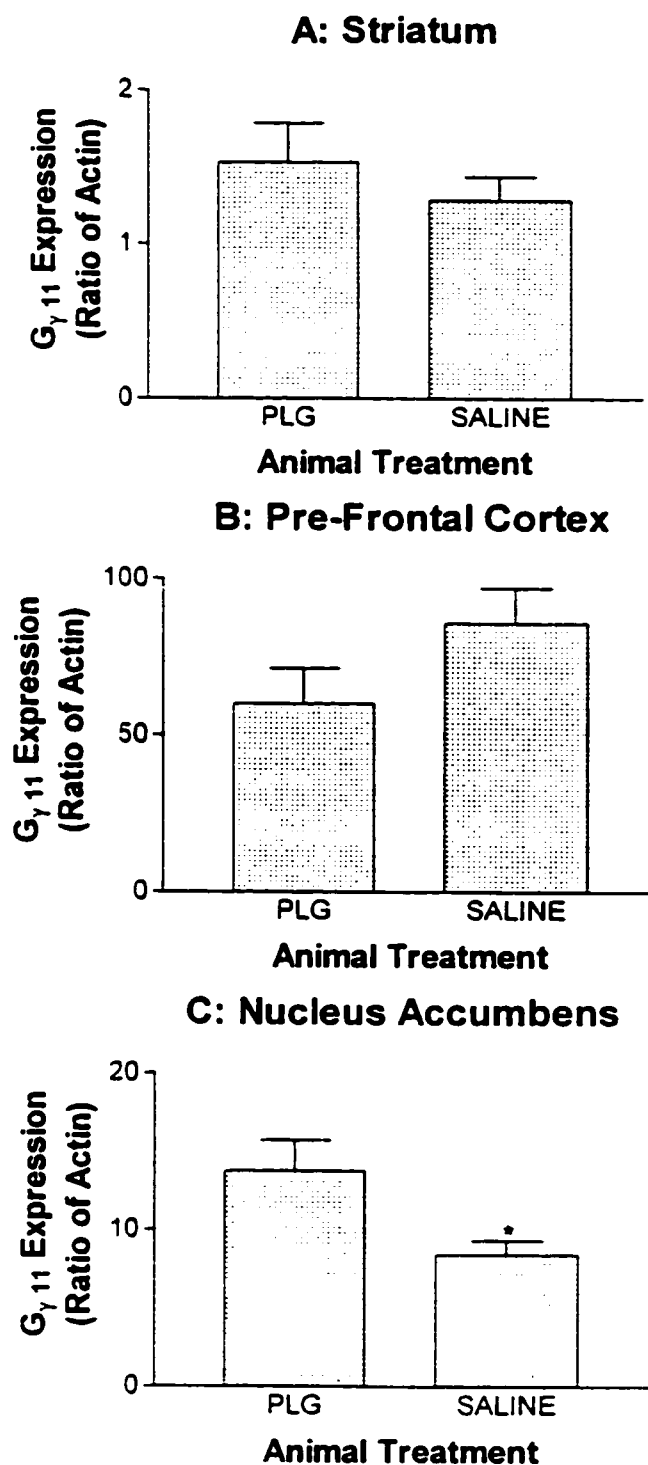
The levels of the  $G_{\gamma 11}$  G-protein subunit were assessed using western blots in rat brain tissues isolated from animals treated with either PLG or saline (section 3.2). As in section 4.6, the PFC, STR and NA were studied. The homogenized tissues (50  $\mu\text{g}$ ) were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed by western blotting. The levels of both  $G_{\gamma 11}$  and Actin (immunoreactive for  $\alpha$ ,  $\beta$  and  $\gamma$  subtypes) were determined in the same blot. Figure 36 is a representative western blot obtained from rat striatal tissue. The  $G_{\gamma 11}$  antibody is reactive with numerous proteins ranging in size from 8 to 98 kDa with a major band at  $\approx 8$  kDa. Previously, this antibody has only been used to detect purified proteins, and its specificity has not been well established (Morishita *et al.* 1998). Although the expected size of the  $G_{\gamma 11}$  protein, deduced from the translation product, is  $\approx 8.5$  kDa, Morishita *et al.* (1998) found that the  $G_{\gamma 11}$  antibody detected a band at  $\approx 5$  kDa. Unfortunately, these authors did not address this inconsistency in their paper.

Figure 37 is a bar graph of the analysis of the levels of expression of  $G_{\gamma 11}$  in tissues obtained from PLG and saline treated rats. This figure shows that  $G_{\gamma 11}$  levels were not significantly different in the PLG treated STR or PFC tissue. In the NA, however,  $G_{\gamma 11}$  levels were reduced by 38.8% in the saline compared to PLG treated animals ( $p < 0.001$ , unpaired t-test). The effect of PLG on the expression of  $G_{\gamma 11}$  in the striatum is consistent with the observed effect on mRNA expression in the striatum (Figure 31).



**Figure 36.** Western blot in rat striatal tissue (50  $\mu$ g/lane) from PLG (28 days with 20 mg/kg, i.p.; lanes 2-5) and Saline (28 days with 1 ml/kg, i.p.; lanes 6-9) treated animals with anti-G $\gamma$ <sub>11</sub> (panel A) and anti-actin (panel B).





**Figure 37.** Levels of expression of  $G_{\gamma 11}$  in rat brain tissues (50  $\mu\text{g}/\text{lane}$ ) from PLG (28 days with 20 mg/kg, i.p.) and Saline (28 days with 1 ml/kg, i.p.) treated animals as determined by western blotting. Data are given for striatum (panel A), pre-frontal cortex (panel B) and the nucleus accumbens (panel C). Values for  $G_{\gamma 11}$  in graph are given as mean  $\pm$  S.E.M. ( $n=4$ ) and are expressed as a ratio of the level of actin ( $G_{\gamma 11}/\text{actin}$ ). \*  $p < 0.001$ , unpaired t-test.

## 5.0 Discussion

There are a large number of reports which show that PLG is capable of modulating dopamine D2 receptor mediated neurotransmission within the central nervous system (Chiu *et al.* 1981b; Srivastava *et al.* 1988; Yu *et al.* 1988; Subasinghe *et al.* 1993; Johnson *et al.* 1986; Johnson *et al.* 1986; Johnson *et al.* 1990; Genin *et al.* 1993; Sreenivasan *et al.* 1993; Ott *et al.* 1996; Smith and Morgan, 1982; Saleh and Kostrzewa, 1989; Kostrzewa *et al.* 1989; Kostrzewa *et al.* 1979a; Kostrzewa *et al.* 1979b; Kostrzewa *et al.* 1976; Kostrzewa *et al.* 1978; Thomas *et al.* 1998). These studies have examined the effects of PLG both in *in vitro* biochemical assays as well as in *in vivo* behavioural experiments. Although PLG's exact mechanism of action is not known, the consensus is that PLG enhances the interaction of dopamine D2 receptors with its agonists. The effect of PLG is observed as either a potentiation of agonist effects (such as apomorphine induced stereotypies) or an attenuation of antagonist effects (such as neuroleptic-induced catalepsy). When dopaminergic agonists or antagonists are given systemically, the modulation of neuroleptic-induced catalepsy, L-DOPA/apomorphine induced stereotypy and rotational behaviour likely result from the effects of PLG on A9 dopaminergic neurons.

The role of PLG in mediating central D2 receptor signalling has intrigued researchers since its discovery in the early 1970's. The interest in PLG as a modulator of dopaminergic function stems from the importance of the dopaminergic neuronal systems in maintenance of proper motor control and psychological state.

### 5.1 Analysis of D2 Receptor Signalling By the [<sup>35</sup>S]-GTP $\gamma$ S Binding Technique

Dopamine receptor signalling within the CNS has been studied extensively using the radioligand binding technique. Although tremendously valuable as a comparative pharmacological technique, receptor radioligand binding primarily provides information about the initial steps in signal transduction, i.e. receptor - ligand interaction. There is

ample pharmacological data to suggest that PLG affects D2 receptor binding characteristics by acting at the level of the G-protein (Srivastava *et al.* 1988; Mishra *et al.* 1990; Baures *et al.* 1994; Baures *et al.* 1997). The importance of the G-protein as a site for regulation of receptor signalling has recently become an expanded area of research with the discovery of a new class of proteins that regulate G-protein function (RGS proteins see Hepler (1999) for review). RGS proteins bind to activated G $\alpha$  subunits and negatively regulated their signalling. This effect is due to their ability to act as GTPase-activating proteins (GAPs) that decrease the life-time of the GTP bound species of G $\alpha$ . RGS proteins are known to bind other proteins, in addition to G $\alpha$  subunits, that are involved in a diverse range of cellular functions (Hepler, 1999). This presents the possibility that PLG could interact with G-proteins directly, or indirectly through RGS proteins or the RGS binding partners.

An alternative method for assessing G-protein coupled receptor pharmacology that has recently been used is the [ $^{35}$ S]-GTP $\gamma$ S binding technique. This method has the advantage that it measures the functional activation of the G-protein, rather than the receptor. Thus, this method can be used to concomitantly gather both pharmacological and functional data.

In this thesis, the [ $^{35}$ S]-GTP $\gamma$ S binding technique was used to assess three aspects of D2 receptor function in the rat striatum. Firstly, the affinity and efficacy of a variety of D2 receptor agonists and antagonist were examined. Secondly, the potential role of PLG in modulating the GDP:GTP exchange step of D2 receptor mediated G $i$  activation was examined. Lastly, the effect of typical and atypical neuroleptics on D2 receptor mediated activation of G $i$  was assessed.

### **5.1.1 Pharmacological Characterisation of Striatal D2 Receptors**

The interaction of ligands, receptors and G-proteins under conditions of equilibrium is described well by the ternary complex model (De Lean *et al.* 1980; Kent *et*

*al.* 1980; Malmberg *et al.* 1998; Costa *et al.* 1992; Lefkowitz *et al.* 1993). According to this model, the action of an agonist (partial or full) is to stabilise the receptor-G protein (RG) complex, enabling the activation of the G protein. Antagonists, however, are thought to bind to receptors in a manner that either does not affect the stability of the RG complex or decreases the stability of the RG complex (Costa *et al.* 1992). The former variety of antagonists are called "silent", whereas the latter are considered negative antagonists or inverse agonists (Costa *et al.* 1992; Malmberg *et al.* 1998; Lefkowitz *et al.* 1993). Whereas silent antagonists do not affect the basal activity of G proteins, negative antagonists can decrease basal G protein activity.

The ternary complex model has recently been "extended" to accommodate observations, such as constitutive activity in mutant receptors, which cannot be accounted for in the original model (Lefkowitz *et al.* 1993; Costa *et al.* 1992). The allosteric ternary complex model proposes that there is an additional "explicit isomerization step regulating the formation of the state of the receptor from R to R\*, which is capable of binding to the G protein" (Lefkowitz *et al.* 1993). Thus, the ability of mutant receptors to interact with and activate G proteins in the absence of a stabilising agonist is explained. However, constitutive activity is not constrained to mutated receptors. For example, Samama *et al.* (1993) found that the basal levels of cAMP increased linearly with increasing expression levels of the cloned wild-type  $\beta_2$ -adrenoceptor. Costa *et al.* (1992) studied negative antagonism and constitutive receptor activity and reasoned that negative antagonism should only be observable in receptor systems that are constitutively active. Thus, negative antagonism is resultant from inhibition of a receptor's constitutive activity. Therefore, the degree of negative antagonism observed reflects the degree of constitutive activity for a given receptor system.

A great deal of the pharmacological data collected to date has relied on receptor binding methodology. Although this technique provides valuable information about drug-receptor interactions, it is limited to measurements of receptor affinity. The

[<sup>35</sup>S]-GTP $\gamma$ S binding technique offers an advantage over the receptor binding technique in that it measures the activity of the G-protein; thereby enabling an estimation of efficacy as well as affinity. Recently, a number of reports have been published which have used the [<sup>35</sup>S]-GTP $\gamma$ S binding technique to examine the pharmacology of individual DA receptor subtypes expressed in transfected cell lines (Gardner *et al.* 1997; Gardner *et al.* 1996; Pregoner *et al.* 1997; Zenner *et al.* 1998; Newman-Tancredi *et al.* 1997; Patel *et al.* 1997; Chabert *et al.* 1994; Malmberg *et al.* 1998). However, only a few studies have been published which used [<sup>35</sup>S]-GTP $\gamma$ S binding to characterise DA receptors endogenously expressed in brain tissue (Costain and Mishra, 1997; Costain *et al.* 1996; Rinken *et al.* 1999). Although studies of homogeneous receptor populations in transfected cell lines provides valuable information about specific receptors, these studies do not preclude the necessity of studying receptor populations in native tissues. Studying receptor systems as they exist in brain tissues (often consisting of multiple receptor subtypes heterogeneously expressed in a given region) provides pharmacological data which is representative of what can be expected *in vivo*. Therefore, I chose to study the coupling of endogenously expressed D2 receptors to G-proteins in the striatum using the [<sup>35</sup>S]-GTP $\gamma$ S binding technique.

In the present study, the usefulness of the [<sup>35</sup>S]-GTP $\gamma$ S binding assay as a method for assessing endogenously expressed D2 receptor mediated activation of G proteins within the striatum (Figure 1) has been validated. Furthermore, it was confirmed that the effect of stimulating D2 receptors is mediated through the Gi G-protein (Figure 5). Although the [<sup>35</sup>S]-GTP $\gamma$ S binding assay has successfully been used to study other receptor systems natively expressed in animal tissues (Hilf *et al.* 1989), there is only one paper (Rinken *et al.* 1999) and two abstracts (Costain and Mishra, 1997; Costain *et al.* 1996) reporting use of this technique to study the natively expressed dopamine receptor systems in the brain. However, several studies have used the [<sup>35</sup>S]-GTP $\gamma$ S binding assay to examine individual D2 class receptor subtypes (D<sub>2S</sub>, D<sub>2L</sub>, D<sub>3</sub> and D<sub>4</sub>) expressed in cell

lines (Gardner *et al.* 1997; Gardner *et al.* 1996; Malmberg *et al.* 1998; Pregoner *et al.* 1997; Patel *et al.* 1997; Chabert *et al.* 1994; Zenner *et al.* 1998; Newman-Tancredi *et al.* 1997). Additionally, a modified [<sup>35</sup>S]-GTP $\gamma$ S binding method has been used to detect D1 and D2 receptor stimulation of Gs and Gi G proteins respectively (Friedman *et al.* 1996; Wang *et al.* 1995). This modified method utilises immunoprecipitation of the G proteins so that [<sup>35</sup>S]-GTP $\gamma$ S binding to specific G protein subtypes can be assessed.

I used the [<sup>35</sup>S]-GTP $\gamma$ S binding assay to examine the pharmacology of D2 receptor agonists in the striatum and obtained the following rank order of potency: NPA >> bromocryptine > apomorphine > dopamine (Figure 2). The rank order of potency is consistent with data collected using receptor binding techniques (Gardner *et al.* 1997; Seeman, 1995; Mishra *et al.* 1990). Comparison of the pEC<sub>50</sub> values obtained in the present study to those obtained in expressed D2 receptors indicates that our results best compare to the results obtained in cells expressing the D<sub>2S</sub> receptor (Gardner *et al.* 1997). This is consistent with the D<sub>2</sub>-receptors subtype being the dominant D2 class receptor subtype expressed within the striatum (Meador-Woodruff, 1994).

In addition to the pharmacological data collected, I also assessed the efficacy of the ligands tested. Other researchers have successfully utilised the [<sup>35</sup>S]-GTP $\gamma$ S binding to detect differences in the efficacy of D2 receptor ligands in individual receptor subtypes expressed in cells (Chabert *et al.* 1994; Gardner *et al.* 1997; Malmberg *et al.* 1998; Newman-Tancredi *et al.* 1997). Although there was observable variation in the maximal responses elicited by the agonists tested, there were no significant differences among them (Table 1). This observation is likely due to the mixed DA receptor population present in the striatum.

The [<sup>35</sup>S]-GTP $\gamma$ S binding assay was also used to pharmacologically characterise a variety of D2 receptor antagonists in bovine striatum. Antagonist dose response effects were examined both in the presence (Figure 3) and the absence (Figure 4) of a D2 receptor agonist (1  $\mu$ M NPA). All of the antagonists tested were able to completely inhibit

NPA stimulated [ $^{35}$ S]-GTP $\gamma$ S binding in a dose dependent manner. The rank order of potency obtained correlates well with values obtained in receptor binding studies (Gardner *et al.* 1996; Seeman and Van Tol, 1994) and is as follows: spiperone > haloperidol > butaclamol > sulpiride > chlorpromazine > clozapine. The pIC<sub>50</sub> values obtained from the analysis of the data in Figure 3 is given in Table 1.

Certain D2 receptor antagonists were observed to inhibit [ $^{35}$ S]-GTP $\gamma$ S binding in the absence of D2 receptor stimulation. Figure 4 shows that haloperidol, butaclamol and chlorpromazine significantly decreased basal [ $^{35}$ S]-GTP $\gamma$ S binding in a dose dependent manner. Analysis of these data sets provided the pIC<sub>50</sub> values presented in Table 2. The rank order of potency of these antagonists is the same in the presence and absence of NPA, indicating that the effect on basal levels of [ $^{35}$ S]-GTP $\gamma$ S binding is a D2 receptor mediated effect. Thus, haloperidol, butaclamol and chlorpromazine behaved as negative antagonists while clozapine, sulpiride and spiperone behaved as silent antagonists.

The phenomenon of negative antagonism was studied by Costa *et al* (1992), and these authors reported that although negative antagonism is observable in membrane preparations it is not observed in intact cells. These authors concluded that uncharacterised intrinsic factors might be responsible for constraining receptors in an inactive state. However, Nilsson and Eriksson (1993) found evidence that haloperidol could act as a negative antagonist in cells expressing D<sub>2</sub> receptors. Although the concept of negative antagonism is intriguing, it is clear that the physiological impact in intact tissues has yet to be resolved.

### **5.1.2 Role of PLG in Modulating Striatal D2 Receptor Activation of Gi**

A number of studies have demonstrated that PLG modulates agonist binding to striatal D2 receptors in *in vitro* receptor binding experiments (Srivastava *et al.* 1988; Mishra *et al.* 1990; Baures *et al.* 1994; Baures *et al.* 1997). Specifically, PLG has been observed to increase D2 receptor agonist binding in a concentration dependent manner.

Further investigation revealed that PLG increases the affinity of the D2 receptor toward its agonists. Importantly, PLG was shown to antagonize the conversion by GTP, or GTP analogues, of high affinity D2 receptor agonist binding sites to low affinity sites. This observation suggested the involvement of the G-protein in mediating the effect of PLG. Two putative sites were identified (Scheme 1) whereby PLG may alter D2 receptor - G-protein interaction: 1/ the activation of the G-protein (GDP:GTP exchange) or 2/ inactivation of the G-protein (GTPase activity). By examining the effect of PLG on [<sup>35</sup>S]-GTP $\gamma$ S binding in the absence and presence of D2 receptor activation, it should be possible to determine whether or not PLG affects the rate of GDP:GTP exchange.

Figure 6 shows the results of an experiment where [<sup>35</sup>S]-GTP $\gamma$ S binding was measured in the absence and presence of maximal and sub-maximal concentrations of the D2 agonist NPA. Measurements of [<sup>35</sup>S]-GTP $\gamma$ S binding were performed in the presence of a range of concentrations of PLG. This was necessary because PLG characteristically displays a bell-shaped dose-response curve (Costain *et al.* 1999), and measurements at a single concentration of PLG could be misleading. The figure shows that PLG did not alter the levels of [<sup>35</sup>S]-GTP $\gamma$ S binding in the absence of D2 receptor stimulation. Nor did PLG alter the levels of [<sup>35</sup>S]-GTP $\gamma$ S binding in the presence of D2 receptor (maximal or sub-maximal) stimulation. If the observed effect of PLG in preventing the conversion of high affinity D2 receptor sites to low affinity sites had been due to altered GDP:GTP exchange, a decrease in the amount of [<sup>35</sup>S]-GTP $\gamma$ S binding would have been expected in the presence of PLG. However, as this was not observed, it must be concluded that PLG does not alter the activation of the G<sub>i</sub> in either a D2 receptor dependent or independent manner.

### **5.1.3 Comparison of Clozapine and Haloperidol Treatment on Striatal D2 Receptor Function**

The effects of neuroleptic treatment on striatal dopaminergic signal transduction



has been extensively studied at the level of the receptor, and less so at the level of G-proteins. A large number of studies have shown that protracted use of typical neuroleptics (such as haloperidol) causes an up-regulation of D<sub>2</sub>-dopamine receptors (Creese and Sibley, 1981); whereas protracted use of atypical neuroleptics does not (Ashby Jr. and Wang, 1996). However, there is no consistent data to support the theory that neuroleptic treatment (typical or atypical) has an effect on the affinity of D<sub>2</sub> receptors towards their agonist (See *et al.* 1990).

Three studies have been published which examined the effect of neuroleptic treatment on striatal G-protein levels. Gupta & Mishra (1993) found that haloperidol treatment (3 mg/kg, 14 days i.p.) decreases the levels of G<sub>i</sub>, G<sub>s</sub>, and β subunit (20-32%) whereas clozapine treatment (20 mg/kg, 14 days i.p.) increases the expression of the same proteins by 34-78%. Similarly, Shin *et al* (1995) found that haloperidol (3 mg/kg, 14 days i.p.) and sulpiride (50 mg/kg, 14 days i.p.) decreased G<sub>o1f</sub> and G<sub>i</sub> in the striatum. The effects of haloperidol are consistent in these two reports with both using the same treatment protocol used in the present study. The only other study published on the topic reports that long-term haloperidol treatment (0.23 mg/day, 32 weeks s.c.) has no effect on G<sub>i</sub> or G<sub>s</sub> levels in the striatum (See *et al.* 1993). The disparity between the reports of See *et al* (1993) and those of Gupta and Mishra (1993) and Shin *et al* (1995) is likely due to the different treatments employed. However, the effects of the haloperidol treatment used here should be consistent with the finding of Gupta and Mishra (1993) and Shin *et al* (1995).

The effect of clozapine on striatal G-protein levels has only been assessed by Gupta and Mishra (1993). Shin *et al* (1995) chose sulpiride as a representative atypical neuroleptic and found that its effects were similar to those of haloperidol. Unfortunately, sulpiride is considered by many to be a typical neuroleptic (Meltzer, 1995) making the data of Shin *et al* (1995) difficult to correlate with the study of Gupta and Mishra (1993).

Here, the finding that haloperidol and clozapine had similar effects on

[<sup>35</sup>S]-GTP $\gamma$ S binding was somewhat unexpected (Figure 7). It was thought that haloperidol and clozapine would have opposite effects on basal [<sup>35</sup>S]-GTP $\gamma$ S binding levels reflecting the differential effects of haloperidol and clozapine on the expression of G<sub>i</sub> (Gupta and Mishra, 1993). However, it is possible that haloperidol and clozapine are utilising different mechanisms in achieving a similar net effect on striatal [<sup>35</sup>S]-GTP $\gamma$ S binding. Although haloperidol is likely causing decreased G-protein expression, increased D2 receptor expression could cause an increase in basal G-protein [<sup>35</sup>S]-GTP $\gamma$ S binding through the constitutive activity of the receptor. With clozapine, receptor expression is not affected but G-protein expression is increased, thereby causing increased basal G-protein [<sup>35</sup>S]-GTP $\gamma$ S binding.

The EC<sub>50</sub> values for NPA observed using the [<sup>35</sup>S]-GTP $\gamma$ S binding assay are presented in Table 3. The NPA EC<sub>50</sub> values reported here are similar to previously reported K<sub>D</sub> values of  $\approx$  0.21 nM for [<sup>3</sup>H]-NPA in receptor binding assays in the striatum (Srivastava *et al.* 1988). The similarity between affinity values for NPA using both the [<sup>35</sup>S]-GTP $\gamma$ S binding assay and the [<sup>3</sup>H]-NPA receptor binding assay supports the validity of the [<sup>35</sup>S]-GTP $\gamma$ S binding assay as an indirect measure of D2 receptor activation. In the present study, haloperidol treatment caused a significant decrease in NPA EC<sub>50</sub> (increased affinity) when compared to control. This effect was not observed as a result of clozapine treatment. While the altered EC<sub>50</sub> in the haloperidol treated group might represent an increased affinity of D2 receptors toward NPA, it is more likely that it is produced by an increase in the number of D2 receptors expressed in the striatum (See *et al.* 1990).

Two other important observations can be made from the present study. While the maximal level of [<sup>35</sup>S]-GTP $\gamma$ S binding was increased in both haloperidol and clozapine treated tissues, this increase was of a lesser magnitude than the increase in basal levels. Thus the magnitude of the response to NPA (maximal - basal) was reduced in both the haloperidol (18%) and clozapine (41%) groups. While no significant differences were observed between the control and neuroleptic treated groups at the maximal concentration

of NPA (1  $\mu$ M,  $p > 0.05$  ANOVA), the tendency towards a decreased response to D2 receptor activation may indicate that neuroleptic treatment reverses a hyper-responsive dopaminergic system in the basal ganglia.

There has been a lot of research directed at defining the differences between typical and atypical neuroleptics in an attempt to increase our understanding of the underlying pathology of schizophrenia. This study was aimed at determining whether or not haloperidol and clozapine treatment had differential effects on D2 receptor stimulation of G-proteins in the striatum. While the net effect of haloperidol and clozapine appears to be similar (increased levels of [ $^{35}$ S]-GTP $\gamma$ S binding) the observation that only haloperidol altered the EC<sub>50</sub> for NPA is evidence that different mechanisms may underlie this effect. Assuming that dopamine receptor blockade is the key event in the antipsychotic action of neuroleptic drugs, the efficacy of the weak D2 receptor antagonist clozapine is apparently anomalous. The present study sheds light on how drugs with such differing pharmacology may have similar net effects on the physiology of a complex system. The results presented here are also consistent with the delay between the initiation of neuroleptic treatment and the onset of antipsychotic action. Compensatory actions in the striatum to the presence of neuroleptic drugs need to occur before the dopaminergic system regains its proper balance. With haloperidol the compensation would be up-regulation of D2 receptors and down regulation of G-proteins. With clozapine the compensation would be up-regulation of G-proteins. While this discussion is limited to the dopaminergic system within the basal ganglia, it does begin to explain how the atypical neuroleptic clozapine may be similar to typical neuroleptics in producing an antipsychotic action.

#### **5.1.4 Clinical Implications of [ $^{35}$ S]-GTP $\gamma$ S Binding Studies**

The findings presented here present several important considerations for the treatment of schizophrenia and Parkinson's disease. Firstly, a drug's affinity towards a

receptor is not necessarily the most important consideration when evaluating its activity. The effect of a drug given to a patient will reflect both the affinity and efficacy of that drug. Secondly, the concept of efficacy is not limited to discussions about agonists. Of particular relevance to schizophrenia is the knowledge that neuroleptic drugs differ in their properties as either silent or negative antagonists. Thirdly, disorders involving alterations in a given neurotransmitter system should be evaluated as more than a phenomenon of altered receptor or G-protein expression. That is, the coupling of a receptor and G-protein, or proteins that affect this, can be altered as well.

## 5.2 Identification and Analysis of PLG Regulated Genes

As a signalling molecule, PLG is a relatively anomalous peptide. Although certain physiological effects of PLG have been well established (Ehrensing *et al.* 1994; Johnson *et al.* 1986; Kostrzewa *et al.* 1978; Mishra *et al.* 1983; Mycroft *et al.* 1987; Ott *et al.* 1996; Rajakumar *et al.* 1987b; Smith and Morgan, 1982), a direct cause and effect relationship has not been established. Attempts to identify a PLG binding site have not been successful, the observable physiological effects cannot be measured directly and pharmacological characteristics of PLG do not produce “typical” dose responses (Chiu *et al.* 1981a; Srivastava *et al.* 1988; Yu *et al.* 1988; Subasinghe *et al.* 1993; Johnson *et al.* 1986; Johnson *et al.* 1986; Johnson *et al.* 1990; Genin *et al.* 1993; Sreenivasan *et al.* 1993). Because PLG does not conform to the classical model of what a neurotransmitter is, it is ideally suited to characterisation with the ddPCR method. Here the use of ddPCR was primarily intended to identify genes that are involved in mediating the physiological effects of PLG. The technique of ddPCR enables the discovery of completely novel genes, and it was hoped that a PLG binding protein could be discovered.

Previous studies have established a role for systemically administered PLG in modulating gene expression within the rat brain. It was demonstrated that co-administration of PLG attenuates the increased levels of expression of the D2

dopamine receptor following protracted haloperidol treatment (Chiu *et al.* 1981b; Chiu *et al.* 1985; Rajakumar *et al.* 1987b). Similarly, an analogue of PLG (PAOPA) has been found to attenuate a haloperidol-induced decrease in the expression of G-proteins (Gi and Golf/Gs) in the rat STR (Costain *et al.* 1997). Although a number of mechanisms could account for these observations, it is plausible that PLG is directly affecting the expression of the mRNA for these proteins. In a study by Ott *et al.* (2000), the effects of haloperidol treatment, in combination with PLG or PAOPA, on the expression of the immediate early gene *c-fos* were examined. These authors found that PLG and PAOPA attenuated the haloperidol-induced up-regulation of *c-fos* and Fos (Ott *et al.* 2000). Furthermore, it was found that PLG or PAOPA administration alone did not affect *c-fos* or Fos. Thus, it is likely that the effect of PLG on *c-fos* and Fos expression was attributable to its modulation of the D2 receptor system, and not a direct effect on *c-fos* or Fos expression.

### 5.2.1 SAP155

The coding regions of genomic DNA (exons) are commonly interrupted by non-coding regions (introns). Nascent pre-mRNA transcripts also often contain intronic regions and must undergo several processing steps within the nucleus prior to becoming mature mRNAs and being transported to the cytoplasm. For intron containing pre-mRNA, these processing steps include 5'-cap formation, methylation, 3'-end cleavage and polyadenylation, and splicing (Liu and Mertz, 1995). A mature mRNA typically consists of a 5' non-coding region followed by an uninterrupted open reading frame and a 3' untranslated region. The splicing reaction joins the exons in an end to end fashion, thereby removing the introns and creating the open reading frame. Splicing takes place in the nucleus and can occur co- or post-transcriptionally (Kramer, 1996). After the mature mRNA has been produced, it is transported out of the nucleus to the cytoplasm where it is translated into a protein.

There are four consensus sequences on the pre-mRNA that are involved in the

splicing process; two at the exon/intron borders (5' and 3' splice sites), one that is usually 18 - 40 nucleotides upstream of the 3' splice site (branch site) and a stretch of pyrimidine residues (polypyrimidine tract) that preceded 3' splice site (Kramer, 1996; Gozani *et al.* 1998; Das *et al.* 1999; Wang *et al.* 1998). These splice sites are recognised by a number of small nuclear ribonucleoproteins (snRNPs) and non-snRNP protein factors that come together to assemble the active splicing complex or spliceosome. A number of spliceosome-associated proteins (SAPs) come together to form what is called the A complex that binds to the branch site. SAP155 is one of the proteins that comprises the A complex. The exact role of SAP155 within the A complex is to bind the mRNA on both sides of the branch site (Gozani *et al.* 1998) and is a critical component of the spliceosome active site (Gozani *et al.* 1998; Wang *et al.* 1998).

Initially, the ddPCR primer combination G3 (Figures 8 and 10) produced a band that was decreased in the PLG treated animals. Cloning of this band revealed the presence of three cDNAs designated ***PRG1***, ***PRG2*** and ***PRG3*** (Table 4). Sequence analysis of ***PRG1*** revealed a very high level of homology with human SAP155 cDNA (95%; Table 5). This ddPCR fragment was extended in both the 5' and 3' directions using RACE-PCR (Section 4.4.2); resulting in the generation of a cDNA of 1683 nucleotides in length (Figure 16). This extended sequence retained a very high level of homology (92%) with the human SAP155 cDNA (Figure 15). Open reading frame analysis of the extended sequence for ***PRG1*** resulted in a protein sequence of 324 amino acids in length with a predicted molecular mass of 35.7 kDa (Figure 16). The protein sequence for ***PRG1*** displayed an even greater level of homology with that of SAP155 (99%) than did the nucleotide sequence (Figure 17). The sequence homology (both nucleotide and amino acid) of ***PRG1*** with that of human SAP155 suggests that ***PRG1*** is the rat homologue of the human SAP155 gene. However, if ***PRG1*** is the rat homologue of SAP155, then full length cloning of this cDNA was not accomplished. The difficulty in obtaining the full length product may have been due to problems inherent to the generation of the rat brain

cDNA that was purchased from Clontech. Specifically, generation of full length cDNAs using avian myeloblast virus reverse transcriptase (AMV RT) is often difficult if the sequence contains regions with high G/C content or complicated secondary structures. In such cases, a truncated version of the cDNA is produced that would limit the amount of extension one could achieve using RACE-PCR (Shimomaye and Salvato, 1989). This is likely to be the problem encountered here. It may be possible to overcome this problem by altering the conditions used to create the cDNA (Shimomaye and Salvato, 1989) or by using MMLV RT.

Analysis of *PRGI* gene expression by northern hybridisation (section 4.5.1) revealed that the *PRGI* probe hybridised with a mRNA species of 4.7 kb (Figure 26) and the levels of expression were increased by  $\approx 54\%$  in PLG treated animals (Figure 27). This change in expression of the *PRGI* mRNA was opposite to what was expected from the initial observation with ddPCR and may be similar to the type of error that occurs during the PCR process that gives rise to false positives.

### 5.2.2 Protein L

Immediately following the initiation of transcription by RNA polymerase II, nascent RNA transcripts are bound by a number of nuclear proteins to form a heterogeneous nuclear ribonucleoprotein (hnRNP) (Piñol-Roma *et al.* 1989; Kramer, 1996). The hnRNP proteins are similar to the proteins that constitute the spliceosome in that their interaction with the nascent mRNA transcripts is necessary for the generation of a mature and translatable mRNA. Some hnRNP proteins have been shown to interact with 5' or 3' splice sites and function in splicing and alternative splice-site selection (Kramer, 1996). However, the hnRNP proteins are considered general RNA-binding proteins that readily bind to single stranded nucleic acids in a manner independent of the presence of functional splicing sites.

hnRNP protein L (protein L) was initially sequenced and characterised by Piñol-

Roma *et al* (1989). As with other hnRNP proteins, these authors found that protein L bound to nascent mRNA transcripts and was localised in the nucleoplasm. However, protein L possessed characteristics that distinguished it from the other hnRNPs, namely a unique pattern of distribution within the cell (i.e. immunofluorescent staining of non-nucleolar structures), association with the bulk of the hnRNP complexes as well as unique transcripts, and possession of an atypical amino acid sequence for an hnRNP protein (Piñol-Roma *et al.* 1989). Garcia-Bassets *et al* (1999) reported that protein L bound to polypyrimidine DNA and RNA sequences and that it could play a role in stabilising single stranded DNA intramolecular triplexes and/or hairpin loops. Furthermore, other proteins found to bind to polypyrimidine sequences, present in the regulatory regions of certain genes, influenced the expression levels of these genes (Takimoto *et al.* 1993; Gaillard *et al.* 1994; Ostrowski *et al.* 1994; Tomonaga and Levens, 1995; Michelotti *et al.* 1996). As an example, the binding of hnRNP-K can act as either an activator or a repressor, depending on the promoter and activator (Michelotti *et al.* 1996; Gaillard and Strauss, 1990; Lee *et al.* 1996; Tomonaga and Levens, 1996; Miao *et al.* 1998). Similarly, hnRNP-I has been shown to interact with the regulatory regions of certain genes (Brunel *et al.* 1991; Jansen-Durr *et al.* 1992; Brunel *et al.* 1996). Furthermore, protein L possesses a glycine rich domain similar to that found in a protein, *Drosophila* sex-lethal, involved in transcription activation in yeast (Wang *et al.* 1997).

It was initially thought that location and function of the hnRNPs were constrained to the nucleus (Piñol-Roma *et al.* 1989). However, it has recently been shown that a number of the hnRNPs, including protein L (Hahm *et al.* 1998b; Shih and Claffey, 1999), shuttle between the nucleus and the cytoplasm (Fan and Steitz, 1998; Nakielny and Dreyfuss, 1996; Piñol-Roma and Dreyfuss, 1992). An interesting function of protein L, reported by Hahm *et al* (1998b), is that of binding to the internal ribosomal entry site (IRES) of Hepatitis C virus and possibly other viral RNAs. By binding to the IRES site, in addition to binding to the polypyrimidine tract-binding protein (PTB) (Hahm *et al.*



1998a), protein L is thought to play a key role in the translation of Hepatitis C virus.

Proteins, including hnRNP-A1, that bind to polypyrimidine rich regions in mRNA species have been shown to affect the turnover/stability of the mRNA (Blaxall *et al.* 2000). Hamilton *et al* (1999) demonstrated that protein L binds a polypyrimidine rich sequence present in the 3' untranslated region of the glucose transporter 1 (Glut1) mRNA and that it complexes tightly with hnRNP-A2. These authors stated that hnRNP-A2, and indirectly protein L, is associated with translational repression of Glut1 and that it is regulated in conditions that lead to increased Glut1 mRNA stability (Hamilton *et al.* 1999). Shih *et al* (1999) found that protein L binds a polypyrimidine rich region in the human vascular endothelial growth factor mRNA (VEGF) that shares a 70% identity to that of the polypyrimidine rich region of Glut1. These authors described a 21 nt RNA binding site for protein L binding, 5'-CACCCACCCACAUACAUAUACAU-3' (Shih and Claffey, 1999). Importantly, Shih *et al* (1999) reported that protein L formed a hypoxia-induced complex with VEGF mRNA in the cytoplasm, and that the binding of protein L stabilised VEGF mRNA during hypoxia. Interestingly, Liu and Mertz (1995) found that protein L plays a role in enabling intron-independent gene expression (pre-mRNA that do not contain introns). Specifically, these authors found that protein L bound to a 119 nucleotide sequence of the herpes simplex virus thymidine kinase gene (HSV-TK), and that the binding of protein L was necessary for cytoplasmic accumulation of the mature HSV-TK mRNA.

***PRG3*** was isolated from a band generated by the ddPCR primer combination G3 (Figures 8 and 10). This ddPCR band was initially identified as being down-regulated by protracted PLG treatment. Sequence analysis of ***PRG3*** revealed a very high level of homology with mouse protein L cDNA (89%; Table 5). This ddPCR fragment was extended in both the 5' and 3' directions using RACE-PCR (Section 4.4.2); resulting in the generation of a cDNA of 835 nucleotides in length (Figure 20). This extended sequence retained a very high level of homology (95% and 90%) with the mouse and

human protein L cDNAs respectively (Figure 19). Open reading frame analysis of the extended sequence for *PRG3* resulted in a protein sequence of 140 amino acids in length with a predicted molecular mass of 15.6 kDa (Figure 20). The protein sequence for *PRG3* was a 100% match with mouse protein L and 98% match with human protein L (Figure 21). The sequence homology (both nucleotide and amino acid) of *PRG3* with that of mouse and human protein L suggests that *PRG3* is the rat homologue of the protein L gene. Similar to what was found for *PRG1*, the *PRG3* sequence obtained is likely to be only a partial sequence of the rat protein L cDNA.

Analysis of *PRG3* gene expression by northern hybridisation (section 4.5.2) revealed that the *PRG3* probe hybridised with a mRNA species of 3.1 kb (Figure 28) and the levels of expression were decreased by  $\approx 65\%$  in PLG treated animals (Figure 29). The levels of protein L were also assessed by western blot and it was found that the levels were decreased by  $\approx 71\%$  and  $61\%$  in the STR and PFC, but not the NA. This change in expression of the *PRG3* mRNA is consistent with what was expected from the initial ddPCR data. Furthermore, the observation that protein L protein levels were affected in both the STR and PFC indicates that PLG is active in both these regions.

Protein L plays many roles in transcriptional and translational events, including: pre-mRNA splicing, alternative splicing, DNA/RNA stabilisation, transcription regulation, mRNA stabilisation, translation regulation, and mRNA export to the cytoplasm. The present observation that protein L expression is down-regulated by PLG indicates that PLG is indirectly capable of altering the expression of genes that rely on protein L for their nucleotide processing. This is consistent with the known effects of PLG on the expression of proteins involved in dopaminergic signal transduction (D2 receptors and Gi G-proteins). Thus, PLG may partly alter dopaminergic function through its effects on such nuclear proteins as SAP155 and protein L.

### 5.2.3 $G_{\gamma 11}$

**PRG4** was isolated from a band generated by the ddPCR primer combination C1 (Section 4.3.1). This ddPCR band was initially identified as being down-regulated by protracted PLG treatment. Sequence analysis of **PRG4** revealed that no significantly homologous nucleotide sequences were present in GenBank (Table 5). The **PRG4** ddPCR fragment was extended in both the 5' and 3' directions using RACE-PCR (Section 4.4.3); resulting in the generation of a cDNA of 609 nucleotides in length (Figure 24). Sequence analysis of the extended sequence revealed a very high level of homology (91%) with the human  $G_{\gamma 11}$  G-protein subunit cDNAs over a 230 bp region of the cDNA (Figure 23). Interestingly, the 3' end of the cDNA sequence was a very poor match with the human  $G_{\gamma 11}$  cDNA, and did not possess significant sequence homology with any gene in GenBank. Open reading frame analysis of the extended sequence for **PRG4** resulted in a protein sequence of 73 amino acids in length with a predicted molecular mass of 8.5 kDa (Figure 24). The protein sequence for **PRG4** was a 100% match with human  $G_{\gamma 11}$  (Figure 25). The sequence homology (both nucleotide and amino acid) of **PRG4** with that of human  $G_{\gamma 11}$  suggests that **PRG4** is the rat homologue of the  $G_{\gamma 11}$  gene. For this gene, the full length clone of the nucleotide sequence was obtained.

Analysis of **PRG4** gene expression by northern hybridisation (section 4.5.2) revealed that the **PRG4** probe hybridised with a mRNA species of 1.1 kb (Figure 30) and the levels of expression were not affected by PLG treatment (Figure 31). The levels of  $G_{\gamma 11}$  were also assessed by western blot and it was found that the levels in the STR and PFC were not affected by PLG treatment, but were increased by  $\approx 39\%$  in the nucleus accumbens. The finding that  $G_{\gamma 11}$  mRNA or protein levels in the STR were not altered as a result of PLG treatment is inconsistent with the initial observation with ddPCR. In light of this, the altered levels of  $G_{\gamma 11}$  in the NA are difficult to reconcile.

With the knowledge of the role of PLG in modulating the G-protein coupled dopaminergic receptor system, it was foreseeable that ddPCR may identify a component of this signal transduction pathway as being affected by PLG treatment. Previous studies

have demonstrated that the expression of D2 receptors and G-protein  $\alpha$ -subunits are modulated by PLG in the striatum (Gupta and Mishra, 1993; Shin *et al.* 1995; Rajakumar *et al.* 1987b; Chiu *et al.* 1985; Chiu *et al.* 1981b; Saleh and Kostrzewa, 1989; Bhargava, 1984a; Bhargava, 1984b). Therefore, the identification of *PRG4* as the G-protein subunit  $G_{\gamma 11}$  following RACE-PCR analysis fit well in the working model of PLG function (Scheme 1). Unfortunately, the effect of PLG treatment on the expression of  $G_{\gamma 11}$  mRNA/protein was not confirmed in the striatum. However, the finding that  $G_{\gamma 11}$  protein expression was reduced in the NA following PLG treatment is something that may warrant further clarification.

### 5.3 Possible Roles of PLG Regulated Genes in PLG's Mechanism of Action

PLG has demonstrated a multifaceted modulatory effect on central D2 dopamine receptor function. PLG has been shown to affect D2 receptor binding properties in *in vitro* binding assay (Srivastava *et al.* 1988; Mishra *et al.* 1990; Baures *et al.* 1994; Baures *et al.* 1997) as well as the regulation of D2 receptor expression *in vivo* (Chiu *et al.* 1981b; Chiu *et al.* 1985; Rajakumar *et al.* 1987b; Bhargava, 1981a; Das and Bhargava, 1985a; Bhargava, 1983a; Rajakumar *et al.* 1987a; Das and Bhargava, 1985b; Chiu *et al.* 1982a; Rajakumar *et al.* 1986). Furthermore, PLG is capable of attenuating haloperidol induced *c-fos* expression (Ott *et al.* 2000). The ability of PLG to modulate D2 receptor affinity and expression may be mediated through a common mechanism. However, it is also possible that separate mechanisms are involved in these two processes.

PLG has consistently demonstrated an ability to counteract the effects of drugs (including those that do not interact with D2 receptors) or conditions that up-regulate the expression of D2 receptors. This general effect on D2 receptor expression suggests that the mechanism responsible may not involve an interaction between PLG and the D2 receptor/Gi-protein complex. Rather, PLG may be affecting D2 receptor expression at the level of transcription or translation. Consistent with this theory is the present observation

that PLG affects the expression of mRNA binding proteins. There is no evidence to suggest that protein L is involved in D2 receptor, or *c-fos* mRNA processing (the protein L mRNA binding consensus sequence, 5'-CACCCACCCACAUAUACAUAU-3', is absent in all dopamine receptor mRNA as well as in *c-fos* mRNA). However, there has not been an exhaustive study of which mRNA species are bound by protein L and the consensus sequence identified by Shih *et al.* (1999) may not be the only sequence recognised by protein L. Furthermore, the consequence of decreased levels of protein L on mRNA processing has not been assessed. Since PLG treatment is associated with an inhibition of D2 receptor upregulation and a decrease in protein L synthesis, it may be that these two phenomenon are causally related; i.e. down-regulation of protein L prevents up-regulation of D2 receptor expression.

Expression of SAP155 was predicted to be decreased in PLG treated animals by ddPCR, but was found to be increased by  $\approx 54\%$  following northern hybridisation analysis. While a result such as this was unexpected, it is not completely incomprehensible as false positives are an inherent obstacle in ddPCR. If ddPCR can imply differences where there are none, it is also possible that it would indicate an expression pattern opposite to the actual pattern of expression.

The identification of SAP155 as a gene that is potentially regulated by PLG (section 5.2.1) indicates that PLG may modulate the splicing of certain genes. The D<sub>2</sub> receptor mRNA undergoes alternate splicing and exists in two forms, D<sub>2short</sub> and D<sub>2long</sub>. Therefore, if PLG does modulate the expression of SAP155, this may affect the stoichiometry of expression for the D<sub>2</sub> receptor splice variants. This could have a subtle effect on D2 receptor signal transduction within the brain. However, there are no reports indicating that D<sub>2short</sub> and D<sub>2long</sub> have significantly different pharmacological profiles or second messenger coupling. Therefore it is difficult to predict how such an effect of PLG could affect central dopaminergic function.

The effect of PLG on D2 receptor pharmacology has been well studied and a

model for its mechanism of action has been proposed (Scheme 1). This model focuses on the G-protein as a site for regulation of D2 signal transduction by PLG. The identification of the  $G_{\gamma 11}$  subunit as potentially being regulated by PLG fit well with this model. Although northern and western blot analysis failed to confirm that the  $G_{\gamma 11}$  subunit was down regulated in the STR (Figures 31 and 37), a PLG-induced increase in protein expression was observed in the NA. The consequence of altered  $G_{\gamma 11}$  expression in the NA is difficult to predict because there is a dearth of information about the function of  $G_{\gamma 11}$ . However, it is known that alterations in the stoichiometry of receptors and G-proteins does affect signal transduction so that a promiscuous system can be biased toward one second messenger over another (Seifert *et al.* 1999). Thus, up-regulation of  $G_{\gamma 11}$  could result in a greater level of activation of  $\beta\gamma_{11}$  dependent processes.

#### **5.4 Clinical Implications of PLG Regulated Genes**

PLG has been examined clinically for treating psychological and neurological disorders such as Parkinson's disease (Plotnikoff *et al.* 1971; Barbeau, 1975; Barbeau *et al.* 1978), depression (Reed *et al.* 1994; Ehrensing and Kastin, 1978; Ehrensing *et al.* 1994; Pignatiello *et al.* 1989) and tardive dyskinesia (Ehrensing, 1974; Ehrensing *et al.*, 1977). The beneficial effects of PLG in treating Parkinson's and tardive dyskinesia are thought to be related to its dopaminergic potentiation and inhibition of neuroleptic-induced D2 receptor up-regulation respectively. The mechanism underlying its anti-depressant effects is unclear. The observations made here present new information regarding the mechanism involved in mediating the effects of PLG. This data provides a new avenue for examining the effects of PLG when used as a treatment for neurological disorders. Similarly, the identification of RNA binding proteins as a potential site for regulation of G-protein coupled receptor function may indicate that members of this class of proteins may be involved in the pathology of certain neurological disorders. Certainly, these concepts warrant further investigation.

## 6.0 Conclusions

1. The [<sup>35</sup>S]-GTPγS binding assay is particularly well suited for the classification of partial agonists, silent antagonists and negative antagonists. Although the physiological significance of negative antagonists remains to be clarified, the [<sup>35</sup>S]-GTPγS binding technique provides valuable insight into the mechanisms of receptor mediated activation of G proteins. Similarly, use of the [<sup>35</sup>S]-GTPγS binding assay in tissue preparations or primary cultures can provide pharmacologists with information about the affinity as well as the efficacy of a given drug.
2. The findings of the present study also indicate that the typical neuroleptic haloperidol and the atypical neuroleptic clozapine have similar effects on the functional coupling of D2 receptors and G-proteins within the STR. Neuroleptic treatment brought about increased levels of striatal G-protein activity as well as decreased responsiveness to the D2 agonist NPA. Although the dopamine theory of schizophrenia is in need of revision (Nestler, 1997), the present findings support the role of neuroleptics in decreasing the over-activity of dopaminergic processes within the basal ganglia. It is important to note that haloperidol and clozapine displayed a tendency toward differential effects on the EC<sub>50</sub> values for NPA. This suggests that typical and atypical neuroleptics may initiate a common functional alteration responsible for their antipsychotic actions through different means.
3. The identification of two nuclear proteins Protein L and SAP155 that are regulated by PLG is particularly interesting. Both of these proteins are involved in mRNA/DNA processing. The fact that ddPCR identified two proteins involved in similar processes strongly suggests that PLG may be involved in regulating

transcriptional/translational events. This is a significant observation, because it opens up the possibility of an entirely new mechanism of action for PLG. Previously, it has been observed that PLG modulates the effect of haloperidol on the expression of D2 receptors. The current observation presents that possibility that this modulation may occur at a level separate from that of the receptor/G-protein. However, a great deal of further work would be required to support this hypothesis.

4. The project described herein has the potential to greatly further our understanding of the mechanism of action PLG and perhaps change the direction in which PLG research is heading. The technique of ddPCR enables researchers to quickly identify numerous genes whose expression is regulated by any given drug treatment. While the technique has its limitations and obstacles, when properly implemented it can provide exciting and novel insight into the mechanism of action of a given pharmacological agent. Thus, by assessing the effects of PLG treatment with ddPCR I attempted to make a significant contribution to our understanding of the mechanism(s) of action of PLG as well as its possible uses in treating neurological disorders.



## 7.0 References

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