FUNCTIONAL GENOMIC ANALYSIS OF NON-IMMUNOSUPPRESSANT NEUROIMMUNOPHILIN LIGAND IN A RAT PARKINSON'S MODEL

A Thesis

Presented to

The Faculty of Graduate Studies

of Carleton University

by

Kathryn B. Payne

In partial fulfillment of the requirements

for the degree of

Master of Science

2004

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-494-00787-7
Our file Notre référence
ISBN: 0-494-00787-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder with the loss of substantia nigra, pars compacta (SNc) neurons as its main pathology. Novel treatments for PD include administration of non-immunosuppressant derivatives of neuroimmunophilin ligands (NILs), such as GPI-1046. Here, we investigate mRNA changes in the striatum of a 6-hydroxydopamine (6-OHDA) model of PD after treatment with GPI-1046, using National Institutes of Health (NIH) 15K mouse probes with two-colour DNA microarray hybridization. We compared the effects of lesioning, treatment and the interaction effects of lesioning and treatment using a 2 x 2 factorial microarray design. Significant changes were observed when comparing lesioning to GPI-1046 treatment but not with both lesioning and treatment. Quantitative polymerase chain reaction (QPCR) was used to confirm the direct interaction of lesioning and treatment versus no lesion and vehicle only treatment. A surprising result of the QPCR assay was a substantial increase in Presenilin 1 in the lesioned treated tissue in a majority of the animals tested.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGEMENTS

The past two years have been filled with new experiences in learning, both academically and in practical techniques. I am especially grateful to my Carleton supervisor, Dr. Mike Poulter for his energy, enthusiasm and patience in teaching me about molecular biology techniques. Dr. Joe Steiner, of Guilford Pharmaceuticals Inc. has a fountain of neuroimmunophilin knowledge, which he has generously and tirelessly shared with me throughout my Master's studies. The Poulter lab has many talented people who have shared their time, experience and knowledge with me. Dr. Lisheng Du and Jin Lu have shared molecular information with me in order to refine techniques. I would like to thank Dr. Bruce Hutcheon and Katie Cleland for demonstrating the 6-OHDA surgery. Dr. Kerstin Schwabe, from Bremen, Germany, helped me make modifications to the stereotaxic surgery and shared her vast experience during her stay, as well as providing much needed respite on the bike paths and ski hills in Ottawa.

I am thankful for my sons, Darryl and Jeremy who have completed their education early in life while supporting me in my academic mid-life crisis. I have also been fortunate to have a friend, Ellie Botner, who has assumed responsibility for many of the details surrounding my plans to relocate to Edmonton, Alberta while I was writing this thesis.

A final word of gratitude I have left for Dr. Bruce Pappas, my undergraduate supervisor and academic mentor, who has continued to encourage me to follow my academic dreams. His faith in my strengths and abilities continues to inspire me. Thank you, everyone, for your help.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF APPENDICES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ABD	Antibody diluting buffer
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
Bet1	Blocked Early in Transport 1
bp	base pair
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-O-Methyltransferase
CsA	Cyclosporin A
CREB	Cyclic-AMP element binding protein
Ct	Cycle threshold
C-terminus	Carboxyl terminal
Cy3	Cyanine 3
DA	Dopamine
DAPI	4',6-Diamidino-2-phenylindole
DAT	Dopamine transporter
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
Disp1	Dispatched protein 1
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DOPA	Dihydroxyphenylalanine
DTT	Dithiothreitol
dTTP	Deoxytimidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine-tetraacetic acid
EGCG	epigallocatechine-3-gallate (green tea polyphenol)
EST	Expressed sequence tags
FKBP	FK506 binding protein
Gas6	Growth Arrest Specific Gene 6
GDNF	Glial cell-derived neurotrophic factor
GPI	GPI-1046
GPI-1046	Glycosylphosphatidylinositol 1046
GSH	Glutathione
$H_2 O_2$	Hydrogen peroxide
HCl	Hydrogen chloride
Hrsp12	Heat responsive protein 12
HSC70	Heat shock cognate 70
Hsp	Heat shock protein
IL-2	Interleukin 2
IP	Intraperitoneal injection

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

IP_3	Inositol triphosphate
LC	Locus ceruleus
L-dopa	3,4-dihydroxyphenylalanine
LES	Unilateral 6-OHDA lesion
LTP	Long term potentiation
MAO-B	Monoamine oxidase B
MPTP	N-methyl-4-phenyl 1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
NF-AT	Nuclear factor of activated T cells
NICD	Notch intracellular domain
NIA	National Institute on Aging
NIH	National Institutes of Health
NILs	Neuroimmunophilin ligands
NMDA	N-methyl-d-aspartate
N-terminus	Amino terminal
Paxip1	Pax transcription activation domain interacting protein 1
PB	Phosphate buffer
PBS	Phosphate buffered saline
PD	Parkinson's disease
PS1	Presenilin 1
Ptp4a3	Protein tyrosine phosphatase 4a3
QPCR	Quantitative polymerase chain reaction
R-APO	R-apomorphine
RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SNc	Substantia nigra, pars compacta
Sptlc1	Serine palmitoyltransferase, long chain base subunit 1
SSC	Saline-sodium citrate
Syt11	Synaptotagmin 11
T	GPI-1046 Treatment
T cells	Thymic lymphocytes
Taq	*Thermus aquaticus* (bacterium)
T_C cell	Cytotoxic thymic lymphocytes
TE	Tris-ethylenediaminetetraacetic acid
TH	Tyrosine hydroxylase
T_H cell	Helper thymic lymphocytes
TX	Triton-X-100
Tyr	Tyrosinase
U	Unlesioned (no 6-OHDA)
UHN	University Health Network
UNL	Unlesioned (no 6-OHDA)
VEH	Intralipid vehicle
VMAT	Vesicle monoamine transporter
v-SNARE	Vesicle bound soluble N-ethylmaleimide-sensitive factor attachment protein receptor
VTA	Ventral tegmental area

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

INTRODUCTION

Parkinson's Disease (PD)

Idiopathic parkinsonism is the most common form of this neurodegenerative disease, accounting for more than 95% of the PD population (Mandel *et al.* 2003). In addition to cytoplasmic Lewy body inclusions, the most notable neuropathological characteristic of PD is degeneration of the neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (SNc) of the basal ganglia (Double *et al.* 2002, Jenner & Olanow 1996, Lai *et al.* 2003, Maimone *et al.* 2001). The SNc neurons have projections that traverse the medial forebrain bundle to stimulate D_1 and D_2 receptors in the caudate and putamen, which is collectively known as the neostriatum (Mink 2003). Hypokinetic extrapyramidal motor disturbances including bradykinesia, rigidity and tremor occur with a reduction in striatal dopamine (DA) of 80% or more (Lai *et al.* 2003, Maimone *et al.* 2001, Sherer *et al.* 2002). Cognitive and psychological impairments are also observed in PD due to degeneration of non-nigral areas such as the dopaminergic ventral tegmental area (VTA), cholinergic innervation from the nucleus basalis Meynert and noradrenergic locus ceruleus (LC) neurons (Blum *et al.* 2001).

The etiology of sporadic PD is unknown, although there is speculation that a combination of environmental and genetic factors as well as accelerated aging may be involved (Gatto *et al.* 2002, Revesz *et al.* 2001, Sherer *et al.* 2002). Only

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

rare PD cases are attributable to genetic causes, such as mutations of the α-synuclein and parkin genes, which are inheritable types of PD (Foltynie *et al.* 2002, Mandel *et al.* 2003, Tanner *et al.* 1999). In addition, the mechanisms underlying dopaminergic cell death in the SNc have not been clearly delineated. Evidence for the involvement of oxidative stress in the pathogenesis of PD has been found in postmortem studies showing increased iron, impaired mitochondrial function and decreased levels of reduced glutathione (GSH) and neurotrophic factors (Gu *et al.* 2002, Jenner & Olanow 1996, Mandel *et al.* 2003, Swerdlow 2002). Since there is no single identifiable cause for PD, symptomatic treatment rather than prevention is the focus of much parkinsonian related research.

Experimental Parkinsonism

Several *in vivo* animal models are currently used to investigate PD, including unilateral 6-hydroxydopamine (6-OHDA) lesions, N-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) injections and systemic infusion of the pesticide rotenone. Although neurotoxins are widely used to investigate neurodegenerative diseases, the usefulness of these models is limited because neurochemical alterations evoked by exogenous neurotoxins may not accurately reflect the progressive neuronal loss of PD or the contribution of non-dopaminergic systems (Mandel *et al.* 2003). However, comparison of neurodegenerative processes of animal models and human PD disease progression is expected to reveal some common biological processes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Systemic administration of MPTP crosses the blood-brain barrier (BBB) and is converted into an active metabolite 1-methyl-4-phenylpyridinium (MPP^+) in glial cells by monoamine oxidase B (MAO-B) (Nicotra & Parvez 2000). MPP^+ is transported into the cell by the dopamine transporter (DAT) where cytosolic MPP^+ enters the mitochondria, inhibiting complex I activity and resulting in decreased adenosine triphosphate (ATP) levels that lead to cell death (Speciale 2002). Other brain areas such as the VTA, LC and hypothalamus are also affected by MPTP treatment (Blum *et al.* 2001, Da Cunha *et al.* 2002). Animals with MPTP lesions also show neuropathologic abnormalities such as rigidity, tremor and akinesia (Nicotra & Parvez 2000).

Rotenone is highly lipophilic and easily enters the brain to inhibit mitochondrial complex I activity. Because rotenone does not depend on the DAT to enter cells, complex I inhibition is uniform in all brain cells. Interestingly, rotenone selectively destroys nigrostriatal dopaminergic neurons and causes the formation of α-synuclein and ubiquitin-containing cytoplasmic inclusions similar to Lewy bodies in SNc cells. Behavioral symptoms such as hypokinesia and rigidity are also observed in rotenone-lesioned experimental animals (Betarbet *et al.* 2000, Sherer *et al.* 2002).

The neurotoxin 6-OHDA is a hydroxylated analogue of dopamine that is taken into catecholamine neurons by the DAT where it destroys the neuron. The mechanism of toxicity is hypothesized to be similar to that of PD, including oxidative stress, elevation in iron levels and impaired mitochondrial function. Because 6-OHDA is unable to cross the blood-brain barrier and is not selective

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

for dopaminergic neurons, 6-OHDA is intracranially injected into the striatum, the substantia nigra or the medial forebrain bundle to preferentially destroy SNc dopaminergic neurons (Blum *et al.* 2001). The advantage of unilateral lesions is that they produce quantifiable asymmetric rotations after systemic injection of amphetamine or apomorphine (Schwarting & Huston 1996) and no obvious physical impairments to the experimental animals as a result of lesioning. In addition, the two hemispheres can serve as both experimental and control in the same animal, although some contralateral effects are possible.

Treatment for PD

Experimental *in vivo* animal models using neurotoxins allow the researcher to explore the degenerating process as well as therapeutic interventions in PD (Speciale 2002). One distinctive feature of PD among neurodegenerative diseases is that it has an effective pharmacological symptomatic treatment (Maimone *et al.* 2001). Levodopa (3,4-dihydroxyphenylalanine or L-dopa), the precursor to dopamine was introduced as a treatment for PD in the 1960s. Although there is evidence that L-dopa may be neuroprotective, L-dopa toxicity can occur with chronic L-dopa supplementation. Symptoms of L-dopa toxicity include motor complications such as dyskinesia and dystonia, which can interfere with normal daily activities. L-dopa may be combined with a dopa decarboxylase inhibitor to reduce peripheral side effects. Notwithstanding the motor

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

complications, L-dopa remains the gold standard symptomatic medical therapeutic for PD (Gatto et al. 2002, Katzenschlager & Lees 2002). Enhancement of L-dopa therapy has been observed using a combination of therapies which may reduce the likelihood of developing L-dopa related complications. Treatment with dopamine receptor agonists is therapeutically less effective but has the advantage of fewer side effects. Selegiline is a selective MAO-B inhibitor that may have neuroprotective properties when combined with L-dopa treatment. Amantadine is an anticholinergic drug that has been shown to enhance dopamine release when used as an adjunct to L-dopa. Glutamate antagonists such as remacemide and riluzole, specific for blocking NMDA receptors have shown some benefit to decrease dyskinesias due to chronic L-dopa treatment (Gatto et al. 2002).

Neuroregeneration in Parkinsonism from growth factors such as brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) has been shown in animal models. However, neurotrophic factors exhibit low penetration of the BBB, lack oral bioavailability and demonstrate abnormal neuronal sprouting (Hamilton & Steiner 1998, Ogawa & Tanaka 2003, Tanaka et al. 2003). Recently, a study using GDNF infusion into the putamen of Parkinson patients was halted as some patients were producing antibodies to the GDNF protein (Gill et al. 2003, Pearson 2004). GPI-1046, a prototype non-immunosuppressant analog of the immunosuppressant drug tacrolimus (FK-506), has been found to exhibit similar benefits to growth factor treatment with fewer adverse events. GPI-1046 and a related molecule GPI-1485 (with oral

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

bioavailability) have high penetration of the BBB with no aberrant sprouting. In addition, these molecules accelerate neurite outgrowth, enhanced neurotrophin manifestation and activation of antioxidant and anti-inflammatory expression in both *in vivo* and *in vitro* experimental conditions (Campbell *et al.*1999, Gold *et al.* 2004, Steiner *et al.* 1997a and 1997b, Tanaka *et al.* 2002 and 2003, Zhang *et al.* 2001).

Neuroimmunophilin Ligands

Immune responses at the cellular level are initiated by white blood cells such as lymphocytes and neutrophils. Activation of immune responses by calcium-activated calcineurin occurs through dephosphorylation of the nuclear factor of activated T cells (NF-AT). Dephosphorylated NF-AT is translocated to the nucleus where it regulates interleukin-2 (IL-2) gene transcription (Dawson 1996). Cytokines such as IL-2 activate helper thymic lymphocytes (T_H cells) as well as activating cytotoxic T lymphocytes (T_C cells) that attack foreign or infected cells (Campbell *et al.* 1999, Liu *et al.* 1991).

After skin grafting or organ transplantation it is desirable to suppress immune responses to prevent tissue rejection. Immunosuppressant drugs such as FK506, cyclosporin A (CsA) and rapamycin have been found to suppress immune responses at the cellular level. The immunophilin ligands have an effector domain for the phosphatase calcineurin as well a docking site for small, cytosolic proteins called immunophilins such as cyclophilin or the 12-kDa FK506-binding protein (FKBP12). Drug-immunophilin complexes are formed by cyclosporin A

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and cyclophilin or by FK506 and FKBP12. Either of these protein complexes will inhibit the phosphatase activity of calcineurin and reduce IL-2 transcription and subsequent T cell activation (Harding *et al.* 1989, Siekierka *et al.* 1989). Another immunosuppressant, rapamycin also binds with FKBP12 without interacting with calcineurin. The rapamycin-FKBP12 complex blocks the actions of IL-2 rather than its transcription (Cameron *et al.* 1995a and 1995b).

In addition to their role in immune response and suppression, immunophilin ligands have been found to play a role in protein folding. The enzymatic activity of cyclophilins and FKBP12 involves catalyzing a slow *cis-trans* (180°) isomerization of proline peptide bonds (peptidyl-prolyl cis-trans isomerase or rotamase) in oligopeptides during protein synthesis (Fischer *et al.* 1989, Helekar *et al.* 1994, Takahashi *et al.* 1989). This rotamase activity may be a regulatory one to stabilize channels and optimize calcium release into the cytoplasm. Inhibition of rotamase activity by FK506/FKBP12 binding may affect the conformation of ion channels or receptors and increase calcium levels in the cell (Costantini & Isacson 2003).

FKBP12 is co-localized with calcineurin and is 10 to 50 fold more abundant in the central nervous system than in the immune system (Steiner *et al.* 1992). High levels of immunophilins are found in the hippocampus, nigrostriatal dopaminergic pathway and granule cells of the cerebellum as well as in peripheral nerve areas (Hamilton & Steiner 1998). Following neuronal injury or lesioning elevated levels of messenger ribonucleic acid (mRNA) for FKBP12 are found surrounding the lesioned area. Immunophilin ligands exhibit high-affinity

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

binding to immunophilins and demonstrate neuroprotective as well as neurotrophic properties at lesion sites (Costantini & Isacson 2000, Gold *et al.* 1998, Gold *et al.* 1999, Guo *et al.* 2001a, Guo *et al.* 2001b, Klettner *et al.* 2001, Sauer *et al.* 1999). Chronic use of immunosuppressive drugs, however, can result in serious complications of neurologic function (Eidelman *et al.* 1991). As a result, glycosylphosphatidylinositol 1046 (GPI-1046), a non-immunosuppressive neuroimmunophilin ligand (NIL) devoid of a binding site for calcineurin was developed to reduce adverse side effects but maintain neurotrophic and neuroprotective characteristics (Tanaka *et al.* 2002).

Current knowledge of how NILS produce neurotrophic effects is limited. It now appears that rotamase activity is not necessary for neuroregenerative activity and research has revealed that other larger FKBPs are involved in some regenerative functions (Klettner *et al.* 2001, Gold *et al.* 1999, Gold *et al.* 2004, Steiner *et al.* 2003). Tanaka *et al.* (2002) pretreated SH-SY5Y human neuroblastoma cells expressing FKBP12 mRNA, and U251 human glioma cell lysate, which did not express FKBP12 mRNA with GPI-1046. The researchers found that after exposing cells to hydrogen peroxide (H_2O_2), GSH levels were increased in cells pretreated with GPI-1046 in both cell lines. Cell viability was also increased compared to the DMSO-treated control group. This would indicate that the antioxidant and neuroprotective properties are not dependent on FKBP12 binding (Tanaka *et al.* 2002). Gold *et al.* (1999) found that high molecular weight NILS such as FKBP52 bind with heat shock protein 90 (Hsp90) implicating the steroid receptor complex in neuroprotection and neuroregeneration.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Tanaka *et al.* (2003) found that GPI-1046 treatment of normal mice increased GDNF and the GDNF family receptor α-1 (GFRα-1) expression levels in the substantia nigra but not the striatum, suggesting that some neurotrophic activity of NILS may be dependent on GDNF activation. The outcome of the *in vivo* studies indicates that NILS may act indirectly by enhancing neurotrophins through a convergence of the neurotrophic and NIL pathways directly, through intracellular calcium release from IP_3 channels to stimulate downstream transcription factors, or through interactions with several FKBPs to produce neurotrophic and regenerative function to damaged areas (Steiner *et al.* 2003).

Microarray Analysis

As outlined above, many pathways may be involved in both the neurodegeneration of PD or neurotoxic lesions and regeneration due to NIL treatment. Recently, complementary deoxyribonucleic acid (cDNA) microarray technology has been developed to study the gene expression profiles of 15,000 gene sequences simultaneously (Tanaka *et al.* 2000). Using microarray technology it is possible to investigate changes in messenger ribonucleic acid (mRNA) expression due to oxidative stress, inflammatory responses, glutamate toxicity, iron accumulation, nitric oxide, reduced neurotrophic factors or other cellular responses concurrently in order to develop better neuroprotective and therapeutic strategies for neurodegenerative disorders such as PD (Cadet *et al.* 2001, Grünblatt *et al.* 2001, Khan *et al.* 1999, Mandel *et al.* 2003, Napolitano *et*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

al. 2002). Microarray studies to date that identify changes in mRNA expression relating to PD include MPTP models (Grünblatt *et al.* 2001, Miller *et al.* 2004, Xu *et al.* 2004, Youdim *et al.* 2002), 6-OHDA lesions (Lai *et al.* 2003, Napolitano *et al.* 2002), methamphetamine neurotoxicity (Cadet *et al.* 2001) as well as human PD studies (Grünblatt *et al.* 2004). In addition, treatments for PD such as L-dopa (Ferrario *et al.* 2004), neuroprotective strategies such as R-apomorphine (R-APO) (Grünblatt *et al.* 2001) and green tea polyphenol epigallocatechine-3-gallate (EGCG) (Youdim *et al.* 2002) as well as melatonin (Weinreb *et al.* 2003) have employed microarrays to identify changes in mRNA expression.

Several articles have addressed functional genomic changes due to MPTP lesioning using cDNA microarrays (Grünblatt *et al.* 2001, Miller *et al.* 2004, Xu *et al.* 2004, and Youdim *et al.* 2002). Grünblatt *et al.* (2001) used the MPTP mouse model of PD to investigate global gene expression in the SN and striatum. Fifty-one genes (out of 1200 cDNA probes) were differentially expressed ($p<.05$) and 18 genes were additionally analyzed using real-time quantitative polymerase chain reaction (RT-QPCR). Results were further validated by pretreatment with R-apomorphine, which was found to attenuate most of the changes due to oxidative stress and inflammation as a result of MPTP lesioning. The authors found that dopaminergic neurodegeneration is complex and involves more systems than oxidative stress could explain. The researchers also revealed evidence of cellular protective measures such as upregulation of neurotrophic factors and anti-inflammatory cytokines. The same researchers (Youdim *et al.* 2002) extended this work by comparing acute and chronic changes in mRNA

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

expression as well as a comparison of neuroprotective drug treatments. Through microarray analysis they were able to identify differences between early effects of MPTP lesioning and late effects leading to neuronal degeneration as well as identifying the neuroprotective effects of R-APO in the PD model. As expected, many of the changes observed involved oxidative stress, inflammatory events, glutamate excitotoxicity, nitric oxide (NO), and iron as well as neurotrophic factors.

The information obtained from microarray studies is clearly based on the type of probes printed on the array as well as the sophistication of the analysis software available. Xu *et al.* (2004), using a mouse MPTP model, found decreased levels of DA, and its metabolites (DOPAC, HVA), dopamine transporters, and vesicle monoamine transporter (VMAT) expression with no change in monoamine oxidases (MAO-A, MAO-B) or catechol O-methyltransferase (COMT). Through microarray analysis, this study concluded that the reduction in DA metabolites was due to reduced monoamine levels rather than increased metabolic enzymes. Furthermore, Miller *et al.* (2004) were able to combine microarray analysis with sophisticated integrative data mining software (Affymetrix Data Mining Tool 3.0) to identify small, yet significant changes in mRNA for cytoskeletal stability and maintenance, synaptic integrity as well as cell cycle and apoptosis dysregulation after MPTP lesioning.

Researchers using the 6-OHDA model of PD have also published the results of microarray experiments. Napolitano *et al.* (2002) used cDNA microarrays to investigate unilateral 6-OHDA lesions and found 50 differentially

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(>2-fold) expressed genes (out of 1176 cDNA probes) in the striatum of Wistar rats. These researchers found profound changes in kinases involved in the phosphorylation state of dopamine signaling molecules rather than protein concentration. Genes regulating serine/threonine and tyrosine protein kinase and phosphatase were down regulated after 6-OHDA treatment. Western blot analysis was used to confirm to phosphorylation status of proteins. It is interesting that this study did not find large changes in protein expression to coincide with the down-regulation of mRNA levels. Many studies to date have used QPCR to validate mRNA changes generated by microarray data without verification that mRNA changes resulted in increased protein levels. As evidenced by this study, one caveat of microarray studies is that mRNA changes do not necessarily translate into functional changes brought about by alterations in protein levels.

A microarray study to examine differential gene expression after L-dopa treatment (Ferrario *et al.* 2004) identified genes involved in neurotrophic mechanisms and plasticity. *In situ* hybridization confirmed that elevated levels of growth factor pleiotrophin, myelin basic protein and calmodulin are expressed in the denervated striatum of L-dopa treated rats. Weinreb *et al.* (2003) demonstrated neuroprotective and antiapoptotic patterns of gene expression at low concentrations (1-10 μM) of R-APO, DA, EGCG and melatonin but proapoptotic activity at higher concentrations (50-500 μM). Real-time QPCR and protein profiles were used to confirm the results. In addition, this research established that similar gene expression and protein profiles exist for the four neuroprotective drugs tested.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The research outlined here is an investigation of the changes in mRNA levels with 6-OHDA lesioned rats that were treated with GPI-1046. As a control, a cohort of lesioned rats was also treated with vehicle (intralipid) only. Previous research using GPI-1046 treatment after unilateral 6-OHDA lesioning in rats has demonstrated regenerative sprouting from spared dopaminergic cells (Steiner *et al.* 1997b), restoration of corticostriatal LTP (Zhang *et al.* 2001), increased striatal tyrosine hydroxylase (TH) immunoreactivity in the ipsilateral striatum in the absence of increased SNc neurons (Ross *et al.* 2001) and a decrease in duration of amphetamine-induced circling (Harper *et al.* 1999). These results indicate that there is substantial rearrangement of striatal neurons after treatment that restores function. Therefore, we were interested in determining what gene expression changes may occur due to immunophilin treatment. Here, we investigated the mRNA changes that may contribute to the physiological and behavioral changes observed by other researchers.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

METHODS

Animal Surgery and GPI-1046 Treatment

Five rounds of surgery were necessary in order to generate tissue samples for sixteen microarray slides. Sprague Dawley rats weighing approximately 250 grams received unilateral microinjections of 6-OHDA according to Carleton University Animal Care Committee Protocol P03-3. Surgical anesthesia was induced with either Halothane (0.5-3% in oxygen) (Rounds 1-4) or Isopropane administration (2.0% in oxygen) (Round 5) alone or in combination with intraperitoneal (IP) Somnotol injection (≤ 40 mg/kg). The injection target was the medial forebrain bundle at stereotaxic co-ordinates bregma, 0.45 cm; lateral, 0.20 cm; dura, 0.78 cm (Paxinos & Watson 1998). All rats received 6-OHDA dissolved in 0.04% ascorbic acid sterile saline injected with a Hamilton syringe at a rate of 0.5 µl/minute.

Two weeks after surgery each rat received an IP injection of the indirect DA agonist, d-amphetamine (2.5 mg/kg in sterile saline) and rats were observed for rotational behavior. Fifteen minutes after amphetamine injection each rat was placed in a large, round, wide-mouth, plastic testing apparatus for fifteen minutes and the number of turns clockwise or counterclockwise were recorded while the tester was blind to treatment group identity. Other observations consistent with amphetamine intoxication, such as piloerection and tail posture were also recorded during this period.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Rats were randomly selected to receive a subcutaneous injection of GPI-1046 (10 mg/kg) treatment or intralipid with 2% ethanol only (vehicle). No more than 5 days after rotational behavior was observed, each animal received seven daily injections of GPI-1046 (10 mg/kg) dissolved in ethanol and mixed with Intralipid (20%). Ten days after the last GPI-1046 injection the rats were again tested for rotational behavior.

Because few rats exhibited rotational behavior in the first two rounds of surgery, we doubled the total 6-OHDA injected by increasing the volume to 4 μl (2 μg/ μl) or increasing the concentration (4 μg/ μl) in 2 μl total volume for the third round of surgery. Since three of the four rats that received 6-OHDA with increased concentration in lower total volume displayed some circling behavior, the total volume of neurotoxin was reduced to 1 μl (4 μg /μl) in the fourth round of surgery. It was expected that the lower volume of toxin would decrease pooling effects in the target area and more precisely target the fibers of the medial forebrain bundle.

Although the experiment had originally been expected to utilize six to eight microarray slides, this number was doubled to sixteen after further discussion with Gary Glonek (February 2004, personal communication), one of the authors of Factorial Designs for Microarray Experiments (Glonek & Solomon 2004). By doubling the number of slides, we were able to substantially increase the residual degrees of freedom available. It was therefore necessary to generate more experimental RNA and therefore a fifth round of surgery was initiated. This time we used 2 μg 6-OHDA in 1 μl of 0.04% ascorbic acid in sterile saline.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Tissue Preparation

Animals in Round 1-3 were sacrificed with carbon dioxide (CO_2), brains were removed and the dorsal striatum isolated, sliced and flash frozen on dry ice. However, only a few rats had large enough lesions to be identified by observing ipsilateral rotations after d-amphetamine injections. Since large lesions usually leave few surviving dopamine cells, it was desirable to find another way to quantify the size of the lesion. Immunostaining TH cells would allow us to estimate the number of surviving cells in the SNc. Initially, we took the freshly harvested midbrain and drop fixed the tissue in Lana's solution. However, high background signals and non-specific staining due to a large number of blood vessels prevented clear identification of TH-stained cells.

Conventional methods of perfusing tissue do not preserve RNA integrity. It seemed logical that since diethylpyrocarbonate (DEPC) - treated water preserves RNA integrity, perfusion with DEPC-treated 0.1M phosphate buffered saline (PBS) would not result in degradation of the RNA. A comparison of the quality of RNA using a QPCR based assay showed that the RNA from DEPC-treated PBS perfused animals was indistinguishable from tissue that was immediately frozen at -80°C without perfusion. We tested samples of RNA extracted from DEPC-treated PBS perfused animals and found that the RNA samples had normal yields and spectrophotometric readings. In addition, DEPC-treated PBS perfused midbrains were found to have clearly defined TH-immunostained cells suitable for counting the number of surviving cells in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

SNc. Therefore, all animals sacrificed in Round 4 and 5 were given 1-2 ml Somnotol and perfused with 60 ml DEPC - treated 0.1M PBS.

After perfusion, the striatum was quickly removed from the brain, taking care that the lesioned and unlesioned sides were placed separately in labeled 1.5 ml centrifuge tubes and saved on dry ice until stored at -80°C. The perfused midbrains were dropped into Lana's solution [containing 20% paraformaldehyde (pH 7.2), 0.1M phosphate buffer (pH 7.2) and saturated picric acid]. After one week, fixed brains were transferred to 30% sucrose and stored at 4°C.

Immunohistochemistry

In order to count cells in the SNc, the midbrains fixed in Lana's solution and stored in 30% sucrose were sliced using a Thermo Shandon electronic cryotome. Each 40-micron slice was stored in cryoprotectant containing ethylene glycol, sucrose and 0.1 M phosphate buffer (PB) and stored at -20°C until used for immunostaining with tyrosine hydroxylase (TH) antibody.

In order to incubate the tissue slices with primary TH antibody, the slices were first washed individually in phosphate buffered solution (PBS) three times for 5 minutes with agitation followed by a 30 minute wash with PBS and 0.2% Triton-X-100 (TX). The TH antibody (mouse monoclonal antibody against TH, DiaSorin, Stillwater MN) was mixed with antibody diluting buffer (ADB) in a ratio of 1:5,000 and mixed with 0.2% TX. The tissue in primary TH antibody was incubated overnight at 4°C with agitation. The next day another 3 washes with

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PBS and TX for 10 minutes at room temperature were followed by 2-hour incubation in darkened conditions with secondary antibody (Cy3-conjugated donkey anti-mouse IgG, Jackson Immuno Research, USA) in PBS in a ratio of 1:10,000. The second incubation was followed by three washes for 10 minutes each in PB (no sodium chloride) and TX. Each slice was then transferred to a microscope slide and a few drops of antifade with DAPI were washed over the slide for 3 minutes with gentle agitation. Coverslips were mounted using a few drops of antifade reagent in PBS. Nail polish was used to seal the coverslip on the slide. TH staining was viewed using a Leica DM RXA fluorescent microscope and cells containing a nucleus were counted and recorded. TH stained cell numbers were counted on both the lesioned and unlesioned side. Tissue samples from three serial sections were counted from each midbrain and those with at least 20-50% surviving cells on the lesioned side were selected for microarray analysis.

RNA Extraction and Isolation

Striatal RNA was isolated using TRIzol reagent (a mono-phasic solution of phenol and guanidine isothiocyanate) according to the manufacturer's protocol (Invitrogen Canada Inc. Burlington ON). Briefly, up to 100 mg of frozen striatum was homogenized in 1 ml of TRIzol using a roto-stator. After 5 minutes at room temperature, 200 µl of chloroform was added to the homogenized mixture and the tube was shaken for 15 seconds. After 3 minutes at room temperature the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

tubes were centrifuged for 15 minutes at 12,000 rpm at 4°C. The mixture separated into three layers with the top, aqueous layer containing the RNA. The top layer was carefully transferred to a clean microfuge tube and mixed with 500µl isopropyl alcohol. After ten minutes at room temperature the tubes were centrifuged for 10 minutes to produce a small RNA pellet. The supernatant was removed by aspiration and the pellet washed with 75% ethanol (in DEPC-treated water). The mixture was centrifuged for 5 minutes, the supernatant removed and the pellet dried at room temperature for 5-10 minutes. The pellet was then redissolved in 20µl RNase free water. A 1 µl sample of total RNA was added to 99 µl of 1X tris-ethylenediaminetetraacetic acid (TE; 10 mM Tris-HCl, 1 mM EDTA) buffer and analyzed using a Bio-Rad Smart Spec 3000 spectrophotometer. RNA samples with a ratio between 1.9 and 2.1 of the wavelength readings at 260 nanometers (nm) and 280 nm on the spectrophotometer were considered to be good quality for further analysis. One sample of RNA could not be sufficiently purified (BH-unlesioned side) and another sample was substituted (BF-unlesioned side). Unused RNA was stored in 50 % ethanol solution at -80 °C.

cDNA Microarray Preparation

Some of the methodologies used in this research were well established at the Institute of Neuroscience at Carleton University. However, microarray technology has developed over the past decade and was not an established technique at the Institute. This research embraced both establishing a working

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

protocol for microarray hybridization and analysis as well as the application of microarray technology. The microarray slides used were purchased from the Microarray Center at The Ontario Cancer Institute, University Health Network (UHN) at the University of Toronto. The 15K mouse cDNA slides were chosen because of the large number of unique sequence probes (15,264 NIA verified sequences), the large size of the probes [average size is 1.4 – 2.5 kilo bases (kb)] and the adjacent duplicate spotting of each sequence. Small sequence differences between the two species (mouse and rat) were not expected to appreciably affect binding of the probes due to the size of the clones. The 15K mouse array came with a protocol for direct labeling of the cyanine dyes to the complementary DNA (cDNA) strands generated from total RNA in a reverse transcription (RT) reaction. However, hybridized arrays initially exhibited weaker fluorescent signals on the cyanine 5 channels after laser scanning using the UHN protocol.

Another type of fluorescent dye is the Alexa dye which is reputed to emit more fluorescence than cyanine dyes and is less likely to lose fluorescence due to photobleaching after laser scanning (Panchuk-Voloshina *et al.* 1999). In an effort to increase the fluorescent signal of the hybridized arrays we followed the protocol for direct labeling with Alexa 546 and Alexa 647 deoxycytidine triphosphate (dCTP) (Molecular Probes, Inc. Eugene OR USA). Several attempts to use the Alexa dyes were unsuccessful with little or no binding of Alexa 546 and no fluorescence with Alexa 647. However, after learning that Alexa fluors are sensitive to pH outside the range of 4-10, we discovered that CyDye products

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

are also sensitive to basic conditions above pH of 8 (Amersham Biosciences, Piscataway NJ USA).

In addition to cyanine and Alexa direct labeling, we experimented with an indirect labeling protocol using 3DNA Array 50 Expression Array Detection Kit (Genisphere, Inc Hatfield PA USA). One benefit of 3DNA labeling is that fluorescent dyes are added after clean-up reactions where high pH could decrease their potency. Unfortunately, high background levels prevented further use of the protocol, but we were able to exploit a portion of the Genisphere protocol by using 0.5M NaOH/50 mM ethylenediamine-tetraacetic acid (EDTA) with the UHN direct labeling. The UHN protocol for RT reaction was left unchanged, but the stop reaction/probe cleanup portion of the protocol was amended as per the Genisphere protocol. This resulted in slightly higher expression levels as well as fairly low background signal (See Table 1).

The following protocol was used for all sixteen experimental microarray hybridizations:

Reverse transcription to directly label cDNA with either Cyanine 3 (Cy3) or Cyanine 5 (Cy5) was done in a 40 µl reaction tube containing 1X First Strand reaction buffer, 3.75 µM anchored dT primer, 1.5 nM deoxynucleotide triphosphates (dNTPs) (500 nM each dATP, dGTP, dTTP), 50 pM dCTP, 25 pM Cy3 or Cy5 dCTP, 10 nM DTT, 10 µg total RNA and RNase free water to 37 µl. The reactions were incubated at 65°C for 5 minutes to denature the RNA and then the reaction was cooled to 4°C while 400 Units of reverse transcriptase enzyme was added along with 20 Units of RNase inhibitor. The enzymatic mixture was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

incubated at 42°C for 2.5 hours. During the RT reaction mRNA strands were reverse transcribed into fluorescently labeled cDNA.

The incubated reactions were placed on ice and 7 µl 0.5M NaOH/50mM EDTA was added to each tube to stop the RT reaction. The tubes were incubated at 65°C for 10 minutes, placed on ice and 10 µl 1M Tris-HCl (pH 7.5) was added. The two tubes containing Cy3 mixture and Cy5 mixture were combined into one tube and 16 µl 10 mM Tris (pH 8.0)/1 mM EDTA was added. A Microcon YM-30 filter device was used to concentrate the 130 µl mixture to ~5 µl by adding 100 µl 1X TE (pH 8.0) to the Microcon reservoir and centrifuging at 14,000 x g for 3 minutes. The RT reaction was then added to the Microcon reservoir and centrifuged for 10 minutes more. The tube was removed from the reservoir and replaced with a clean one. Next, 5-µl 1X TE buffer (10 mM Tris-HCl, pH 8.0/ 1 mM EDTA) was added to the reservoir and the sides of the reservoir were tapped gently. The reservoir was placed upside down in the clean microfuge tube and centrifuged for 2 minutes. The concentrated cDNA samples were then kept on ice while the hybridization solution was prepared according to UHN protocol by combining 5µl yeast transfer RNA and 5-µl calf thymus DNA with 100 µl DIG Easy Hyb solution. This was heated for 2 minutes at 65°C then 60µl of the Easy Hyb mixture was added to the concentrated cDNA (~5µl). This was incubated for 2 minutes at 65°C and the heated mixture was pipetted onto a preheated microarray slide. After applying a coverslip the slide was placed in a dark, moist hybridization chamber and incubated overnight (8-18 hours) at 37°C.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The next day the slides were rinsed in 1X saline-sodium citrate buffer (SSC) and then washed 3 times with gentle agitation for 10 minutes at 50°C in 1X SSC, containing 0.1% sodium dodecyl sulfate (SDS). After washing, the slides were rinsed in 1X SSC, centrifuged briefly to dry and scanned using a GenePix 4000B Microarray Scanner (Axon Instruments Inc., Union City CA). GenePix Pro 4.0 Array Acquisition and Analysis Software (Axon Instruments Inc., Union City CA) was used to generate microarray data including the ratios of the 532 nm wavelengths for Cy3 and 635 nm wavelengths for Cy5.

Experimental Design

The experiment was designed as a 2 x 2 factorial microarray experiment, with four possible conditions, having two factors (Lesioning and Treatment) with two levels of each factor (6-OHDA lesion, right side or no lesion, left side) (GPI-1046 treatment or intralipid vehicle treatment). For statistical analysis each gene is analyzed separately, but the analysis is done simultaneously for each gene on the two-colour array. We were interested in the genes changing due to treatment alone, lesioning alone and any interaction effects of lesioning and treatment. The design allows for both cyanine 3 and cyanine 5 readings at each juncture and a direct comparison of each experimental condition (Glonek & Solomon 2004, Yang & Speed 2003) (See Appendix I). In addition we used a dye swap for six of the eight possible microarray combinations in order to directly compare any dye effects.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A similar design was used by Jin *et al.* (2001) using SASISTAT software for analysis of the ANOVA model. Using 2 fixed factors (sex and genotype) and two time points, these authors used 24 microarray slides (with dye flips) in order to identify significant changes in expression with a 1.2 fold difference.

Statistics

The first step in microarray analysis involved a visual inspection of the array image and flagging of spots that were irregular. Next, absolute expression levels were inspected with an automatic flag feature built into the GenePix programme.

The raw data information was then imported to Acuity 4.0 software (Axon Instruments Inc, Union City CA) and group data was normalized. Normalization is necessary due to the possibility that systematic error may occur when analyzing 15,000 genes at once. Systematic error may be due to dye bias, high background levels due to irregular processing of the microarray slide or spots that are larger or smaller than normal due to printing irregularities. Normalization can be done on a local or a global basis. Local normalization is usually considered more accurate but global normalization can be used when background is uneven. Since all spots are duplicated (adjacently) the average of the duplicates is calculated to obtain the spot intensity on the slide for each gene. Acuity 4.0 software compared the ratio of Cy5/ Cy3 over a number of microarray slides for each spot and tested the significance of genes expressed differently (either increased or decreased) across

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

experiments using a simple t-test. P-values < .05 were used to determine if the spot differences between slides were significant. Those genes found to be significantly different were considered for further testing using QPCR.

Expression levels for Cy3 and Cy5, as measured by the microarray scanner, range between 0 and 65,000. With the wide range of possible expression levels one consideration in our data analysis was the problem that genes with low expression levels have greater inherent error and higher ratios than highly expressed genes. Highly expressed genes are less likely to meet the 2-fold cut off even when they have large differences in expression levels. A large number of replications of the microarray experiments are not possible due to the high cost of the printed slides. An article by Mutch *et al.* (2002), which considers absolute expression levels in calculating fold change, provided useful information about the limitations of the 2-fold cutoff in choosing which genes to test with QPCR. Since we were interested in novel genes, which could have both high and low expression level changes, p-values were chosen over fold change to indicate significant changes in mRNA expression in this research.

Real-Time QPCR

Genes identified as changing are typically confirmed as being different by several methods including *in situ* hybridization, Western blot analysis and real-time quantitative polymerase chain reaction (QPCR) (Cadet *et al.* 2001, Grünblatt *et al.* 2001). From a list of 77 genes that were considered differently expressed in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

our initial analysis, we selected ten mRNA sequences for QPCR validation. Primers of 18-22 base pairs were designed to detect and amplify a 100 base pair (bp) amplicon. In order to verify that the primer was able to faithfully reproduce amplicons, a standard curve was generated for each primer. Six dilutions (1/10 – $1/10^{-5}$) (in duplicate) of 100 ng whole rat brain RNA or unlesioned striatal RNA were used to test primer efficiency. Primers with 100% efficiency were expected to demonstrate a decrease in the cycle threshold of approximately three cycles with each dilution. Primers with efficiency less than 80% were redesigned and another standard curve generated. Primers for synaptophysin were used to normalize all the QPCR data.

We used ~ 2 µg of total RNA preserved from the microarray hybridization to prepare cDNA in a 20 µl reverse transcription reaction. QPCR assays were run in a 25 µl reaction containing iTAQ SYBR Green supermix with ROX [Bio-Rad Laboratories (Canada) Ltd. Mississauga ON] (2X reaction buffer containing 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.8 mM dUTP, iTAQ DNA polymerase, 50 units/ml, 6mM Mg^{+2}, SYBR Green I dye,1 µM ROX internal reference dye, and stabilizers), 1 µM sense and antisense primers, 2 ng cDNA and DNase/RNase free water to dilute supermix to 1X concentrate.

The reaction was initially heated to 95°C for three minutes to activate the iTAQ DNA polymerase, followed by 15 seconds at 95° C then the temperature was lowered to 55°C for 45 seconds. The latter two cycling conditions were repeated for 45 cycles. Heating the reaction to 95°C for 15 seconds denatures the two strands of the DNA double helix. The bacterium *Thermus aquaticus* (Taq)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DNA polymerase is an enzyme that catalyzes the synthesis of cDNA strands from the primers and nucleotides in the reaction tube. Because the Taq bacteria live in hot springs, they are not destroyed by the denaturing temperatures and they are active at a temperature above the annealing temperature. The optimal temperature for Taq is 72°C when nucleotides are selectively added onto the 3' end of the primer to replicate the desired DNA segment. Since ~ 50,000 nucleotides can be added in a minute, it takes only a fraction of a second to produce the 100 bp strand. When the temperature is further reduced to 55°C, two complementary DNA strands anneal to form a DNA double helix (Becker *et al.* 2000).

Each cycle doubles the amount of DNA in the tube and the sequence of interest is amplified exponentially. After each cycle a fluorescence reading of SYBRGreen, a nucleic acid stain that fluoresces when bound to double strand DNA, is recorded by the Stratagene MX4000 software. Amplification curves generated by the QPCR plot number of cycles versus fluorescence and indicate the cycle threshold (Ct). The fold change levels in RNA can be calculated based on differences in the number of cycles required to reach the cycle threshold.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

RESULTS

Lesion Assessment

In this experiment there was a great deal of variability in rotational behavior. Large lesions leave few surviving cells but are identifiable by quantifiable ipsilateral (to the lesion) rotations. It was necessary to have a small number of surviving neurons in the SNc in order to effectively treat with GPI-1046 so lesion size was further defined by counting the numbers of tyrosine hydroxylase (TH) immunoreactive cells in the SNc. Rats were selected for microarray analysis that had at least 20% TH stained neurons in the lesioned SNc.

In order to best match the lesion between groups we chose three serial sections from each dissected brain. The sections were stained with TH antibody and examined with 40X magnification in order to measure the lesion size. Immunostaining indicated 100% lesions on two rats in Round 4 so these were also not used for microarray hybridization. Round four generated enough tissue for eight microarray hybridizations. In Round 5, rats displaying some preference for ipsiversive turning and with dopamine cell loss in the SNc estimated between 50-80% were selected for microarray analysis (See Figure 1).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

RNA Isolation, Microarray Hybridization and Analysis

A total of 16 microarray slides, from three separate UHN printing batches were used for the experiment. Some of the hybridized microarray slides demonstrated high Cy3 background in the lower quadrant of the slide near the barcode. Since background is calculated on a local rather than a global basis, spot signals in this area of the slide were usable. One slide was over six months old when used and background was very high on this array. All arrays were checked for quality control. Flagged features (unfound) due to low signal intensity, high background noise or substandard printing were usually 20-50% of the total spots.

Data generated by Gene Pix Pro 4.0 was imported to Acuity 4.0 and p-values for significant changes between experimental conditions were generated from Gene Pix Pro ratios. The group data was normalized in the Acuity programme (See Table 2). The highest number of changes in gene expression levels was observed in the interaction of the two main effects, lesioning and treatment. Fifty-five sequences showed significant changes when both lesioning and treatment were considered (See Table 3). Twenty sequences were unknown or expressed sequence tags (ESTs). It is interesting to note that ratios increased in 26/55 and decreased in 29/55 of the cases where only one of lesioning or treatment was considered, but levels returned to normal when both or neither were included (unlesioned/vehicle vs. lesioned/treated), suggesting an equilibrium effect of treatment after lesioning, but not in the absence of cellular stress. Eight

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mRNA sequences were selected from this group for QPCR assays. Nineteen genes were differently expressed as a main effect of treatment with 14 increasing due to treatment and five decreasing after treatment. Seven of these were expressed sequence tags (ESTs) with no known function. Two of the known sequences from the treatment group, Growth Arrest Specific Gene 6 (Gas 6) and Blocked Early in Transport 1(Bet1) were selected for QPCR testing. When differences due to the main effect of lesioning were considered, only three mRNA sequences had significant changes, two were ESTs. No mRNA sequences were selected from this group for QPCR testing.

Rationale for Specific Genes Investigated

See Appendix 2

Real-Time QPCR

From the list of genes changing due to main effects or interaction effects, ten gene products were selected for their biological significance and tested in a QPCR assay. Primers were designed from rat mRNA sequences due to their biological interest. Only the cross design Unlesioned/Vehicle (UNL/VEH) versus Lesioned/GPI-1046 treated (LES/GPI) was tested in the QPCR assay. As noted earlier, this interaction effect seems to modulate the disruption observed by either lesioning or treatment in the LES/VEH vs. UNL/GPI crossover. Therefore, no significant differences were expected with the QPCR assay. In nine out of the ten

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

assays, there were no significant differences in the UNL/VEH vs. LES/GPI testing (See Figure 2). However, there was a six cycle earlier threshold with the assay for Presenilin 1 (PS1) in the treated group in 4 out of 6 samples tested (See Figure 3). Although the original group did not reach significance (p=.11) it warranted further testing. Therefore, six additional RNA samples were tested from both groups to confirm the results. Again, four of the six additional treated samples had PS1 mRNA upregulation and this time the difference was significant (p=.01) (See Table 5). This result was unexpected and was not predicted by the microarray studies.

Blocked Early in Transport 1 (Bet1)

The microarray analysis indicated that lesioning without treatment slightly increased Bet1 mRNA levels and treatment significantly increased Bet1 mRNA in the absence of lesioning (~4 fold) (See Table 4). Although we did not directly test changes due to GPI-1046 treatment on the QPCR assay, there were no significant differences in Bet1 levels between the unlesioned/vehicle and lesioned/treated groups (p = .11) (See Table 5).

Dispatched Protein 1 (Disp1)

Levels of Disp1 mRNA were significantly increased in tissue of treated animals on the unlesioned side (~ 6 fold), but were reduced in treated animals on

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the lesioned side on the microarrays (See Table 4). QPCR results did not show significant mRNA changes between the lesioned/treated and unlesioned/vehicle (p=.17) (Table 5).

Growth Arrest Specific Gene 6 (Gas6)

Gas6 levels were somewhat decreased after lesioning and only slightly increased after treatment (Table 4). Similar to Bet1 we did not directly test the effect of genes changing due to treatment with a QPCR assay. Fold change on the microarray was not significant with treatment or vehicle and there were also no significant changes on the QPCR assay of the interaction effects of GPI-treated/lesioned levels of Gas6 mRNA and the vehicle-treated/unlesioned tissue (p=.77) (Table 5) (Figure 2).

Heat responsive protein 12 (Hrsp12)

Levels of Hrsp12 were significantly reduced in unlesioned/GPI-1046 treated tissue when compared with lesioned/vehicle treated tissue (~2 fold), but these reductions became more moderate when LES/GPI treated and UNL/VEH treated tissue (Table 4). No significant differences were found with QPCR (p=.32) (Table 5).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Heat-Shock Protein 40 (Hsp40)

Levels of Hsp40 were significantly increased in unlesioned/treated tissue (~3 fold) but were only slightly changed in tissue that was both lesioned and treated (Table 4). QPCR assay for LES/GPI vs. UNL/VEH was not significantly different.

Pax transcription activation domain interacting protein (Paxip1)

Paxip1 mRNA levels increased significantly in GPI-treated but unlesioned tissue when compared with lesioned/untreated tissue (~5 fold), but no significant changes were noted in GPI-treated/unlesioned tissue compared to unlesioned /untreated tissue on the microarray slide (See Table 4). The latter comparison was confirmed by QPCR (p=.13) (See Table 5).

Presenilin (PS1)

PS1 levels were increased in unlesioned/GPI-treated tissue on the microarray slides (~2 fold), but not on the lesioned/GPI treated tissue (Table 4). A comparison of Lesioned/GPI-treated versus Unlesioned/Vehicle treated tissue with QPCR did not verify the microarray results. A large increase in PS1 levels was detected with the QPCR in the lesioned/GPI-treated tissue when compared with unlesioned/vehicle treated tissue (p=.01) (Table 5) (Figure 3).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Serine Palmitoyltransferase, long chain base subunit 1 (Sptlc1)

In the microarray analysis Sptlc1 was considerably elevated in GPI-treated/unlesioned tissue over vehicle-treated/lesioned tissue (~3 fold), but showed a minor decrease in lesioned/GPI-treated assays as compared to unlesioned/vehicle treated tissue (Table 4). There were no significant differences in these groups on the QPCR analysis (p=.15) (Table 5).

Synaptotagmin 11 (Syt11)

Two clones on the UHN microarray indicated changes in Syt11 levels. H3100B02 was decreased in the unlesioned/GPI-1046 treated tissue when compared to lesioned/vehicle treated mRNA levels of Synaptotagmin. H3026B06 showed an increase in lesioned/GPI-1046 treated mRNA when compared with untreated/unlesioned tissue (~2 fold). In both clones there was no difference in Syt11 mRNA levels for lesioned/GPI-1046 treated and unlesioned/vehicle treated ratios (See Table 4). QPCR assay differences were also not significantly different (p= .89) (Table 5).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Tyrosinase (Tyr)

The microarray study results indicated that GPI-1046 treatment in the absence of lesioning decreased Tyr mRNA when compared with untreated/lesioned tissue (~2 fold). However, both lesioning and GPI-1046 treatment restored Tyr to a level similar to the unlesioned/untreated tissue (Table 4). QPCR assay for Tyr indicated no significant changes with both lesioning and treatment ($p=.18$) (Table 5).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 1: Comparison of CY3/5 Dyes and Genisphere

BARCODE	Mean 635	Mean 532	Bkgrd 635	Bkgrd 532
Cy 3/5	**With 10N NaOH**			
12451990	707	1197	120	159
12451989	123	1092	46	116
	With 0.5M NaOH/50mM EDTA			
12693539	828	847	57	108
12600038	868	1193	59	100
Genisphere				
12453365	1201	1265	145	296
12453366	819	959	113	256

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 2: Group normalization constants for data analysis in Acuity 4.0 for each slide in experiment. Large differences in normalization constants may indicate dye effects or background intensity differences. 635 indicates the wavelength reading for cyanine 5 labeling and 532 indicates the wavelength reading for cyanine 3. LES = lesioned UNL = unlesioned GPI = GPI-1046 treatment VEH = intralipid vehicle only

Acuity Normalization Constants

Barcode	635 Constant	Tissue Sample	532 Constant	Tissue Sample
Round 4				
12600038	1.24356	LES GPI	0.804141	LES VEH
12600040	0.82912	LES GPI	1.2061	UNL GPI
12600041	1.56476	UNL VEH	0.639076	LES VEH
12602837	1.13036	UNL GPI	0.884672	UNL VEH
12693538	1.05272	LES GPI	0.94992	UNL VEH
12693539	1.07718	LES VEH	0.928348	LES GPI
12693543	1.32007	UNL GPI	0.757533	LES VEH
12693544	0.590415	UNL VEH	1.69372	UNL GPI
Round 5				
12602838	1.69346	UNL VEH	0.590507	LES VEH
12748414	1.45844	LES VEH	0.685665	UNL VEH
12748415	0.914283	UNL VEH	1.09375	UNL GPI
12748416	1.36021	LES GPI	0.735182	LES VEH
12748676	0.89566	UNL GPI	1.1165	LES GPI
12748677	1.64847	LES VEH	0.606672	UNL GPI
12748678	1.28506	UNL VEH	0.778173	LES GPI
12748679	1.28392	LES GPI	0.778864	UNL VEH

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 3: List of gene sequences identified as changing in different experimental conditions from microarray studies. **Bold** Type indicates mRNA sequences tested with QPCR assay.

		genes changing due to lesioning and treatment	p-value
H3129G02	**Sptlc1**	**serine palmitoyltransferase, long chain base subunit 1**	**0.0006**
H3029C08	**Paxip1**	**Pax transcription activation domain interacting**	**0.0007**
H3074G01	**Hsp40**	**DnaJ (Hsp40) homolog, subfamilyA, member 3**	**0.0001**
H3027B08	EST		0.0009
H3100B02	**Syt11**	**synaptotagmin 11**	**0.0008**
H3116C08	EST		0.001
H3002H03	EST		0.0006
H3037D04	EST		0.002
H3085D04	EST		0.001
H3140C07	EST		0.0002
H3150D02	**PS-1**	**Presenilin 1**	**0.0006**
H3095H09	EST		0.0012
H3026B06	**Syt11**	**synaptotagmin 11**	**0.0023**
H3108G09	Ctbp1	C-terminal binding protein 1	0.0015
H3154D09	**Tyr**	**tyrosinase**	**0.0027**
H3091A09	Not found		0.0051
H3051C10	EST		0.0015
H3030C07	myd	like-glycosyltransferase	0.0016
H3085F03	Paip2	Pabp-interacting protein 2	0.0037
H3012B06	Ppp1ca	Protein phosphatase 1, catalytic subunit	0.0012
H3140G11	Not found		0.0012
H3005H06	Pcdha11	protocadherin alpha 11	0.002
H3035H08	EST		0.003
H3025H03	Mtif2	Mitochondrial translational initiation factor 2	0.0035
H3133C02	LNR42	L-name related protein	0.0003
H3091C07	Speer4b	Spermatogenesis associated glutamate (#)-rich protein 4b	0.0007
H3043E06	Pdha1	pyruvate dehydrogenase E1 alpha subunit	0.0012
H3070C07	Atp6v1f	ATPase, H+ transporting, V1 subunit F	0.0009
H3016F06	Myo1c	Myosin IC	0.0018
H3135G07	Ppox	protoporphyrinogen oxidase	0.0002
H3037H08	Mov10	Moloney leukemia virus 10	0.0057
H3042E01	D1Ertd228e	DNA segment, Chr 1,	0.0008
H3133A08	EST		0.0055
H3010H09	Trim7	Tripartite motif protein 7	0.0024
H3087A04	Hsd3b2	Hydroxysteroid dehydrogenase-2 delta <5>-3-beta	0.002
H3151E02	EST		0.0059
H3121A02	Disp1	Dispatched homolog 1	0.0055
H3138C01	Rps6ka2	ribosomal protein S6 kinase 90 kD polypeptide 2	0.0017
H3143D08	Stx5a	syntaxin 5a	0.0068
H3101G06	Cdca1	cell division cycle associated 1	0.0025

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

H3041E08	EST		0.003
H3041F10	Neu3	Neuraminidase 3	0.0051
H3062C07	Phc3	Polyhomeotic-like 3	0.003
H3130B03	EST		0.0082
H3122B07	**Hrsp12**	**Heat-responsive protein 12**	**0.0028**
H3131H12	Rpl37a	Ribosomal protein L37a	0.0019
H3158H09	EST	similar to phophoinositide 3-kinase	0.0082
H3028A09	Bach2	BTB and CNC homology 2	0.0032
H3117G08	Casp8ap2	Caspase 8 associated protein 2	0.0083
H3077H01	Rnf38	Ring finger protein 38	0.0016
H3104G08	Not found		0.0071
H3001H08	Adm	Adrenomedullin	0.0044
H3089F04	EST		0.0018
H3109E04	Car9	Carbonic anhydrase 9	0.0046
H3104E07	Ramp2	Receptor-activity modifying protein 2	0.0069

genes changing due to treatment

H3045A04	D1Ertd259e	DNA segment, Chr 1 ERATO Doi 259	0.0058
H3100G08	Acat1	Acetyl-coenzyme A acetyltransferase 1	0.0028
H3015A07	EST		0.004

genes changing due to lesioning

H3002G02	EST		0.0003
H3145G10	D13Wsu64e	DNA segment Chr 13, Wayne State University 64	0.0001
H3142A05	Ulk2	Unc-51 like kinase 2	0.0002
H3126G04	Not found		0.0014
H3138A05	EST		0.0006
H3135A04	Not found		0.0021
H3136G10	mBSC2	sodium/potassium/chloride transporters	0.0048
H3049B02	Ptp4a3	Protein tyrosine phosphatase 4a3	0.0021
H3001G09	JAK3	weakly similar to JAK3 tyrosine-protein kinase	0.004
H3158C03	Mea1	male enhanced antigen 1	0.0004
H3151E04	Not found		0.004
H3142E10	Pacsin3	Protein kinase C and casein kinase substrate in neurons 3	0.0041
H3062A06	EST		0.0168
H3143A12	**Bet1**	**Blocked early in transport 1 homolog golgi vesicular membrane trafficking protein p18**	**0.0007**
H3153C12	Hnrpu	heterogeneous nuclear ribonucleoprotein U nuclear matrix protein sp120	0.0046
H3159C10	Pspc1	Paraspeckle protein 1	0.0091
H3054C06	Png	Phospholipase c neighboring	0.0248
H3116A01	EST		0.0015
H3154E07	**Gas 6**	**Growth arrest specific 6**	**0.0053**

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table 4: **Ratios [Cy5 (635nm)/Cy3 (532 nm)]**generated by microarray studies of genes selected for QPCR Testing. U=Unlesioned L=6-OHDA Lesioned V=Intralipid Vehicle Treatment T= GPI-1046 Treatment

Genes Changing Due to Lesioning and Treatment

Clone ID	Name	12693543	12748677	12748678	12748679	12693538
		UT/LV	UT/LV	LT/UV	LT/UV	LT/UV
H3129G02	Sptlc1	2.74	3.092	0.795	0.746	0.668
H3029C08	Paxip1	5.624	6.485	1.302	1.269	1.07
H3074G01	Hsp40	3.524	3.599	0.685	0.875	0.726
H3100B02	Syt11	0.698	0.736	1.107	1.084	1.05
H3150D02	PS-1	1.738	1.69	1.023	1.081	0.969
H3026B06	Syt11	2.053	1.831	1.045	1.128	1.074
H3121A02	Disp1	5.65	7.712	1.029	0.795	1.242
H3122B07	Hrsp12	0.341	0.419	0.797	0.792	0.885
H3154D09	Tyr	0.435	0.509	1.203	1.16	1.362

Genes Changing Due to GPI-1046 Treatment

		12600041	12602838	12748414	12748676	12600040
		UV/LV	UV/LV	UV/LV	UT/LT	UT/LT
H3143A12	Bet1	0.749	0.931	0.581	4.047	3.593
H3154E07	Gas 6	0.782	0.695	0.681	1.394	1.21

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

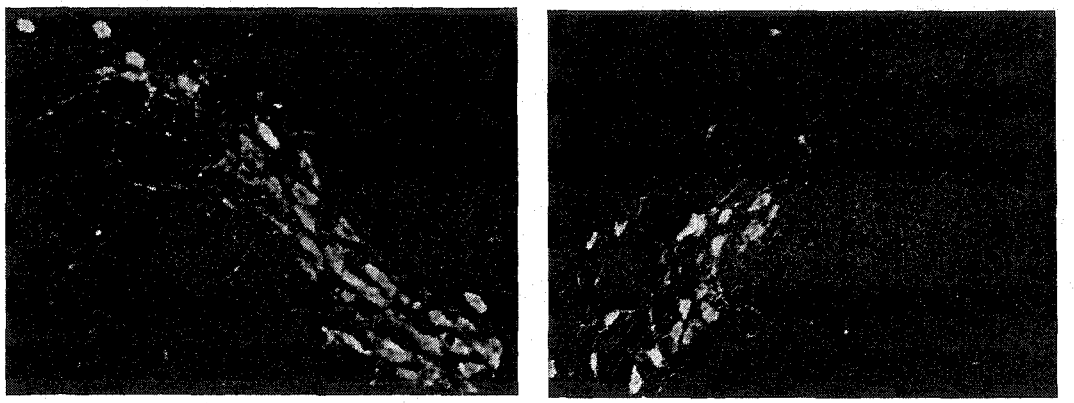
Table 5: Cycle Thresholds and significance generated by QPCR assay to compare Lesioned/GPI-treated tissue to Unlesioned/Intralipid Vehicle treated animals.

Primer Name	Identified Mouse Sequence	Rat Sequence for RT-QPCR	Avg. Ct LES/ GPI	Avg. Ct UNL/ VEH	Ct Std. Dev.	n =	p-value
Blocked early in transport 1 homolog	H3143A12	NM019251	2.57	2.28	.32/ .25	12	.11
Dispatched homolog 1	H3121A02	XM213964	5.85	5.43	.45/ .54	12	.17
Growth Arrest Specific gene 6	H3154E07	BC070881	2.23	2.14	.66/ .70	24	.77
Heat Responsive Protein 12	H3122B07	NM031714	2.42	2.73	.48/ .54	12	.32
Heat Shock Protein 40 Dnaja3 (Hsp40) homolog subfamily A member 3	H3074G01	XM233767	2.41	1.52	1.73/ 1.69	12	.19
Pax Transcript-tion activation interacting	H3029C08	XM231271	12.19	13.02	.93/ .91	12	.13
Presenilin 1	H3150D02	BC070887	7.57	12.97	3.86/ 6.44	12	.11
Presenilin 1	H3150D02	BC070887	6.99	12.76	2.50/ 6.39	24	.01
Serine Palmitoyl-transferase Long chain base subunit 1	H3129G02	XM341495	2.88	2.93	.53/ .73	24	.15
Synapto-tagmin 11	H3100B02 H3026B06	NM031667	-.17	-.20	.47/ .18	12	.89
Tyrosinase	H3154D09	XM238901	11.05	11.81	.64/ 1.06	12	.18

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 1: Tyrosine Hydroxylase (TH) staining
of substantia nigra pars compacta (SNc)
LHS shows unlesioned SNc with TH staining
RHS shows lesioned SNc with TH staining

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



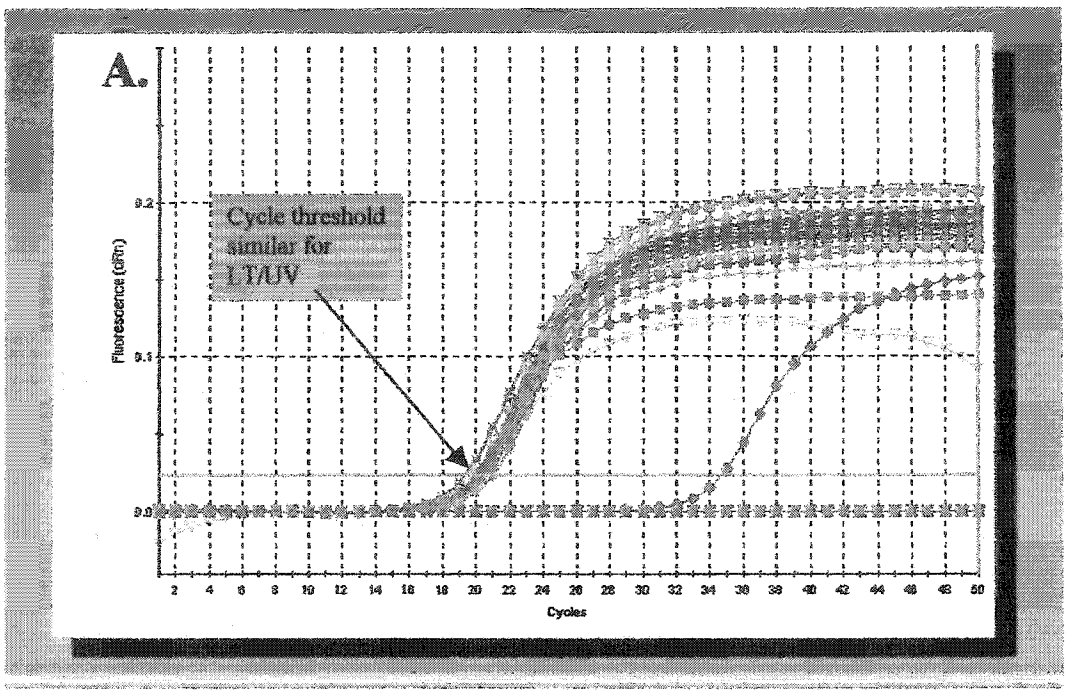
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

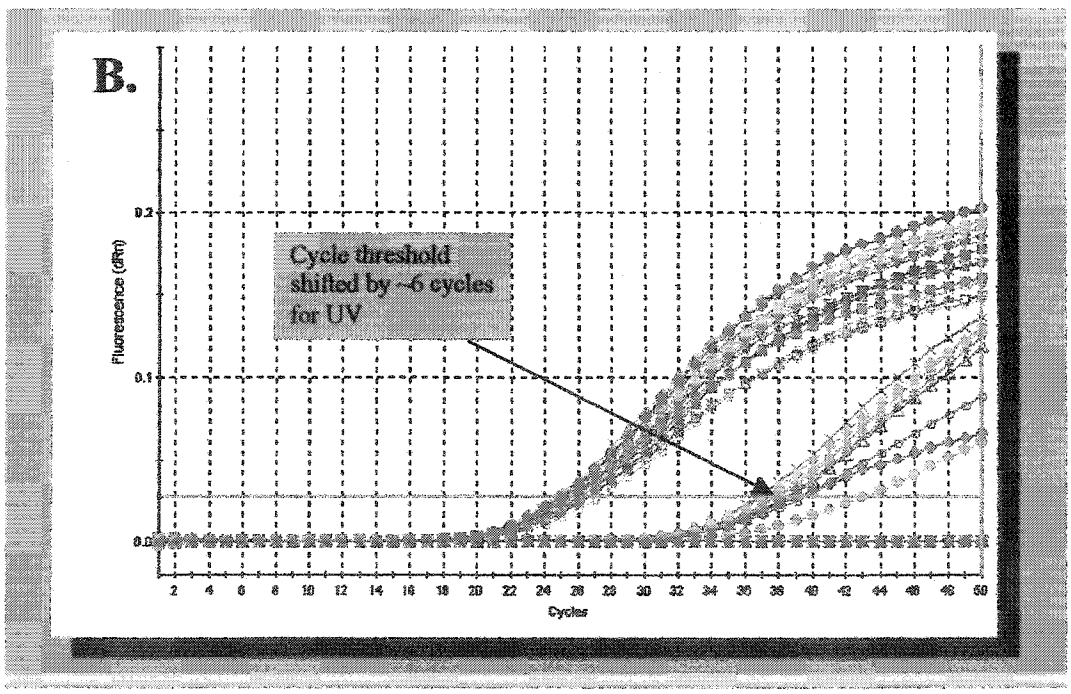
Figure 2: QPCR Amplification Curves

A. Growth Arrest Specific Amplification Curves from QPCR Assay showing no difference in cycle threshold (reflecting mRNA expression) between Lesioned/GPI-1046 treated and Unlesioned/Vehicle treated tissue

B. Presenilin 1 mRNA levels increased in Lesioned/GPI-1046 treated tissue as indicated by lower cycle threshold (6 cycles) than Unlesioned/Vehicle treated sample.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.





Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DISCUSSION

Microarray Analysis

Our microarray analysis found that depending on the comparison under consideration, between three and 55 gene products were differently expressed. We chose from the list those genes that had a biological function that was of interest with respect to GPI-1046 treatment. Although one of the strengths of microarray analysis is to identify unknown genes we excluded EST validation from our QPCR analysis as it was beyond the scope of the current study to clone 30 or more gene products. From the list of gene products that were consistently different between arrays we selected ten mRNA sequences that were deemed biologically interesting for validation by QPCR methodology.

The number of genes changing due to lesioning was small (3) which is not surprising since we were lesioning the SNc and testing tissue from the striatum. Also sufficient time had passed since lesioning that early immediate gene changes would no longer be active in the affected region. It is possible that the same temporal and spatial rationale can be applied to the moderate number of genes changing due to treatment alone (19). It is interesting to note that proteins involved in phosphorylation activity were well represented in the treatment group (Gas6, Ptp4a3, Ulk2, and JAK3), similar to the study by Napolitano *et al.* (2002).

The microarray experiment was designed so that differences in gene expression could be assessed at each level of 6-OHDA lesioning

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(lesioned/unlesioned) and each level of treatment (GPI-1046 treated/intralipid vehicle only). An interesting finding of the microarray study was that significant changes (> or < 2 fold differences) that were noted due to GPI-1046 treatment on the unlesioned side returned to normal levels (~1:1) on the lesioned side in nine of the ten gene products tested with QPCR assays. From these results it would appear that GPI-1046 might work by restoring physiological levels of gene products to the lesioned area (whether up- or down regulated). As noted in the introduction, other novel treatments for PD, such as BDNF can produce abnormal sprouting and connectivity, a problem not encountered with GPI-1046 treatment. It would appear that although treatment may disrupt the equilibrium on the unlesioned side, it is somehow able to restore normal levels of gene function to the lesioned striatum and suggests that global transcription regulation is altered.

QPCR Assay

Several of the genes selected for QPCR assay testing related to cell growth, proliferation, regionalization and differentiation (Disp1, Hsp40, Paxip1, and Sptlc1). Levels of these gene products were found to be elevated greater than two fold when comparing the cross design involving a single factor (Lesioning only vs. Treatment only). However, gene products were not significantly different (~1:1) when both lesioning and treatment vs. neither lesioning nor treatment (vehicle) were compared. Given the assumption that unlesioned/vehicle samples represent normal physiological levels of the gene products, there is some

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

indication that GPI-1046 treatment may restore stable levels of mRNA products to the striatum ipsilateral to the lesion, while disrupting levels on the contralateral side.

Two genes decreased greater than two fold in the microarray analysis (Hrsp12 and Tyr) when comparing lesioning only to treatment only. Both sequences did not show significant differences between the control (unlesioned/untreated) and experimental groups (lesioned/GPI-1046 treated) groups. The homogeneity of control vs. experimental comparisons was confirmed by QPCR in nine of ten sequences tested.

The one anomaly to our QPCR findings was presenilin 1, which was elevated in the lesioned/GPI-1046 treated tissue in 8/12 animals tested for QPCR testing, but not in the microarray results. Although PS-1 abnormality is usually associated with Alzheimer's disease there is some evidence that amyloid precursor protein (APP) synthesis and secretion is regulated by neuroimmunophilin ligands (Lee & Wurtman 2000). Furthermore, researchers have suggested that PD and AD share the accumulation of neuronal inclusions in the pathology of both diseases and that a common pathway may involve mutations in amyloid precursor protein and the presenilins (Bertoli-Avella *et al.* 2004). As stated in Appendix II, presenilin is important for Notch1 initiated transcription as well as normal processing of APP. Since PS1 levels are increased in the lesioned/GPI-1046 treated tissue it would appear that APP processing may be increased in these cells through elevated levels of PS1.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Lee & Wurtman (2000) found that APP secretion was increased with immunosuppressants such as cyclosporin A and FK-506 and proposed that analogues such as GPI-1046 would also produce this neurotrophic effect. Presenilins are a component of the γ-secretase enzyme complex and mutations of presenilins are thought to be responsible for the release of pathogenic beta amyloid (Aβ) from the APP molecule through a proteolytic cleavage (Koo & Kopan 2004). However, it was recently reported that presenilin deficient mice exhibited memory impairments as well as deficits in N-methyl d-aspartate (NMDA) receptor mediated responses. It was further reported that loss of presenilin resulted in reduced levels of cyclic-AMP element binding protein (CREB) and its target genes such as brain-derived neurotrophic factor (BDNF). The authors concluded presenilins play an essential role in neuron survival, synaptic plasticity as well as learning and memory (Saura *et al.* 2004). Although excessive levels of presenilin mutations cause the accumulation of pathologic Aβ accumulation, the up-regulation of non-mutated presenilins demonstrated here may have a neurotrophic function in the striatum. This may be due to increased processing of APP molecules, and confirmation of this result through Western blot analysis would be desirable to determine if only PS1 mRNA is present, or if the protein product is increased in the cell, as well. Although presenilin did not reveal changes in expression according to the microarray results, it is possible that the mouse sequence on the microarray was different than the rat sequence used to test PS1on the QPCR and this could account for the discrepancy in the two methods.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Summary and Conclusions

At the outset, we wanted to establish a working protocol for microarray hybridization in order to determine which genes were changing as a result of immunophilin treatment by using microarray technology. We have demonstrated that overall the protocol can be used to produce data showing that gene expression changes can be predicted with some reliability. We were surprised to learn that greater than or less than two-fold changes were rarely occurring when comparing the control (unlesioned, vehicle treated) to the experimental tissue (lesioned, GPI-1046 treated). It would appear that GPI-1046 treatment without lesioning disturbs mRNA expression in the striatum; lesioning with GPI-1046 treatment does not in the gene sequences we tested.

Future Directions

In order to verify disruptions in mRNA levels when only lesioning or treatments are compared, it would be desirable to test these ratios using a QPCR assay. Microarray analysis using SNc cells would give more information about the changes in the cells rather than the connectivity investigated here. In addition, time course experiments would give more information about the early and late effects in mRNA expression. Future work in this area could also involve more complex analysis of the data in order to predict groups of genes that are

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

functionally related and up-or down-regulated together. Custom microarrays may also be used to further identify groups of genes that may be coordinately regulated. As mentioned in the introduction, not all mRNA changes result in changes in protein expression so Western blots may be used to determine if changes in mRNA expression result in altered protein levels. Although we were not able to fully explore the ANOVA model design due to software limitations, this work will be ongoing and additional small changes in the GPI-1046 treated/lesioned versus vehicle treated/unlesioned may become significant when the full power of the experimental design is utilized.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

REFERENCES

Alberici A, Moratto D, Benussi L, Gasparini L, Ghidoni R, Gatta LB, Finazzi D, Frisoni GB, Trabucchi M, Growdon JH, Nitsch RM & Binetti G (1999) Presenilin 1 protein directly interacts with Bcl-2 *The Journal of Biological Chemistry* **274**(43): 30764-30769

Barr FG (2001) Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma *Oncogene* **20**: 5736-5746

Becker WM, Kleinsmith LJ & Hardin J (2000) *The World of the Cell 4th Edition* The Benjamin/Cummings Publishing Company pp 540-545

Bernardini C, Fantinati P, Zannoni A, Forni M, Tamani C & Bacci ML (2004) Expression of HSP70/HSC70 in swine blastocysts: effects of oxidative and thermal stress *Molecular Reproduction and Development* **69**:303-307

Bertoli-Avella AM, Oostra BA & Heutink P (2004) Chasing genes in Alzheimer's and Parkinson's disease *Human Genetics* **114**: 413-438

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV & Greenamyre JT (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neuroscience* **3**(12): 1301-1306.

Blum D, Torch S, Lambeng N, Nissou M-F, Benabid A-L, Sadoul R & Verna J-M (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Progress in Neurobiology* **65**: 135-172

Bowtell D & Sambrook J (2003) *DNA Microarrays: A Cloning Manual* Cold Spring Harbor Laboratory Press Cold Spring Harbor NY p 519

Cadet JL, Jayanthi S, McCoy MT, Vawter M & Ladenheim B (2001). Temporal profiling of methamphetamine-induced changes in gene expression in the mouse brain: evidence from cDNA array. *Synapse* **41**: 40-48.

Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV & Snyder SH (1995a). Calcineurin associated with the Inositol 1,4,5-trisphosphate receptor–FKBP12 complex modulates Ca^{2+} flux. *Cell* **83**: 463-472.

Cameron AM, Steiner JP, Sabatini DM, Kaplin AI, Walensky LD & Snyder SH (1995b). Immunophilin FK506 binding protein associated with inositol 1,4,5-triphosphate receptor modulates calcium flux. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 1784-1788.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Campbell G, Holt JKL, Shotton HR, Anderson PN, Bavetta S & Lieberman AR (1999). Spontaneous axonal regeneration after optic nerve injury in adult rat. *NeuroReport* **10**: 3955-3960.

Chauhan BK, Reed NA, Yang Y, Cermák L, Reneker L, Duncan MK & Cvekl A (2002). A comparative cDNA microarray analysis reveals a spectrum of genes regulated by Pax6 in mouse lens *Genes to Cells* 7:1267-1283.

Cho EA, Prindle MJ & Dressler GR (2003) BRCT domain-containing protein PTIP is essential for progression through mitosis *Molecular and Cellular Biology* **23**(5): 1666-1673

Cohen FE & Kelly JW (2003) Therapeutic approaches to protein-misfolding diseases *Nature* **426**:905-909

Cohen MM Jr (2003) The hedgehog signaling network *American Journal of Medical Genetics* **123A**: 5-28

Costantini LC & Isacson O (2000). Neuroimmunophilin ligand enhances neurite outgrowth and effect of fetal dopamine transplants. *Neuroscience* **100**(3): 515-520.

Costantini LC & Isacson O (2003). Chapter 3. Immunophilin ligands and dopamine neurons *specific effects in culture and in vivo*. **In:** *Immunosuppressant Analogs in Neuroprotection*. Borlongan CV, Isacson O & Sanberg PR (Editors).Humana Press Inc. Totowa, NJ. pp 49-66.

Crosier KE & Crosier PS (1997) New insights into the control of cell growth; the role of the Axl family *Pathology* **29**:131-135

Da Cunha C, Angelucci MEM, Canteras NS, Wonnacott S & Takahashi RN (2002). The lesion of the rat substantia nigra par compacta dopaminergic neurons as a model for Parkinson's disease memory disabilities. *Cellular and Molecular Neurobiology* **22**(3): 227-237.

Dawson TM (1996). Immunosuppressants, immunophilins, and the nervous system. *Annals of Neurology* **40**(4): 559-560.

Denny PW & Smith DF (2004) Rafts and sphingolipid biosynthesis in the kinetoplastid parasitic protozoa *Molecular Microbiology* **53**(3): 725-733

Double KL, Ben-Shachar D, Youdim MBH, Zecca L, Riederer P & Gerlach M (2002). Influence of neuromelanin on oxidative pathways within the human substantia nigra. *Neurotoxicology and Teratology* **24**: 621-628.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Eidelman BH, Abu-Elmagd K, Wilson J, Fung JJ, Alessiani M, Jain A, Takaya S, Todo SN, Tzakis A, Van Thiel D, Shannon W & Starzl TE (1991). Neurologic complications of FK506. *Transplantation Proceedings* **23**(6): 3175-3178.

Ferrario JE, Taravini IR, Mourlevat S, Stefano A, Delfino MA, Raisman-Vozari R, Murer MG, Ruberg M & Gershanik O (2004). Differential gene expression induced by chronic levodopa treatment in the striatum of rats with lesions of the nigrostriatal system. *Journal of Neurochemistry* **90**(6): 1348-1358

Figueroa DJ, Morris JA, Ma L, Kandpal G, Chen E, Li Y-M, Austin CP (2002) Presenilin-dependent gamma-secretase activity modulates neurite outgrowth *Neurobiology of Disease* **9**:49-60

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T & Schmid FX (1989). Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**: 476-478.

Foltynie T, Sawcer S, Brayne C & Barker RA (2002). The genetic basis of Parkinson's disease. *Journal of Neurology, Neurosurgery and Psychiatry* **73**: 363-370.

Gatto EM, Riobó N, Carreras MC, Poderoso JJ & Micheli FE (2002) Neuroprotection in Parkinson's disease; a commentary. *Neurotoxicity Research* **4**(2): 141-145.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN & Heywood P (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nature Medicine* **9**(5): 589-595.

Glonek GF & Solomon PJ (2004) Factorial and time course designs for cDNA microarray experiments *Biostatistics* **5**(1): 89-111

Gold BG, Densmore V, Shou W, Matzuk MM & Gordon HS (1999). Immunophilin FK506-bingind protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *The Journal of Pharmacology and Experimental Therap*eutics **289**: 1202-1210

Gold BG, Udina E, Bourdette D & Navarro X (2004) Neuroregenerative and neuroprotective actions of neuroimmunophilin compounds in traumatic and inflammatory neuropathies *Neurological Research* **26**(4): 371-380

Gold BG, Zeleny-Pooley M, Chaturvedi P & Wang M-S (1998). Oral administration of a nonimmunosuppressant FKBP-12 ligand speeds nerve regeneration. *NeuroReport* **9**: 553-558.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Grünblatt E, Mandel S, Jacob-Hirsch J, Zeligson S, Amariglo N, Rechavi G, Li J, Ravid R, Roggendorf W, Riederer P & Youdim MB (2004) Gene expression profiling of parkinsonian substantia nigra pars compacta; alterations in ubiquitin-proteasome, heat shock protein, iron and oxidative stress regulated proteins, cell adhesion/cellular matrix and vesicle trafficking genes. *Journal of Neural Transmission* **111**(12): 1543-1573.

Grünblatt E, Mandel S, Maor G & Youdim MBH (2001). Gene expression analysis in N-methly-4-phenyl-1,2,3,6-tetrahydropyridine mice model of Parkinson's disease using cDNA microarray: effect of R-apomorphine. *Journal of Neurochemistry* **78**:1-12.

Gu G, Reyes PF, Golden GT, Woltjer RL, Hulette C, Montine TJ & Zhang J (2002). Mitochondrial DNA deletions/rearrangements in Parkinson disease and related neurodegenerative disorders. *Journal of Neuropathology and Experimental Neurology* **61**(7): 634-639.

Guo X, Dawson VL & Dawson TM (2001a). Neuroimmunophilin ligands exert neuroregeneration and neuroprotection in midbrain dopaminergic neurons. *European Journal of Neuroscience* **13**: 1683-1693.

Guo X, Dillman JF III, Dawson VL & Dawson MD (2001b). Neuroimmunophilins: novel neuroprotective and neuroregenerative targets. *Annals of Neurology* **50**(1): 6-16.

Hamilton GS & Steiner JP (1998). Immunophilins: beyond Immunosuppression. *Journal of Medicinal Chemistry* **41**(26):5119-5143.

Harding MW, Galat A, Uehling DE & Schreiber SL (1989) A receptor for the immuno-suppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**: 758-760.

Harper S, Bilsland J, Young L, Bristow L, Boyce S, Mason G, Rigby M, Hewson L, Smith D, O'Donnell R, O'Connor D, Hill RG, Evans D, Swain C, Williams B & Hefti F (1999). Analysis of the neurotrophic effects of GPI-1046 on neuron survival and regeneration in culture and in vivo. *Neuroscience* **88**(1): 257-267.

Helekar SA, Char D, Neff S & Patrick J (1994) Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels *Neuron* **12**: 179-189

Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A & van der Kooy D (2002) Notch pathway molecules are essential for the maintenance, but not the generation of mammalian neural stem cells *Genes & Development* **16**(7): 846-858

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hitoshi S, Seaberg RM, Koscik C, Alexson T, Kusunoki S, Kanazawa I, Tsuji S, van der Kooy D (2004) Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling *Genes & Development* **18**(15): 1806-1811

Hoffmeister A, Ropolo A, Vasseur S, Mallo GV, Bodeker H, Ritz-Laser B, Dressler GR, Vaccaro MI, Dagorn J-C, Moreno S & Iovanna JL (2002) *The Journal of Biological Chemistry* **277**(25):22314-11319

Husseini AE-D & Bredt DS (2002) Protein palmitoylation: a regulator of neuronal development and function *Nature Reviews Neuroscience* **3**:791-802

Jenner P & Olanow CW (1996) Oxidative stress and the pathogenesis of Parkinson's disease *Neurology* **47**(Supplement 3): S161-S170.

Jin W, Riley RM, Wolfinger RD, White KP, Passador-Gurgel G & Gibson G (2001) The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster, Nature Genetics* **29**(4): 389-395

Katzenschlager R & Lees AJ (2002) Treatment of Parkinson's disease: levodopa as the first choice. *Journal of Neurology* **249**(Supplement 2): II/19-II24.

Khan J, Bittner ML, Chen Y, Meltzer PS & Trent JM (1999) DNA microarray technology: the anticipated impact on the study of human disease. *Biochimica et Biophysica Acta* **1423**: M17-M28.

Kipnis P, Tomas N, Ovalle R & Lipke PN (2004) The ER-Golgi v-SNARE Bet1p is required for cross-linking alpha agglutinin to the cell wall in yeast. *Microbiology* **150**(Pt10): 3219-3228.

Klettner A, Baumgrass R, Zhang Y, Fischer G, Bürger, Herdegen T & Mielke K (2001). The neuroprotective actions of FK506 binding protein ligands: neuronal survival is triggered by de novo RNA synthesis, but is independent of inhibition of JNK and calcineurin. *Molecular Brain Research* **97**: 21-31.

Koo EH & Kopan R (2004) Potential role of Presenilin-regulated signaling pathways in sporadic neurodegeneration *Nature Medicine* **10**(7): S26-S33.

Lai SK, Tse YC, Yang MS, Wong CKC, Chan YS & Yung KKL (2003). Gene expression of glutamate receptors GluR1 and NR1 is differentially modulated in striatal neurons in rats after 6-hydroxydopamine lesion. *Neurochemistry International* **43**: 639-653.

Lee RK & Wurtman RJ (2000) Regulation of APP synthesis and secretion by neuroimmunophilin ligands and cyclooxygenase inhibitors *Annals of the New York Academy of Sciences of the United States of America* **920**: 261-168

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I & Schreiber SL (1991). Calcineurin is common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes *Cell* **66**: 807-815.

Maimone D, Dominici R & Grimaldi LME (2001). Pharmacogenomics of neurodegenerative diseases. *European Journal of Pharmacology* **413**: 11-29.

Mandel S, Weinreb O & Youdim MBH (2003). Using cDNA microarray to assess Parkinson's disease models and the effects of neuroprotective drugs. *Trends in Pharmacological Sciences* **24**(4): 184-191.

Melaragno MG, Fridell Y-WC & Berk BC (1999) The Gas6/Axl system: A novel regulator of vascular cell function. *Trends in Cardiovascular Medicine* **9**(8):250-253.

Milani V, Noessner E, Ghose S, Kuppner M, Ahrens B, Scharner A, Gastpar R & Issels RD (2002) Heat shock protein 70: role in antigen presentation and immune stimulation *International Journal of Hyperthermia* **18**(6): 563-575.

Miller RM, Callahan LM, Casaceli C, Chen L, Kiser GL, Chui B, Kaysser-Kranich TM, Sendera TJ, Palaniappan C & Federoff HJ (2004) Dysregulation of gene expression in the 1-methyl-4-phenyl-1,2,3,6-Tetrahydropyridine-lesioned mouse substantia nigra. *The Journal of Neuroscience* **24**(34): 7445-7454

Mink JW (2003) The Basal Ganglia **In**: *Fundamental Neuroscience 2nd Edition.* Squire, LR, Bloom FE, McConnell SK, Roberts JL, Spitzer NC & Zigmond MJ (Editors) Academic Press, San Diego, USA pp 816-839

Mutch DM, Berger A, Mansourian R, Rytz A & Roberts M-A (2002) The limit fold change model: a practical approach for selecting differentially expressed genes from microarray data. *BMC Bioinformatics* **3**(17):1-12

Nair SC, Rimerman RA, Toran EJ, Chen S, Prapapanich V, Butts RN & Smith DF (1997) Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor *Molecular and Cellular Biology* **17**(2): 594-603.

Napolitano M, Centonze D, Calce A, Picconi B, Spiezia S, Gulino A, Bernardi G & Calabresi P (2002). Experimental parkinsonism modulates multiple genes involved in the transduction of dopaminergic signals in the striatum. *Neurobiology of Disease* **10**: 387-395

Nicotra A & Parvez SH (2000) Cell death induced by MPTP, a substrate for monoamine oxidase B. *Toxicology* **153**: 157-166.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Nishiki T & Augustine GJ (2004) Synaptotagmin 1 synchronizes transmitter release in mouse hippocampal neurons *The Journal of Neuroscience* **24**(27):6127-6132

Ogawa N & Tanaka K-I (2003) Chapter 2 Cyclosporin-mediated amelioration of degeneration of dopaminergic neurons in experimental models of parkinsonism. **In**: *Immunosuppressant Analogs in Neuroprotection.* Borlongan CV, Isacson O & Sanberg PR (Editors).Humana Press Inc. Totowa, NJ pp35-48

Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung W-Y & Haugland RP (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates *The Journal of Histochemistry & Cytochemistry* **47**(9): 1179-1188

Paxinos P & Watson C (1998) *The Rat Brain in Stereotaxic Coordinates, 4th edition* Academic Press San Diego CA

Pearson H (2004) Parkinson's trial halted. news@nature.com 5 October 2004 http://www.nature.com/news/2004/041004/pf/041004-6_pf.html

Perry DK (2002) Serine palmitoyltransferase: role in apoptotic de novo ceramide synthesis and other stress responses *Biochimica et Biophysica Acta* **1585**: 146-152

Peters C, Wolf A, Wagner M, Kuhlmann J & Waldmann H (2004) The cholesterol membrane anchor of the Hedgehog protein confers stable membrane association to lipid-modified proteins. *Proceedings from the National Academy of Sciences of the United States of America* **10**(23):8531-8536

Petrescu SM, Popescu CI, Petrescu AJ & Dwek RA (2003) The glycolsylation of tyrosinase in melanoma cells and the effect on antigen presentation *Advances in Experimental Medicine and Biology* **535**: 257-269

Pratt WB & Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones *Endocrine Reviews* **18**(3): 306-360

Pratt WB & Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery *Experimental Biology and Medicine* **228**:111-133.

Revesz T, Gray F & Scaravilli F (2001) Chapter 9 Parkinson's disease **In**: *Pathology of the aging human nervous system 2nd Edition* Duckett S & De La Torre JC (Editors) Oxford University Press NY pp 264-270.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Ross DT, Guo H, Howorth P, Chen Y, Hamilton GS & Steiner JP (2001). The small molecule FKBP ligand GPI 1046 induces partial striatal re-innervation after intranigral 6-hydroxydopamine lesion in rats. *Neuroscience Letters* **297**(2): 113-116

Samuel SJ, Tzung SP & Cohen SA (1997) Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver. *Hepatology* **25**(5): 1213-1222

Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, Shankaranarayana RBS, Chattarji S, Kelleher RJ III, Kandel ER, Duff K, Kirkwood A & Shen J (2004) Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration *Neuron* **42**(1):23-36

Sauer H, Francis MJ, Jiang H, Hamilton GS & Steiner JP (1999). Systemic treatment with GPI1046 improves spatial memory and reverses cholinergic neuron atrophy in the medial septal nucleus of aged mice. *Brain Research* **842**: 109-118.

Schmiedeknecht G, Büchler C & Schmitz G (1997) A bidirectional promoter connects the p14.5 gene to the gene for RNase P and RNase MRP protein subunit hPOP1. *Biochemical and Biophysical Research Communications* **241**: 59-67.

Schwarting RKW & Huston JP (1996). The unilateral 6-hydroxydopamine lesion model in behavioral brain research. Analysis of functional deficits, recovery and treatments. *Progress in Neurobiology* **50**:275-331.

Sherer TB, Betarbet R & Greenamyre JT (2002). Environment, mitochondria, and Parkinson's disease. *Neuroscientist* **8**(3): 192-197.

Siekierka JJ, Hung SHY, Poe M, Lin CS & Sigal NH (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**: 755-757.

Speciale SG (2002) MPTP Insights into parkinsonian neurodegeneration. *Neurotoxicology and Teratology* **24**: 607-620.

Steiner JP, Connolly MA, Valentine HL, Hamilton GS, Dawson TM, Hester L & Snyder SH (1997a). Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporine A. *Nature Medicine* 3(4): 421-428.

Steiner JP, Dawson TM, Fotuhi M, Glatt CE, Snowman AM, Cohen N & Snyder SH (1992). High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**: 584-587.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Steiner JP, Hamilton GS, Ross DT, Valentine HL, Guo H, Connolly MA, Liang S, Ramsey C, Li J-H, Huang W, Howorth P, Soni R, Fuller M, Sauer H, Nowotnik AC Suzdak PD (1997b). Neurotrophic immunophilin ligands stimulate structural and functional recover in neurodegenerative animal models. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 2019-2024.

Steiner JP, Ross DT, Sauer H, Morrow T & Hamilton GS (2003). Chapter 4. Effects of neuroimmunophilin ligands on Parkinson's disease and cognition. **In**: *Immunosuppressant Analogs in Neuroprotection.* Borlongan CV, Isacson O & Sanberg PR (Editors).Humana Press Inc. Totowa, NJ. pp 67-91.

Swerdlow RH (2002). Mitochondrial DNA-related mitochondrial dysfunction in neurodegenerative diseases. *Archives of Pathology and Laboratory Medicine* **126**: 271-280.

Tai WCS & Banfield DK (2001) AtBS14a and AtBS14b, two Bet1/Sft1-like SNAREs from *Arabidopsis thaliana* that complement mutations I the yeast SFT1gene. *FEBS Letters* **500**: 177-182.

Takahashi N, Hayano T & Suzuki M (1989). Peptidyl-prolyl *cis-trans* isomerase is the cyclosporine A-binding protein cyclophilin. *Nature* **337**: 473-476.

Tanaka K-I, Fujita N, Higashi Y & Ogawa N (2002). Neuroprotective and antioxidant properties of FKBP-binding immunophilin ligands are independent on the FKBP12 pathway in human cells. *Neuroscience Letters* **330**: 147-150.

Tanaka K-I, Fujita N & Ogawa N (2003). Immunosuppressive (FK506) and non-immunosuppressive (GPI1046) immunophilin ligands activate neurotrophic factors in mouse brain. *Brain Research* **970**: 250-253.

Tanaka TS, Jaradat SA, Lim MK, Kargul GJ, Wang X, Grahovac MJ, Pantano S, Sano Y, Piao Y, Nagaraja R, Doi H, Wood WH III, Becker KG & Ko MSH (2000). Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proceedings of the National Academy of Sciences of the United States of America* **97**(16): 9127-9132.

Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R & Langston JW (1999). Parkinson disease in twins: an etiologic study. *The Journal of the American Medical Association* **281**(4): 341-346

Thinakaran G & Parent AT (2004) Identification of the role of presenilins beyond Alzheimer's disease *Pharmacological Research* **50**:411-418

Tsaioun KI (1999) Vitamin K-dependent proteins in the developing and aging nervous system *Nutrition Reviews* **57**(8):231-240

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Weinreb O, Mandel S & Youdim MB (2003) Gene and protein expression profiles of anti-and pro-apoptotic actions of dopamine, R-apomorphine, green tea polyphenol (-) – epigallocatechine-3-gallate, and melatonin *Annals of the New York Academy of Sciences* **993**: 351-361

Wehr R & Gruss P (1996) *Pax* and vertebrate development *The International Journal of Developmental Biology* **40**: 369-377

Xu Z, Cawthon D, McCastlain KA, Slikker W Jr, Ali SF (2004) Selective alterations of gene expression in mice induced by MPTP. *Synapse* **55**(1): 45-51.

Yanagita M (2004) The role of the vitamin K-dependent growth factor Gas6 in glomerular pathophysiology *Current Opinion in Nephrology and Hypertension* **13**: 465-470.

Yang YH & Speed T (2003). Part 1: Design of microarray expression experiments. **In:** Bowtell D & Sambrook J. *DNA Microarrays: A molecular cloning manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp 513-525.

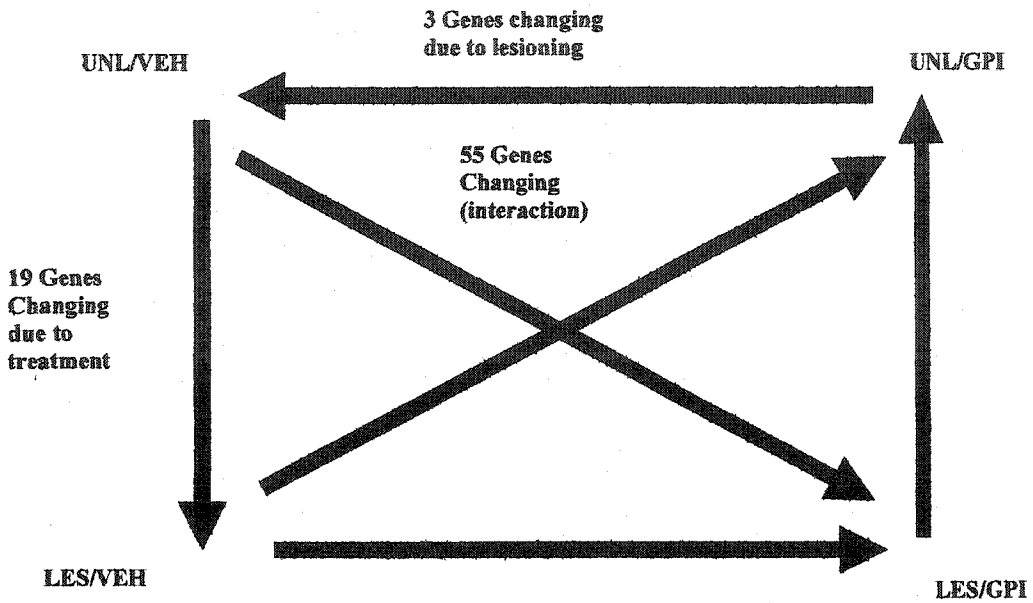
Yenari MA (2002) Heat shock proteins and neuroprotection. *Advances in Experimental Medicine and Biology* **513**:281-299.

Youdim MBH, Grünblatt E, Levites Y, Maor G & Mandel S (2002) Early and late molecular events in neurodegeneration and neuroprotection in Parkinson's disease MPTP model as assessed by cDNA microarray; the role of iron. *Neurotoxicity Resea*rch **4**(7-8): 679-689.

Zhang C, Steiner JP, Hamilton GS, Hicks TP & Poulter MO (2001). Regeneration of dopaminergic function in 6-hydroxydopamine-lesioned rats by neuroimmunophilin ligand treatment. *The Journal of Neuroscience* **21**: RC156 (1-6) From Bowtell & Sambrook 2003 p.519

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Appendix 1: Experimental Design



Each line represents one microarray slide. The arrow-head represents cyanine 5 labeling and the arrow-tail of the line represents cyanine 3 labeling. Statistical analysis compares two (or more) lines rather than comparing different labeling within the same line.

Adapted from Bowtell & Sambrook (2003) p.519

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Appendix 2: Rationale for Genes of Interest

Blocked Early in Transport 1 (Bet1)

Blocked early in transport 1 encodes ER-Golgi v-soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (v-SNARE) protein to transport vesicles from the endoplasmic reticulum (ER) to the Golgi apparatus (Kipnis *et al.* 2004). In order for vesicles to dock on the correct target membrane SNARE proteins, such as Bet1, on both the transport vesicle and target compartment, must be paired (Tai & Banfield 2001). Disruption of transport of essential cellular proteins, such as G-proteins, could be harmful to the cell and so changes in the levels of Bet1 mRNA are biologically important.

Dispatched Protein 1 (Disp1)

Hedgehog (Hh) proteins are involved in cell growth and differentiation and are important organizers during animal development. Hh proteins are secreted molecules that undergo posttranslational modification to covalently attach a cholesterol moiety on the carboxyl-terminal (C-terminus) and a palmitate at the amino-terminal (N-terminus) (Cohen 2003). The hydrophobic cholesterol-modification serves to anchor Hh to the plasma membrane and limit the range of activity of Hh proteins. Dispatched 1 interacts in partnership with Hh proteins to delete the cholesterol modification and to permit more potent and longer-range intercellular Hh protein signaling (Peters *et al.* 2004).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Growth Arrest Specific Gene 6 (Gas6)

As mentioned in the introduction, Napolitano *et al.* (2002) found some cellular changes were at the level of phosphorylation but unchanged in protein expression levels. Growth arrest specific gene 6 phosphorylates the tyrosine kinase domain of Axl, a receptor tyrosine kinase (RTK) (Yanagita 2004). Gas6 is a vitamin K-dependent secreted protein, structurally similar to anticoagulation factor protein S, which is expressed at high levels in growth-arrested cells and is distributed throughout the central nervous system (CNS) (Melaragno *et al.* 1999, Tsaioun 1999). There is evidence that Gas6 is found in precursor T-cells and monocytes that are activated during an initial inflammatory response following injury. In contrast, Gas6/Axl at high concentrations is reported to have an anti-inflammatory effect so the outcome of Gas6/Axl interactions may be dependent on the timing or abundance of Gas 6 expression (Melaragno *et al.* 1999). In the developing nervous system protein-tyrosine phosphorylation is important for the regulation of survival, including cell division, differentiation, proliferation and migration (Crosier & Crosier 1997). Gas 6 binding provides a structural link between cell adhesion receptors and growth factor-related cell differentiation. One RTK, Tyro3 (also called Dtk) has a wide pattern of expression in the brain and may function as a neurotrophic factor receptor. In conditions of stress, Gas 6 increases cell survival in the presence of Axl and its anti-apoptotic properties may be important for neuron survival in normal and pathologic aging (Tsaioun 1999).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Heat responsive protein 12 (Hrsp12)

Heat responsive protein 12 kDa has significant sequence similarity to heat shock protein 70 (Hsp70) with an atypical response to heat shock that demonstrates oscillatory patterns of expression after an initial increase in Hrsp12 mRNA rather than continuous increases as demonstrated by other heat shock proteins. Hrsp12 is a translational inhibitor that may work to inhibit protein synthesis and DNA-proliferation. Levels of Hrsp12 mRNA have been observed to decrease dramatically in highly proliferating cells (Samuel *et al.* 1997, Schmiedeknecht *et al.* 1997)). Although the function of Hrsp12 is unknown, it is expressed at high levels in the kidney and liver as well as in the visual system, particularly in the lens where many structural proteins are closely related to heat shock proteins. A recent cDNA microarray study found that Hrsp12 mRNA is up-regulated in the lens of the visual system of transgenic mice over-expressing Pax6 (paired box gene 6), a gene identified with development of the visual system (Chauhan *et al.* 2002).

Heat-Shock Protein 40 (Hsp40)

Heat-shock protein (Hsp) mRNA changes are interesting in the context of NIL treatment since immunophilins such as FKBP52 and FKBP51 are co-factors within Hsp90/receptor heterocomplexes with steroid-binding activity. In a

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

stepwise assembly, Hsp40 and Hsp 70 combine with adenosine triphosphate (ATP), Hsp90 and steroid receptors to permit high-affinity steroid binding (Pratt & Toft 2003). Constitutive heat shock proteins such as heat shock cognate 70 (HSC70) perform housekeeping functions and are involved in chaperone functions to assist in protein folding as well as protein translocation of misfolded proteins into the cytoplasm for proteolysis (Cohen & Kelly 2003), antigen presentation and steroid receptor function (Nair *et al.* 1997, Pratt & Toft 1997, 2003). Constitutive Hsp may also be activated by oxidative stress but not with heat shock stress (Bernardini *et al.* 2004). Inducible forms of heat shock proteins, such as HSP70 are diagnostic markers for stressful stimuli such as fever, ischemia, inflammation, neurodegenerative disease or exposure to excitotoxins. In addition, heat shock proteins are associated with cell cycle, proliferation and differentiation and may have anti-apoptotic influence (Milani *et al.* 2002). Inducible Hsp mRNA is observed in the area peripheral to the injury within hours. Hsp protein expression follows several hours later and persists for days in cell populations that are resistant to injury but Hsp protein is not highly expressed in degenerating neurons (Yenari 2002).

Pax transcription activation domain interacting protein (Paxip1)

Pax transactivation domain-interacting protein interacts with the paired-box gene (Pax) family of transcription regulators. Paxip1 has been shown to inhibit the transcription of glucagons by the Pax 2 transcription factors

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(Hoffmeister *et al.* 2002). The Pax family of transcription factors has a highly conserved DNA sequence found in many vertebrates as well as *Drosophila melanogaster* suggesting a fundamental role for these genes common among many different species. Since Pax gene expression is spatially and temporally restricted, it may be involved in brain regionalization during development, as well maintaining regional identity in the adult brain (Barr 2001, Wehr & Gruss 1996). Paxip1 is essential to normal development as it appears to affect chromatin condensation when cells are entering mitosis. In Paxip1-null mutants DNA is replicated but shows evidence of damage and few Paxip1-null embryos survive more than a few days. Therefore, Paxip1 may be a component of the DNA damage/repair pathway that facilitates cell proliferation during development. In addition, Paxip1 is omnipresent in all cells suggesting that it is an essential component of universal cellular processes (Cho *et al.* 2003).

Presenilin (PS1)

Presenilin mutations have a pathogenic influence in the generation of β-amyloid plaques in Alzheimer's disease (AD), but unaltered PS has an important proteolytic function in the cell for regulating turnover of signaling molecules during development as well as maintaining neuronal survival in adulthood (Koo & Kopan 2004). PS interacts with calcium-binding proteins such as calsenilin, sorcin and calmyrin, and has a modulatory effect on calcium homeostasis. Presenilin-modulation of intracellular calcium levels also affects glutamatergic

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

systems of uptake and response in neurons. Presenilin is the catalytic component of the γ-secretase protease complex that is involved in Notch signaling. Notch proteins are transcription co-activators that are membrane-bound until released by γ-secretase. After release the Notch intracellular domain (NICD) moves from the cytoplasm to the nucleus to co-activate transcriptional machinery. Notch is important in the process of cell fate determination through lateral inhibition during development (Koo & Kopan 2004, Thinakaran & Parent 2004) and NICD modulates neurite extension and maintenance in the adult (Figueroa *et al.* 2002). In addition, PS and Notch are necessary for the maintenance, though not the generation, of neural stem cells in the adult CNS (Hitoshi *et al.* 2002, 2004) and PS has been shown to interact with the anti-apoptotic molecule Bcl-2 (Alberici *et al.* 1999).

Serine Palmitoyltransferase, long chain base subunit 1 (Sptlc1)

Palmitoylation is a post-translational modification of a protein through the addition of a lipid palmitate to increase hydrophobicity, facilitate interaction with the lipid bilayer of cell membranes or to alter protein function. The thioester bond that links palmitate to the post-translational protein is reversible and labile, which is important for regulating neuronal development and synaptic function. Palmitoylation is important during development to control neurite extension and cell differentiation. In the adult brain palmitoylation modifies synaptic vesicles at the presynaptic nerve terminal (Husseini & Bredt 2002).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Serine Palmitoyltransferase is the rate-limiting enzyme in the production of ceramide, a product stored in the Golgi apparatus and a substrate for sphingolipid synthesis (Denny & Smith 2004). Ceramide is elevated in apoptosis, growth arrest and cell differentiation. Serine palmitoyltransferase is up-regulated in response to stress and apoptosis but it has also been found to be required for normal cellular function (Perry 2002).

Synaptotagmin 11 (Syt11)

Synaptotagmin is thought to be the calcium (Ca^{2+}) sensor responsible for Ca^{2+}-dependent neurotransmitter release. These integral membrane proteins have binding sites for calcium which change its biochemical properties and cause binding to membrane fusion proteins such as SNARE proteins and membrane lipids. Synaptotagmin1 does not however, control the amount of neurotransmitter release, but rather synchronizes the rapid release of neurotransmitters associated with elevated Ca^{2+} levels in the cell (Nishiki & Augustine 2004).

Tyrosinase (Tyr)

Tyrosinase is a membrane glycoprotein that regulates the synthesis of pigment in mammalian cells. Tyrosinase enzymatically catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and oxidation to DOPA quinine, the first two steps in the synthesis of melanin, which protects the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cell from radiation. Tyrosinase activity depends on interaction with the chaperone protein calnexin for proper folding and maturation (Petrescu *et al*. 2003).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.