The Role of Astroglial HO-1 in the Pathogenesis of Parkinson's Disease

Linyang Song

Department of Neurology and Neurosurgery
McGill University, Montreal
October 2005

A thesis submitted to McGill University in partial fulfilment
of the requirements of the degree of Master of Science

Your file Votre référence
ISBN: 978-0-494-24804-1
Our file Notre référence
ISBN: 978-0-494-24804-1

TABLE OF CONTENTS

ABSTRACT

The mechanisms responsible for the progressive loss of dopaminergic neurons and pathological iron deposition in the substania nigra pars compacta of patients with Parkinson disease (PD) remain incompletely understood. Heme oxygenase-1 (HO-1), the rate-limiting enzyme in the degradation of heme to biliverdin, carbon monoxide, and ferrous iron, is up-regulated in affected PD astroglia and may contribute to aberrant mitochondrial iron sequestration in these cells. To determine whether glial HO-1 hyperexpression is inimical to nearby neuronal constituents, we co-cultured dopaminergic PC12 cells atop monolayers of human (h) HO-1 transfected, sham-transfected or non-transfected primary rat astroglia. We observed that PC12 cells grown atop hHO-1 transfected astrocytes, but not the astroglia themselves, were significantly more vulnerable to dopamine (1μM) + H_2O_2 (1μM)–induced death (measured by nuclear ethidium monoazide bromide staining and anti-TH immunofluorescence microscopy) relative to control preparations. In the experimental group, PC12 cell death was significantly attenuated by the administration of the HO inhibitor, SnMP (1.5μM), the antioxidant, ascorbate (200μM), or the iron chelators, deferoxamine (400μM) and phenanthroline (100μM). Exposure to conditioned media derived from HO-1 transfected astrocytes also augmented PC12 cell killing in response to dopamine (1μM) + H_2O_2 (1μM) relative to control media. In PD patients, over-expression of HO-1 in nigral astroglia and attendant iron liberation may facilitate the bioactivation of dopamine to neurotoxic free radical intermediates and predispose nearby neuronal constituents to oxidative damage.

RÉSUMÉ

La maladie de Parkinson (MP) est un trouble du mouvement caractérisé par la dégénérescence des neurones dopaminergiques, d'astrogliose et de déposition pathologique du fer dans la substance noire pars compacta. L'heme oxygenase-1 (HO-1), enzyme limitante du taux de dégradation de l'heme en biliverdine, oxyde de carbone et fer ferreux, est sous régulation positive dans les astroglies affectés, et, peut contribuer à la séquestration aberrante du fer dans leurs mitochondries. Afin de déterminer si la surexpression de l'HO-1 chez les glies est hostile aux constituents neuronaux avoisinants, nous avons co-cultivé des cellules dopaminergique PC12 au dessus de monocouches d'astrocytes soit humains transfectés à l'hHO-1 ou transfectés témoin, soit d'astrocytes primaires de rat non-transfectés. Nous avons constaté que les cellules PC12 cultivées au dessus d'astrocytes transfectés à l'hHO-1, et non les astroglies eux-mêmes, étaient significativement plus vulnérables à la mort cellulaire en présence de dopamine (1μM) et d'H_2O_2 (1μM) (mesuré par marcage de noyaux au bromure d'ethidium monoazide et d'anti-TH en microscopie d'immunofluorescence) par rapport aux témoins. Dans le groupe expérimental, la mort cellulaire des PC12 a été atténuée de façon significative par l'ajout de l'inhibiteur d'HO SnMP (1.5μM), de l'antioxydant ascorbate (200μM), ou de chélateurs ferriques tel le déféroxamine (400μM) et le phenamathroline (100μM). Leur exposition à du milieu conditionné par des astrocytes tranfectés à l'HO-1 a également augmentée la mort cellulaire des PC12 en réponse à la dopamine (1μM) et l'H_2O_2 (1μM) par rapport au milieu témoin. Chez les patients parkinsonien, la surexpression de l'HO-1 dans les nigro-astroglies et la libération du fer résultante, pourrait faciliter la bioactivation neurotoxique de la dopamine en ses radicaux libres intermédiaires, et ainsi prédisposer

les constituent neuronaux avoisinants aux dommages oxidatifs.

ACKNOWLEDGEMENTS

I would like to express appreciation to Department of Neurology and Neurosurgery, McGill University which provided me with the chance to pursue my interests as well as to extend my knowledge. I sincerely thank my thesis supervisor, Dr. Hyman M. Schipper, for introducing me to the field of oxidative neurochemistry, giving me the opportunity to work on such an interesting project, and providing me with excellent guidance in the process of the accomplishment of this work. During the three years spent in his laboratory, I accumulated valuable experience in experimental design and communicative skills as a scientist. I also want to thank my thesis mentor, Dr. Andrea LeBlanc, and my thesis committee members, Dr. James Brawer and Dr. Hemant Paudel, for their advice and guidance.

Dr. Wei Song and Mrs. Adrienne Liberman assisted me with experimental design and laboratory techniques. Finally, I wish to thank all the current and former members of the Schipper lab for providing a stimulating work environment.

INTRODUCTION

Parkinson's disease (PD) is a chronic movement disorder characterized by progressive degeneration of dopaminergic neurons, astrogliosis, pathological iron sequestration, and mitochondrial insufficiency in the pars compacta of the substantia nigra (DiMonte 1998). The mechanisms responsible for the progressive loss of dopaminergic neurons and pathological deposition of brain iron in Parkinson's disease remain poorly understood. There is considerable literature implicating oxidative stress as a major factor in the pathogenesis of PD. Lipid peroxidation and other oxidative markers are elevated in the PD substantia nigra compared with aged-matched control tissues. Free radical scavenging enzymes and reducing substances are deficient in the basal ganglia of PD patients (Ambani 1975; Perry et al., 1982.). Excessive concentrations of hydrogen peroxide (H_2O_2) are generated by monoamine oxidase B (MAO-B) - mediated deamination of dopamine (DA) in idiopathic PD brain and experimental parkinsonism. MPTP and manganese, triggers of parkinsonism in animal models, exert their toxic effects, in part, through the generation of free radicals (Barbeau 1984; Cadet et al., 1989).

Abnormally high levels of tissue iron have been consistently documented in the substantia nigra of PD subjects. The excessive nigral iron is primarily deposited within nonneuronal compartments, including astrocytes, microglia, macrophages, and microvessels. There is little or no histochemical evidence for significant iron sequestration in PD-affected nigral or striatal neurons (Faucheux et al., 1995; Leveugle et al., 1996). High concentrations of tissue iron in the basal ganglia of PD patients can serve as a major generator of reactive oxygen species by (a) reducing H_2O_2 to highly

reactive hydroxyl radical and (b) behaving as a non-enzymatic peroxidase activity capable of oxidizing dopamine to neurotoxic semiquinone intermediates. Mitochondrial insufficiency, predominantly implicating Complex I of the electron transport chain, is another consistent feature of PD-affected neural tissues (Schapira 2001; Beal 2003).

Heme oxygenase-1 (HO-1) is a cellular stress protein expressed in brain and other tissues in response to oxidative challenge and other noxious stimuli. It is the rate-limiting enzyme in the degradation of heme to biliverdin, carbon monoxide, and ferrous iron. Cytoplasmic Lewy bodies, a pathological hallmark of PD, observed in affected dopaminergic neurons are prominently decorated with HO-1 staining (Castellani etal., 1996; Schipper et al., 1998). The proportion of GFAP-positive astrocytes that co-express HO-1 in PD-affected substantia nigra is significantly increased relative to age-matched controls. Upregulation of HO-1 and iron deposition primarily implicate the astroglial compartment of the PD-affected substantia nigra (Schipper et al., 1998). Overexpression of the human HO-1 gene in cultured rat astroglia by transient transfection stimulated mitochondrial ^{55}Fe deposition, an effect that was attenuated by the administration of the HO inhibitors, tin mesoporphyrin (SnMP) or dexamethasone (DEX). These investigations suggested that stress-induced upregulation of HO-1 in astroglia may contribute to the abnormal patterns of iron deposition and mitochondrial insufficiency in PD-affected brain regions (Schipper et al., 1999).

In present study, we investigated whether catecholamine-secreting PC12 cells grown atop HO-1 transfected astroglial monolayers exhibit elevated vulnerability to oxidative

stressors in comparison with PC12 cells cocultured with control astroglia. Non-differentiated PC12 cells were cocultured with HO-1 transfected or sham-transfected (control) astrocytes and subjected to dopamine/H_2O_2 challenge. In some experiments, HO-1 inhibitors, antioxidants, or iron chelators were administrated to the cocultures. PC12 cytotoxicity was assessed by anti-tyrosine hydroxylase immunostaining in conjunction with EMA staining. In further experiments, PC12 cells cultured alone were exposed to conditioned media from HO-1 transfected and control astrocytes. The extent of PC12 cell death was examined by Trypan blue staining. Our observations suggest that up-regulation of HO-1 expression in nigral astroglia may render nearby dopaminergic neurons increasingly vulnerable to oxidative injury.

BACKGROUND

Parkinson's Disease

In 1817, a London physician, Dr. James Parkinson, initially described the clinical and pathological features of a chronic neurological disorder characterized by involuntary tremulous motion and rigidity of the limbs, trunk and head (the "shaking palsy"). This disorder was subsequently referred to as Parkinson's disease (PD) (Allam et al., 2005). PD is a slowly progressive movement disorder that afflicts 1-2% of the population. The average age of onset of PD is approximately 60 years. Early-onset PD is defined when occurring before the age of 40 and afflicts approximately 5% of the total PD population. Idiopathic PD is somewhat more prevalent in men than women. Although the majority of cases are sporadic, some cases are familial (Morris, 2005). The primary symptoms of this common neurodegenerative disorder include: muscular rigidity, resting tremor, difficulty with movement initiation, slowness of voluntary movement, difficulty with balance, and difficulty with walking. Additional symptoms include autonomic instability, sleep disorder, mood abnormalities and cognitive dysfunction. The clinical diagnosis of PD is based on the identification of combinations of the aforementioned symptoms.

Although some pharmacotherapies, including the administration of L-DOPA, dopamine receptor agonists and anticholinergics, are helpful in improving symptoms of PD, there currently exists no treatment that unequivocally attenuates neuronal depletion and clinical decline in this debilitating condition.

Loss of Dopaminergic Neurons in PD Brain

PD is believed to be caused, in large measure, by the deficiency of nigrostriatal dopamine. The basal ganglia play an important role in controlling voluntary movement. Signals from the cortex are conveyed through the basal ganglia to the thalamus, which, in turn, provides feedback control to motor centers in the cerebral cortex. In postmortem studies, investigators found that the substantia nigra had lost its pigment (neuromelanin) in PD patients. Subsequent studies showed that dopamine levels in the striatum were drastically (>80%) reduced. Because the basal ganglia contain most of the dopaminergic neurons of the brain, these observations suggested that the dopaminergic pathway between substantia nigra and striatum are degenerated in PD patients. The depletion of dopamine disturbs the direct and indirect pathways from the striatum, which causes the thalamus to be over-stimulated. As a result the frontal cortex is less activated which would account for many of the Parkinsonian symptoms.

Tyrosine hydroxylase (TH) is the rate-limiting step in the biosynthesis of dopamine and other catecholamines. There are at least two pathways (enzymatic and non-enzymatic) for dopamine oxidation in the brain (Figure 1). In the enzymatic pathway, monoamine oxidase B (MAOB) catalyzes the oxidation of DA to generate DOPAC, NH_3, and hydrogen peroxide. In the non-enzymatic pathway, in the presence of hydrogen peroxide, ferrous iron behaves as a pseudoperoxidase activity that converts dopamine to neurotoxic quinones and ortho-semiquinone radicals.

Heme Oxygenase-1

Heme oxygenase-1 is a 32-kDa member of the stress protein superfamily that mediates the oxidative catabolism of heme in brain and other tissues. The HO-1 gene contains heat shock elements and AP1, AP2, NFkB binding sites in its promoter region, thereby making it highly inducible by a wide range of pro-oxidant and inflammatory stimuli such as oxidative stress, dopamine, H_2O_2, UV light, metal ions, and pro-inflammatory cytokines (Scapagnini et al., 2002). Mammalian cells express two isoforms of heme oxygenase, HO-1 and HO-2. These two isoforms are encoded by distinct genes and exhibit significant differences in molecular weight, electrophoretic mobility, and cellular distribution and regulation. Whereas HO-1 exists primarily in microsomes, HO-2 is mainly localized in mitochondria (Morse et al., 2005). HO-1 is the rate-limiting enzyme that catalyzes the oxidative degradation of heme to biliverdin, free ferrous iron and carbon monoxide (CO). Biliverdin is metabolized further to the bile pigment, bilirubin by the action of biliverdin reductase (Figure 2).

Synthetic metalloporphyrins, such as tin mesoporphyrin (SnMP) is competitive inhibitor of the heme oxygenase reaction whereas dexamethasone (DEX) suppresses HO-1 expression at the transcriptional level (Schipper et al., 1999).

Dual Effects of HO-1

Bliverdin, bilirubin, free ferrous iron and carbon monoxide (CO), products of the heme oxygenase reaction, are all biologically active molecules. HO-1 has exhibited both deleterious and protective effects in various models of tissue injury and disease. In some situations, upregulation of HO-1 in response to oxidative stress may protect cells by accelerating the catabolism of pro-oxidant heme, to bile pigments that have potent free radical scavenging capabilities (Suttner and Dennery, 1999). In other situations, free iron and carbon monoxide released during heme degradation have been shown to increase intracellular oxidative stress and predispose the mitochondria to free radical damage (Song et al., in press). The extent and duration of HO-1 induction and the status of the local redox microenvironment may determine whether the antioxidant benefits or the deleterious pro-oxidant effects predominate.

HO-1 Expression in PD Brain

Numbers of neuroglia immunoreactive for HO-1 in cortical and subcortical regions of the normal human brain progressively increase with advancing age. In 1998, Schipper used immunohistochemical techniques to assess HO-1 expression in various postmortem human brain specimens derived from PD and control subjects matched for age and post-mortem interval. In the substantia nigra of both PD and control specimens, moderate HO-1 immunoreactivity was consistently observed in dopaminergic neurons (Schipper et

al., 1998). In the PD samples, affected dopaminergic neurons could be readily identified by the presence of cytoplasmic Lewy bodies that were prominently decorated with annular HO-1 labeling. Neuronal HO-1 staining was faint or nondetectable in the other brain regions surveyed in both PD and control specimens. The percentages of glial fibrillary acidic protein (GFAP)-positive astrocytes expressing HO-1 in PD substantia nigra were significantly greater than that observed in age-matched control subjects. In the other regions examined, percentages of GFAP-positive astroglia coexpressing HO-1 were relatively low and were not significantly different between control and PD specimens. Upregulation of HO-1 in the substantia nigra of PD subjects supports the opinion that the affected tissue is experiencing chronic oxidative stress. In addition, excessive cellular levels of free iron and carbon monoxide resulting from HO-1 overexpression may contribute to the pathogenesis of PD (Schipper et al., 1998; Rogers et al., 2003).

The fact that expression of HO-1 is enhanced in PD-affected brain regions is consistent with the induction of other stress proteins in this pathologic condition, including heat shock protein 27, 72, and ubiquitin (Wang et al., 1995). The genes encoding HO-1 and the other stress proteins contain various consensus sequences in the promoter regions that mediate transcriptional regulation by redox sensitive transcription factors (Mehindate et al., 2001). Local overexpression of these proteins is possibly secondary to the elevated level of oxidative stress existing in PD-affected brain regions. As described below, stress-induced up-regulation of HO-1 in astroglia may be responsible for the abnormal patterns of brain iron deposition and mitochondrial insufficiency documented in PD.

Iron Sequestration in PD Brain and Stressed Astroglia

Metal ions are vital elements for life and they participate in many metabolic processes. Disturbance of normal metal ion homeostasis and distribution can precipitate cellular dysfunction and death and thereby contribute to the pathogenesis of a host of neurological and systemic diseases. Abnormally high levels of tissue iron have been consistently documented in the substantia nigra and basal ganglia of PD subject (Ponka et al 1982). The excessive nigral iron appeared to be primarily deposited within nonneuronal substrates, including astrocytes, microglia, macrophages, and microvessels. Although minor amounts of iron have been detected in neuronal neuromelanin using microanalytical techniques, there is little histochemical evidence for significant iron sequestration in PD-affected nigral or striatial neurons (Castellani et al., 1996).

In rat primary astrocyte cultures, cysteamine, H_2O_2 and dopamine rapidly induce HO-1 expression (mRNA and protein) followed by sequestration of non-transferrin-derived ^{55}Fe by the mitochondrial compartment (Schipper et al., 1999). The effect of dopamine on HO-1 expression was inhibited by ascorbate implicating a free radical mechanism of action. Dopamine induced mitochondrial iron sequestration was attenuated by administration of SnMP, DEX, or antioxidants indicating that HO-1 upregulation is necessary for subsequent mitochondrial iron deposition in these cells (Schipper et al., 1999). Overexpression of the human HO-1 gene in cultured rat astroglia by transient transfection also stimulated mitochondrial ^{55}Fe deposition and upregulation of manganese

superoxide dismutase (MnSOD) mRNA and protein, an effect that was also attenuated by SnMP or DEX administration (Frankel et al., 2000). These data suggest that free ferrous iron and carbon monoxide generated by HO-1 mediated heme degradation may promote mitochondrial membrane injury and the deposition of redox-active iron within this organelle.

Mitochondrial Insufficiency in PD Brain

Mitochondria provide the energy cells need to move, divide, contract, and secrete. Mitochondria are the centers of cellular respiration and energy metabolism and contain various enzymes and electron transporters needed in the process of cellular respiration. The electron transport chain (ETC) is located in the inner mitochondrial membrane where carbohydrate is combusted with oxygen to produce ATP. The produced energy is stored in the high-energy phosphate bonds of ATP and transported to a variety of cellular compartments.

Mitochondrial insufficiency involving complex I of the ETC is characteristic of PD-affected brain tissues (Beal, 1995). There is abundant evidence showing mitochondrial insufficiency in PD human nigra and PD animal models, such as reduced complex I activity (Jenner, 2003), increased mitochondrial DNA mutations in both neurons and glia (Soong et al 1992), and decreased glucose utilization and elevated lactate production (Bowen et al., 1995). The glial mitochondriopathy may accelerate free radical production within damaged ETC, suppress the cellular ATP levels and ATP-dependent processes, and

release cytochrome c and other pro-apoptotic factors (Hattori et al., 1991). The development of effective therapies to slow or arrest the neurodegenerative process in the brain of PD subjects presupposes a thorough understanding of the mechanisms responsible for pathological iron deposition and oxidative mitochondrial injury in this debilitating condition.

RATIONALE, OBJECTIVE, AND SPECIFIC AIMS

Rationale

1. Loss of dopaminergic neurons, HO-1 overexpression, iron deposition, and mitochondrial insufficiency are characteristic patholgical features of the PD substantia nigra. HO-1 overexpression and mitochondrial iron sequestration have been shown to implicate primarily the astroglial compartment (Schipper, 2000).

2. Transient transfection of HO-1 in rat primary astrocytes promotes the sequestration of iron by the mitochondrial compartment and the compensatory upregulation of manganese superoxide dismutase mRNA and protein (Schipper et al., 1999).

3. HO-1 transfected primary astrocytes exhibit enhanced oxidative mitochondrial damage relative to nontransfected and sham-transfected controls, and the effects are attenuated by the administration of the HO inhibitor, SnMP. Specifically, elevated levels of protein carbonyls, 8-epiPGF2α and 8-OHdG, markers of oxidative protein, lipid and nuclear acid damage, respectively, were observed in mitochondrial fractions of HO-1 transfected astroglia (Song et al., in press).

4. Mitochondrial iron sequestration within the astroglial substratum enhances the vulnerability of co-cultured PC12 cells to oxidative killing (Frankel and Schipper, 1999).

Objective

The major objective of this thesis was to test the following hypothesis: PC12 cells grown atop HO-1 overexpressing astrocytes will exhibit augmented vulnerability (cell death) to oxidative stressors. Experiments described below were designed to investigate the events that may contribute to loss of dopaminergic neurons, pathological iron sequestration, and mitochondrial insufficiency in the pars compacta of the substantia nigra in PD patients. The specific aims are intended to examine the hypothesis that originates from work in Dr. Schipper's laboratory implicating astroglial HO-1 overexpression in the susceptibility of dopaminergic neurons to oxidative stressors.

Specific Aims

1. To examine cell death of PC12 cells cocultured with nontranfected, sham, HO-1 transfected, and HO-1 transfected + SnMP astrocytes in the face of the oxidative stressors, dopamine (1μM) + H_2O_2 (1μM).

2. To determine whether deleterious effects of HO-1 transfected astroglia on co-cultured PC12 cells can be inhibited by antioxidants and iron chelators.

3. To examine PC12 cell death in response to dopamine (1μM) + H_2O_2 (1μM) following exposure of the PC12 cells to conditioned media derived from HO-1 transfected astroglia.

MATERIALS AND METHODS

Materials

Neonatal Sprague Dawley rats (2 days after birth) were obtained from Charles River Breeding Farms (St. Constant, Quebec, Canada). PC12 rat pheochromocytoma cells, a catecholamine-secreting cell line, were obtained form American Type Culture Collection (Manassas, VA). Ham's F-12, high glucose Dulbecco's modified Eagle's medium (DMEM), and opti-MEM I reduced serum medium were purchased from GIBCO BRL (Life Technologies, Burlington, Canada). Fetal bovine serum (FBS) and horse serum (HS) were purchased from WISENT (St-Bruno, Canada). Tin mesoporphyrin (SnMP) was purchased from Porphyrin Product (Logan, UT). Human heme oxygenase-1 (Xho1-EcoR1) (1Kb) in pBluescript SKII(+) was obtained from Dr. S. Shibahara (Tohuku University, Japan), pcDNA3.1(+)/Zeo.flag from Dr. R. Lin (Lady Davis Institute for Medical Research, Montreal, Quebec) and pEGFP.C1 from Clontech (Palo Alto, CA). Dopamine, poly-D-lysine, anti-glial fibrillary acidic protein (GFAP) antisera, trypan blue, penicillin-streptomycin, ascorbate, deferoxamine (DFO), and phenanthroline were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture flasks, cover slips, chamber slides, well dishes and hydrogen peroxide (H_2O_2) were purchased from Fisher Scientific Ltd. (Montreal, Canada). Ethidium monoazide bromide (EMA) was purchased from Molecular Probes, Inc. (Eugene, OR). FITC anti-rabbit antisera and FITC-conjugated goat anti-mouse antibody were purchased from Jackson Immunoresearch Lab (Baltimore, MD). Anti-tyrosine hydroxylase (TH) antibody was purchased from Boehringer-Mannheim (Laval, Quebec, Canada).

Transfection of Human HO-1 cDNA

Neonatal rat astrocytes were transiently transfected with human HO-1 (hHO-1) cDNA and control vectors using the Lipofectamine method. The hHO-1 construct consisted of pcDNA3.1/Zeo.CMV.Flag.hHO-1 containing the entire protein-coding region (866 bp) of the human HO-1 gene. Identical plasmids without the hHO-1 cDNA were used as vector controls (sham- or flag transfection). Upon reaching >95% confluence, 1×10^6 cells were transiently transfected with 4.0 μg of plasmid DNA-Lipofectamine 2000 complex according to manufacturer instructions (Invitrogen): 4.0 μg of plasmid DNA and 15 μl of Lipofectamine 2000 reagent were diluted individually in 250 μl opti-MEM I and incubated for 5 minutes at room temperature with gentle mixing. The two solutions were combined, incubated at room temperature for 20 minutes to promote formation of DNA-lipid complexes and administered to the cells. Following incubation for 6 hours at 37°C, the transfection mixture was replaced with complete media without antibiotics. At 12 hours post-transfection, some cultures were treated with tin mesoporphyrin (SnMP; 1.5 μM), a competitive inhibitor of heme oxygenase activity (with light-shielding to prevent metalloporphyrin photoactivation). At concentrations below 5 μM, the metalloporphyrin specifically inhibits the enzymatic activity of heme oxygenase, but not nitric oxide synthase or other heme-containing enzymes (Appleton et al., 1999). Cells were harvested at 72 hours post-transfection for HO-1 mRNA/protein expression and HO enzyme activity as described below. Transfection efficiency was determined by assessment of

green fluorescence protein (GFP) expression in astrocytes co-transfected with hHO-1 cDNA plasmid and pEGFP.C1 vector. The cells were seeded on coverslips precoated with 0.01% poly-D-lysine. On day 3 post co-transfection with plasmids containing hHO-1 and EGFP (1:1), the coverslips were rinsed twice with PBS and mounted on glass slides with 95% glycerol + 5% dH_2O. Transfection efficiency was assessed by evaluation of 9 coverslips in three separate experiments and expressed as no. transfected cells (counted under FITC fluorescence) /total cells (counted under phase contrast) × 100%.

Western Blot Analysis

On transfection day 3, cells were rinsed twice with ice-cold phoaphate buffer saline and scraped on ice in lysis buffer consisting of 1% Nonidet P-40, 50 mM Tris.HCl (pH 7.4), 30 mM NaCl, 25 mM beta-glycerophosphate, 10 mM EDTA, 1 mM $MgCl_2$, and protease inhibitors. The supernatant fraction was obtained by centrifugation at 15000 rpm for 15 min at 4°C and protein content was determined with Bradford reagent assay. 20μg aliquots plus 6X SDS-PAGE loading buffer were subjected to 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride transfer membranes. Nonspecific binding was blocked by incubation in Tris buffer saline containing 3% nonfat milk, and 0.1% Tween 20 for 1h at room temperature. Blots were probed with mouse anti-flag monoclonal antibody or anti-β actin monoclonal antibody (loading control). The secondary antibody was a 1:4000 diluted horseradish peroxidase-conjugated goat anti-mouse antibody. Protein bands were visualized by the enhanced chemiluminescence using ECL western blotting reagents.

Heme Oxygenase Enzyme Activity Assay

Cytoplasmic extracts were prepared for HO activity measurement using a method previously described (Ryter et al 2000). Cells were scraped in ice-cold PBS-EDTA, 1mM, containing 50μm/ml protease inhibitor (AEBSF), centrifuged at 150 g and resuspended in 20 mM Tris-HCl, 0.25 M sucrose containing protease inhibitors. Cell suspensions were sonicated on ice 2 × 15 s at 20w and centrifuged for 20 min at 15000g. Protein concentrations of resulting supernatant were determined by Bradford reagent assay. HO activity was determined by rates of bilirubin production. Final reaction concentrations were 25 μM heme, 2 mM glucose 6-phosphate, 2 units glucose 6-phosphate dehydrogenase, 1mM β-NADPH, 0.5 mg/ml cytoplasmic extracts, and 2 mg/ml partially purified rat liver biliverdin reductase preparation. Reaction mixtures were incubated at 37°C in a water bath in the dark for 60 min with vortex every 10 min. The reactions were terminated by administration of 1 volume chloroform. Bilirubin concentration in the chloroform extracts was determined spectrophotometrically by measuring optical density at 464-530 nm. HO activity was calculated as picomoles bilirubin per milligram protein per min, assuming an extinction coefficient of 40 mM^{-1} cm^{-1} in chloroform.

Coculture

Primary Astrocyte Cultures

Primary neural cell cultures were prepared by mechanoenzymatic dissociation of cerebral tissues as previously described (Chopra et al., 1995). Cells were grown in Ham's F12 and high glucose DMEM (1:1) supplemented with 10 mM Hepes, 5% inactivated horse serum and 5% inactivated fetal bovine serum and penicillin-streptomycin. The neural cells were plated in T75 culture flasks precoated with 0.01% poly-D-lysine at a density of 1×10^6 cell/ml. At 6 hours, the cultures were intensely shaken 20 times to remove adherent oligodendroglia and microglia from the astroglial monolayers. The cultures were incubated for 6 days at which time they consisted of >98% astroglia as determined by GFAP immunostaining.

PC12 Cell Cultures

PC12 rat pheochromocytoma cells were kept at -135°C in sterilized freezing media until plating. Culture flaskes were precoated with 0.01% poly-D-lysine. PC12 cells were plated at a density of 1×10^6 cell/ml in F12K (modification of Ham's F12 medium) supplemented with 15% horse serum, 2.5% fetal bovine serum, and penicillin-streptomycin. Cells were sub-cultivated twice weekly and replated as mentioned above. The cultures were incubated at 37°C in a humidified 95% air-5% CO_2 chamber.

PC12/Astrocyte Cocultures

At one day post-transfection, the astrocytes were trypsinized and plated on coverslips or chamber slides precoated with 0.01% poly-D-lysine at a density of 1×10^6 cells/ml. The astrocytes were given an additional 24h to adhere to the substrata before addition of PC12 cells. PC12 cells were removed from the flasks by gentle, repetitive pipetting of the culture media, concentrated by centrifugation, resuspended in fresh medium, and plated on top of the astrocytes at a density of 2×10^4 cells/ml. The cocultures were incubated at 37°C in a humidified 95% air-5% CO2 chamber and allowed 48h to settle in preparation for the cytotoxicity experiments as described below. The ratio of PC12/astrocytes in the cocultures at the initiation of the cytotoxicity studies was approximately 1:50, consistent with neuron/astrocyte ratios reported in situ (Desagher et al., 1996).

Pharmacologic Treatment

On day 10 *in vitro*, PC12 cell in co-culture with astrocytes (nontransfected, flag, HO-1-transfected, and HO-1 + SnMP) were challenged with the following conditions: (1) dopamine (1 μM) and H_2O_2 (1 μM) (DH), (2) dopamine (1 μM) alone, (3) H_2O_2 (1 μM) alone, (4) DH with ascorbic acid (200 μM), (5) DH with deferoxamine (400 μM), or (6) DH with phenanthroline (100 μM). All drugs were administered in prewarmed culture media for 24h. The cocultures were subsequently washed with 0.1 M phosphate-buffered saline and stained with ethidium monoazide bromide (EMA 10 μg/ml) for 10 min at room temperature under UV light exposure. EMA selectively labels cells with damaged

nuclear membranes. It binds to the DNA of dead nuclei covalently, thereby preventing dye leakage after fixation (Berns et al., 2000).

Immunofluorescence Confocal Microscopy

Immunofluorescent staining

The cultures were rinsed with PBS containing calcium and magnesium ion and prior to fixation. After fixation in 4% paraformaldehyde for 20 min at room temperature, the cultures were permeablized in Triton X-100 buffer (sodium phosphate 10 mM, NaCl 0.5 M, 0.2% Triton X-100, and 0.5 BSA) for 10 min, and then blocked with blocking solution for 30 min at room temperature. Cultures not stained with EMA were incubated with mouse-derived anti-tyrosine hydroxylase monoclonal antibody (1/200 dilution) overnight at 4°C and secondary antisera consisting of rhodamine-conjugated anti-mouse antibody (1/50 dilution) for 1 hour at room temperature. Astrocytes were immunolabeled with rabbit-derived polyclonal anti-GFAP antisera (1/400 dilution) overnight at 4°C followed by FITC-conjugated anti-rabbit secondary antibody (1/50 dilution) for 1 hour at room temperature. Cultures for cytotoxicity quantification (stained with EMA) were incubated with mouse-derived anti-tyrosine hydroxylase monoclonal antibody (1/200 dilution) overnight at 4°C followed by FITC-conjugated anti-mouse secondary antibody (1/50 dilution) for 1 hour at room temperature. Astrocytes were left unstained. Cultures were mounted with 95% glycerol + 5% dH_2O, coverslipped, and sealed with nail polish.

Confocal Microscopy

The fluorescence-labeled slides were examined using a Bio-Rad MRC-600 laser scanning confocal microscope. This system is equipped with a krypton/argon laser which excites samples with lines at 488, 568, and 647 nm. The emission wavelengths were detected from the samples using the K1 and K2 filter block set. The latter allows the detection of wavelengths between 522 and 554 nm on the green channel and 585 nm or greater on the red channel. Both channels were opened to the 2/3 level. Images scanned on the two channels (green and red) were merged to produce a single profile. In merged images, yellow fluorescence indicates co-localization of red and green emitters. After scanning, the images were collected using COMOS software package.

Cell Death Assays

Cocultures

In PC12/astrocyte cocultures dually labeled with EMA and anti-TH (FITC-conjugated), the extent of PC12 cell death under the various experimental conditions was determined in 25 × fields as the proportion of total PC12 cells (labeled fluorescent green with anti-TH) that exhibited bright red, EMA-positive nuclei. Four sister cultures were evaluated for each experimental group. A minimum of 100 cells were assessed per culture.

Astrocytes

Cytotoxicity of astrocytes cultured alone under various experimental conditions was

ascertained by trypan blue staining. Astrocytes were plated in 24-well dishes which were precoated with 0.01% poly-D-lysine at a density of 1×10^6 cells/ml. Cells were transfected with vectors when reaching 95% confluence and exposed to pharmacologic treatment 4 day post-transfection. Twenty four hours later, cells were trypsinized, gently harvested, and incubated with trypan blue. Four wells were evaluated for per experimental group. Astrocyte cell death was calculated (with the aid of a hemocytometer and light microscope) as the proportion of total astrocytes exhibiting trypan blue staining.

Conditioned media

Potentially deleterious effects of astroglial HO-1 overexpression on dopaminergic PC12 cells were further investigated when astrocytes and PC12 cells were cultured separately. Astrocytes were plated in 24-well dishes precoated with 0.01% poly-D-lysine. Cultures were transfected with vectors when reaching 95% confluence and exposed to pharmacologic treatments 4 day after transfection. PC12 cells were plated in 96 well dishes precoated with 0.01% poly-D-lysine. Cells were allowed 48h to settle and then media were replaced by supernatant derived from the various astrocyte cultures (conditioned media). The PC12 cells were exposed to glial conditioned media for a total 36 hours with reapplication of refresh glial supernatant every 12 hours. PC12 cells were typsinized, gently harvested, and incubated with trypan blue. Four wells were evaluated for per experimental group as described above. PC12 cell death was calculated as the proportion of total PC12 cells exhibiting trypan blue staining.

Statistics

Statistical analyses were performed using a two-factor between-subjects analysis of variance (ANOVA) test with $P<0.05$ indicating significance. A Tukey's post-hoc test was used to assess the significant main effects within a given group.

RESULTS

Transfection efficiency, Protein Expression, and Enzyme Activity

Cells successfully transfected with pEGFP.C1 vector were identified by their bright green color under fluorescence microscopy (figure 3). Transfection efficiency was determined to be 40.2 ± 1.7% (mean ± SE). Transient transfection with human heme oxygenase-1 cDNA resulted in up-regulation of exogenous HO-1 protein expression in a dose-dependant manner. Addition of SnMP had little or no effect on exogenous HO-1 expression in the HO-1 transfected glial cultures (figure 4). Astrocyte HO-1 enzyme activity increased 4-5 fold after hHO-1 transfection in comparison with non-transfected and sham-transfected astrocytes. Heme oxygenase activity was significantly inhibited by the addition of SnMP (figure 5).

Morphology

In PC12/astrocyte cocultures dually labeled with anti-GFAP (FITC-conjugated) and anti-TH (rhodamine-conjugated), the majority of astrocytes (green fluorescence) exhibited polygonal shapes with radiating processes and formed sheet-like monolayers beneath the overlying PC12 cells (red fluorescence). Some astroglia were stellate and extended short, fine processes over adjacent cells in the glial substratum. The PC12 cells were distributed singly or in small clusters atop the glial substratum and exhibited spherical,

cuboid, or elongated profiles. Some PC12 cells extended fine short processes. There were little or no morphological differences in the growth characteristics of astrocytes and PC12 cells among the various experimental groups in the absence of pro-oxidant stressors (Figure 6, 7). In cocultures stained with EMA, PC12 cell death was characterized by the presence of large, round EMA-positive nuclei (Figure 8, 9). Shrunken or compacted PC12 cell nuclei and apoptotic bodies were not observed, suggesting that necrosis, rather than apoptosis, was the mechanism of cell death in this paradigm.

Cell Death Assay

PC12/Astrocyte Cocultures

In the cytotoxicity experiments, all PC12 cells were stained with anti-TH using a FITC-conjugated secondary antibody (green fluorescence), dead PC12 cells were identified by the presence of red, EMA-positive nuclei and the astroglial substratum remained unstained. Healthy PC12 cells exhibited green cytoplasm staining and unstained nuclei, whereas dead PC12 cells exhibited green fluorescent cytoplasm and red nuclei. Dead astrocytes exhibited red fluorescent nuclei and unstained cytoplasm (figure 8). In the absence of oxidative stressors, fractions of dead PC12 cells in co-culture with HO-1 transfected astroglia were not significant different from PC12 cells co-cultured with sham- and non-transfected astroglia ($P>0.05$) (Figure 10). However, the addition of SnMP significantly reduced cell death of PC12 cocultured with HO-1 transfected astrocytes ($P<0.01$).

PC12 cells grown on the HO-1 transfected astroglial monolayer were much more

vulnerable to killing by 24h exposure to dopamine (1μM) and H_2O_2 (1μM) (DH) relative to similarly challenged PC12 cells co-cultured with sham- or non-transfected astrocytes ($P<0.05$ for each comparison) (Figure 11). The administration of SnMP significantly attenuated DH-induced cell death in PC12 cells cocultured with HO-1 transfected astroglia ($P<0.05$), and the extent of cell death was not significantly higher than PC12 cells cocultured with non- or sham-transfected astrocytes ($P>0.05$ for each comparison). Dopamine (1μM) or H_2O_2 (1μM) administered alone was not significantly more toxic to PC12 cells when the PC12 cells were grown atop HO-1 transfected in comparison with sham-transfected astroglial monolayers. PC12 cell death under these two conditions was significantly less than that seen in cultures exposed to both dopamine and H_2O_2 ($P<0.05$ for each comparison). The antioxidant, ascorbate, (200μM) eliminated DH-induced cell death of PC12 cells grown atop the HO-1 transfected astroglia ($P<0.05$), and the extent of PC 12 cell death under this condition was not significantly different from the unchallenged condition ($P>0.05$). The iron chelators deferoxamine (DFO) and phenanthroline significantly protected PC12 cells grown atop HO-1 transfected astroglia from DH toxicity ($P<0.05$). There was no significant difference between DFO and phenanthroline ($P>0.05$) in their ability to protect against DH-induced PC12 cytotoxicity in this coculture model (Figure 11). EMA positive astrocytic nuclei were extensively observed under any of the experimental conditions described in this study.

Astrocyte Cocultures

There was no significant difference found among any experimental groups when astrocytes were cultured alone, although HO-1 transfected astrocytes showed greater

levels of cell death that did not achieve statistical significance ($P>0.05$) in comparison with non-transfected and flag-transfected astrocytes (Figure 12). DH administrations did not significantly increase cell death of astrocytes transfected with HO-1 cDNA relative to control groups ($P>0.05$).

Conditioned media

Incubation of PC12 cells in astrocyte-conditioned media resulted in similar patterns of PC12 cell death observed in coculture experiments except on a smaller scale (Figure 13). PC12 cell death was significantly elevated when incubated with supernatants from HO-1 transfected astrocytes subjected to DH challenge relative to the control groups ($P<0.05$). Exposure to conditioned media derived from HO-1 transfected astrocytes treated with SnMP did not show augmented PC12 cell killing ($P>0.05$). Ascorbate protected PC12 cells from death induced by incubation of conditioned media from DH-challenged HO-1 transfected astrocytes ($P<0.05$). The addition of both DFO ($P<0.05$) and phenanthroline ($P<0.05$) abrogated PC12 cell death induced by exposure to conditioned media from DH-challenged, HO-1 transfected astroglia.

DISCUSSION

The precise roles that astrocytes play in promoting or ameliorating neuronal degeneration in PD and other aging-related neurodegenerative disorders remain unclear. Further investigation of the behaviour of these cells in aging and degenerating neural tissues would facilitate our understanding of the pathophysiology of these conditions. Astrocytes, the most abundant glial cell types in the mammalian brain, provide metabolic and trophic support to neurons and modulate synaptic activity. Some astrocytes extend processes to the surface of the CNS to form a glial membrane that serves as a protective covering fro the CNS (Kandel and Schwartz, 1991). Astrocytes are involved in the guidance and migration of neuronal cells and growth cones during embryogenesis. Astrocytes are responsible for the maintenance of ion homeostasis, production of proinflammatory and immunomodulatory cytokines and metabolism of various neurotransmitters (Hertz, 1981; Fedoroff and Vernadakis, 1986; Benveniste, 1992). Astrocyte apoptosis may contribute to the pathogenesis of many acute and chronic neurodegenerative disorders, such as cerebral ischemia, Alzheimer's disease and Parkinson's disease. A previous study demonstrated that pure PC12 cell cultures without astrocytes were entirely destroyed by dopamine/H_2O_2 exposure in doses utilized in the current study (Frankel et al., 1999). Therefore, alterations in normal physiological conditions of astrocytes can dramatically influence neuronal survival and function. We have focused on the astroglial compartment because (a) iron deposition in the aging and degenerating human central nervous system has primarily implicating astroglial and other nonneuronal compartments (Schipper, 1998); and (b) a subpopulation of astroglia in the mammalian hippocampus, basal ganglia, and other subcortical regions progressively accumulates iron-laden cytoplasmic

inclusions originated from oxidatively damaged mitochondria (Schipper, 2004). Our findings raise the likelihood that the trophic effects of astroglia in brain may be gradually compromised by oxidative and other stressors (e.g., alterations in HO-1 expression) in PD and other neurodegenerative disorders. Importantly, exposure to dopamine and H_2O_2 in doses promoting marked PC12 cell death in the current coculture paradigm had little or no effect on the viability of the HO-1 transfected, sham-transfected, and non-transfected astroglia, when cultured alone. This is in agreement with the fact that, in contrast to the often severe neuronal depletion, astrocyte survival and hypertrophy (reactive gliosis) occur in the face of increased oxidative stress that is characteristic of every major aging-dependent human neurodegenerative disorder (Beal 1995; Norenberg1998). The capacity of stressed astroglia to elaborate robust antioxidant defenses and cytoprotective heat shock proteins, as well as their ability to adapt to anaerobic metabolism, may explain the prolonged survival of these cells in the degenerating human CNS.

There is ample evidence for HO-1 mediated neuroprotection in various animal and tissue culture model of CNS injury and disease. Both neuronal and nonneuronal brain cells exhibit a capacity to rapidly upregulate HO-1 (and other heat shock protein) at the transcriptional level in response to noxious stimuli (Suttner et al., 1999). HO-1 overexpression has been shown to confer neuroprotection to cultured cerebellar granule cells exposed to excitotoxin, to neuroblastoma cells treated with β-amyloid or H_2O_2 (Le, et al., 1999; Takeda et al., 2000), and in the animal model of ischemic or traumatic brain injury (Fukuda et al., 1996). On the other hand, there also exists considerable literature attesting to the potentially deleterious effects of HO-1 activity. Metalloporphyrin

suppression of heme oxygenase activity was documented to ameliorate tissue necrosis and edema after focal cerebral ischemia in intact rats (Kadoya, et al., 1995). Upregulation of the HO-1 activity in cultured rat astroglia by stress stimuli increases uptake of non-transferrin-derived $^{59/55}$Fe by the mitochondrial compartment, an effect that was attenuated by tin mesoporphyrin (SnMP) or dexamethasone (DEX) administration (Schipper 1999). Recent work performed in our laboratory (Song et al., 2005) showed that primary rat astrocytes transfected with human HO-1 cDNA exhibit augmented levels of protein carbonyls (protein oxidation), 8-epiPGF2α (lipid peroxidation), and 8-HOdG (DNA damage) in both whole cell and mitochondrial compartments relative to non-tranfected and sham-transfected controls. Therefore, the cell type in question, the extent and duration of HO-1 induction, and the status of the local redox microenvironment may determine whether the antioxidant benefits of heme-derived bilirubin or the neuroendangering effects of free iron and CO predominate.

In the present study, we demonstrated that exposure to dopamine + H_2O_2 induced high levels of cell death in PC12 cells grown atop HO-1 transfected astroglia in comparison with PC12 cells grown on sham- or non-transfected astroglia. DH-induced cytotoxicity was significantly attenuated by the administration of 1.5μM SnMP, attesting to the biological potency of the h*ho-1* gene product in these preparations. These findings support our contention that glial HO-1 overexpression *in situ* may enhance the vulnerability of nearby dopaminergic neurons to oxidative injury. Furthermore, the combination of dopamine and H_2O_2 were much more toxic to PC12 cells cocultured with HO-1 transfected astrocytes compared with equimolar concentrations of dopamine or

H_2O_2 administrated alone, consistent with the role of peroxidase-dependent (iron-mediated) catechol bioactivation in the observed neurotoxicity (pathway 2 in figure 1) (Schipper, 1998). These data suggested that it is conversion of dopamine into neurotoxic intermediates that is responsible for the PC12 cell killing, not the toxic effects of dopamine or H_2O_2 alone. This finding is in accordance with other studies demonstrating that dopamine and norepinephrine can be oxidized to semiquinone radicals with proven neurotoxic capabilities via peroxidase-mediated reactions (Metodiewa et al., 1989). In the presence of monoamine oxidase-B (MAO-B), dopamine is metabolized to DOPAC and ammonia and the pro-oxidant species, H_2O_2. In the presence of ferrous iron, H_2O_2 is converted to hydroxyl radicals, one of the most damaging free radicals generated in living tissues (pathway 2 in figure 1) (Schipper, 1998). In both of the human and rat brain, iron-rich glial inculsions progressively accumulate with advancing age (Schipper et al., 1981). The iron-mediated pseudoperoxidase activity characteristic of these glial granules may play an important role in the generation of free radicals in the course of natural brain aging and in neurodegenerative diseases. H_2O_2 generated from dopamine oxidation in the presence of MAO-B may serve as a co-factor for further dopamine oxidation to neurotoxic semiquinone radicals by peroxidase-mediated reactions in these senescent astroglia. In additon to dopamine, redox-active glial iron may also facilitate the non enzymatic bioactivation of the pro-toxins, MPTP and DOPA (Schipper 1999).

Considerable evidence suggested that intracellular oxidative stress may be the ultimate pathway responsible for the transformation of normal astrocyte mitochondria to peroxidase-positive (iron-rich) inclusions *in vitro* and *in vivo* (Mydlarski et al., 1993;

Desjardins et al., 1992; Manganaro et al., 1995; Srebro, 1971). Previous work in our laboratory showed that administration of ascorbate, melatonin or resveratrol eliminated the late, compensatory induction of the redox-sensitive mnsod gene in astrocytes transiently transfected with hHO-1 cDNA (Frankel et al., 2000). This observation raised the possibility that HO-1 up-regulation in astroglia, in contrast to other neural tissues, promotes intracellular oxidative stress. The current observation that ascorbate abrogate DH-induced cytotoxicity further implicates oxidative stress in this neurodegenerative process. Ascorbate (vitamin C) has previously been postulated to serve as an antioxidant in human neurodegenerative disorders such as amyotrophic lateral sclerosis (Kok, 1997). The role of ascorbate as an important endogenous antioxidant in humans is supported by a) a correlation between advancing age and decreased ascorbate plasma levels, b) the ability of ascorbate to prevent the formation of peroxynitrite radicals, and c) a close correlation between CNS ascorbate levels and susceptibility to neural injury. Ascorbate may sometimes behave as a pro-oxidant by increasing intracellular H_2O_2 generation and promoting the redox cycling of ferric iron to the more reactive ferrous state (Mendiratta et al, 1998). Whether ascorbate behaves as a pro-oxidant or an antioxidant may be determined by its cellular concentrations and the status of the redox microenvironment. The 200μM dose of ascorbate utilized in the present study clearly behaved as an antioxidant. Both free ferrous iron and CO released in the course of HO-1 mediated heme degradation are likely mediators of oxidative injury in this paradigm (Schipper 2004).

Neuronal dysfunction, gliosis, mitochondrial insufficiency, and iron deposition are

consistent neuropathological features of PD and other human neurodegenerative disorders. These derangements also occur, to a lesser extent, in the course of normal brain aging. The precise mechanisms responsible for the abnormal mobilization of iron, a potentially important source of damaging ROS, in the PD-affected regions remain unclear. Previous experiments performed in our laboratory have implicated astroglial HO-1 induction as a common pathway mediating oxidative mitochondrial injury and iron sequestration in senescent and degenerating neural tissues. CSH, dopamine, TNFα and IL-1ß upregulated HO-1 mRNA, protein and activity levels in primary rat astroglial cultures. After 3-6 days of exposure to these stimuli, uptake of non-transferrin-derived $^{59/55}$Fe by the mitochondrial compartment was significantly increased in comparison with control cultures (Ham et al., 1998; Mehindate et al., 2001; Schipper, 1999; Wang et al., 1995). Administration of SnMP or DEX significantly reduced mitochondrial iron deposition in cultured astroglia exposed to dopamine, TNFα or IL-1ß (Schipper et al., 1999; Mehindate et al., 2001). Similarly, administration of SnMP or DEX eliminated pathological accumulation of mitochondrial ^{55}Fe observed in rat astrocyte culture overexpressing human HO-1 by transient transfection. These observations suggested that up-regulation of HO-1 is a critical event in the cascade leading to mitochondrial iron sequestration in astroglia exposed to oxidative challenge. Deferoxamine (DFO), an iron chelator, has been used in a variety of clinical settings including tumor therapy, acute iron poisoning, heart disease, and in animal models of MPTP neurotoxicity. In these condition, DFO inhibited iron accumulation thereby ameliorating free radical-related tissue damage. Phenanthroline is another commonly used iron chelator which has high affinity for ferric iron and readily gains access to the intracellular milieu. In the current

study, DH-induced killing of PC12 cells co-cultured with HO-1 transfected astrocytes was significantly attenuated by the addition of DFO and phenanthroline. These data further support our contention that dopamine bioactivation in our *in vitro* model is facilitated by the pseudoperoxidase behaviour of excessive glial iron derived from the HO-1-mediated breakdown of cellular heme. To the extent that these findings may be extrapolated to events prevailing in the PD substantia nigra, HO-1-related deposition of glial iron in the affected nigra may foster oxidative damage to nearby dopaminergic neurons and thereby contribute to clinical deterioration in patients with PD.

Potentially deleterious effects of astroglial HO-1 upregulation on PC12 cells were further ascertained by incubating PC12 cells with the conditioned media derived from HO-1 transfected and control astrocytes cultured separately. Exposure of PC12 cells to astrocyte-conditioned media resulted in similar patterns of PC12 cell death observed in the coculture experiments except on a smaller scale (Figure 13). For example, exposure to conditioned media derived from HO-1 transfected astrocytes exposed to dopamine/H_2O_2 challenge induced 16% PC12 cell death, whereas 45% PC12 cell death was observed in PC12/astrocyte cocultures under similar experimental conditions. Exposure to conditioned media derived from HO-1 transfected astrocytes treated with DH and SnMP did not augment PC12 cell killing. This observation further attests to the biological potency of the hHO-1 construct in our model. The administration of ascorbate, DFO or phenanthroline abrogated PC12 cell death induced by exposure to conditioned media from DH-challenged, HO-1 transfected astroglia. These findings suggested that there are oxidative mechanisms contributing to neuronal cell death via extracellular

pathways distinct from those accruing from direct neuronal-glial interactions. The astroglial mitochondriopathy may compromise several ATP-dependent processes and predispose to neuronal degeneration by (a) augmenting extracellular glutamate concentration and (b) curtailing GSH delivery to the neuronal compartment (Aschner, 2000). As alluded to previously, the astroglial mitochondrial iron may behave as nonenzymatic peroxidase activity that facilitates the oxidation of dopamine and MPTP to toxic o-semiquinone radicals and MPP+, respectively (Schipper 1999). Both MPP^+ and superoxide anion derived from the redox cycling of ortho-semiquinones (Kontos et al., 1985) may be extruded to the extracellular space and exert their dystrophic effects on susceptible neuronal targets (Kontos et al., 1985). Alternatively, HO-1 derived iron may be the culprit sensitizing the PC12 cells to DH toxicity because (i) there is some evidence of active iron extrusion from cells over-expressing heme oxygenase activity (Ferris et al., 1999) and (ii) as postulated for mitochondrial iron, the pseudoperoxidase activity of extracellular iron may accelerate the conversion of extracellular dopamine to neurotoxic semiquinones in the presence of H_2O_2 (Schipper 1999). Another candidate molecule is carbon monoxide liberated in the course of heme degradation. Carbon monoxide shares some chemical and biological properties with nitric oxide and has physiological functions as a signaling molecule. Under pathological conditions, CO may exacerbate free radical production by the mitochondrial compartment and thereby contribute to cellular injury (Zhang and Piantadosi, 1992). Further studies will be required to delineate the role of heme-derived CO in the pathophysiology of dopaminergic cell death and dysfunction in human and experimental PD.

The frequent co-appearance of oxidative stress, iron deposition and mitochondrial insufficiency in PD suggests that these pathological features may constitute a single neuropathological lesion (Schipper 2004). In accord with the prevailing Free Radical-Mitochondrial Theory of Aging, our laboratory has proposed the following model for pathological brain iron deposition and oxidative mitochondrial injury in PD: In the aging and degenerating basal ganglia, oxidative stressors such as dopamine and TNFα induce a cellular stress response in astroglia characterized by up-regulation of HO-1. Free iron and carbon monoxide released from HO-1 mediated heme degradation perpetuate intracellular oxidative stress, which, in turn, promotes opening of mitochondrial permeability transition pores and influx of non-transferrin iron into the mitochondrial compartment. In agreement with this model are reports that excess brain iron and mitochondrial electron transport chain deficits are present in the brains of PD subjects (Connor et al., 1990; Olanow, 1992). The excessive mitochondrial ^{55}Fe behaves as a non-enzymatic peroxidase activity that oxidizes catechols and xenobiotics (e.g. MPTP) to dopaminergic neurotoxins. Astroglial bioenergetic failure (ATP depletion) resulting from the mitochondriopathy decreases export of reduced glutathione to nearby dopaminergic neurons and uptake of glutamate from the extracellular space. In this way, primary metabolic derangements within astroglia further predispose indigent neurons to oxidative and excitotoxic injury. Release of dopamine from degenerating nigrostriatal neurons perpetuates HO-1 induction in the astroglial compartment completing a vicious cycle of pathological neuronal-glial interactions in PD and possibly other aging-related neurodegenerative and inflammatory disorders (Figure 14).

In summary, the results of the experiments described herein may help unify several pathological features of PD, viz., oxidative stress, iron deposition and mitochondrial insufficiency, into a single, cohesive "lesion" contingent upon the sustained up-regulation of astroglial HO-1. Our data are consistent with the Free Radical- Mitochondrial Theory of Aging and may provide a novel link between normal brain aging and a host of senescence-dependant neurodegenerative disorders. As a key transducer of mitochondrial iron deposition under oxidative stress, suppression of glial HO-1 activity by pharmacological or other methods may prove to be a rational therapeutic objective in the management of PD and other aging-related neurodegenerative conditions. Several metalloporphyrin inhibitors of heme oxygenase activity are already in clinical use for the management of neonatal hyperbilirubinemia (jaundice) and certain adult liver conditions and, if warranted, could be adapted for the treatment of CNS disorders. A note of caution would seem in order, however, given the Janus-faced behavior of HO-1 as a determinant of neuronal survival in various experimental paradigms (Mawal et al., 2002). Further testing of the hypotheses set forth in present study, using transgenic and other appropriate whole animal models, will be required before modulation of central HO-1 expression can be advocated as a potential therapeutic modality for diseases of the aging human CNS.

CONCLUSIONS

1. Overexpression of the human *ho-1* gene in primary rat astroglia, correnspondingto enhancement of bilirubin production in the range of 4-5 folds above control levels, resulted in significantly increased cell death in co-cultured PC12 cells following exposure to the oxidative stressors, dopamine (1μM) + H_2O_2 (1μM). DH-induced cytotoxicity was significantly attenuated by the administration of 1.5 μM SnMP, attesting to the biological potency of the h*ho-1* gene product in these preparations. On the basis of nuclear morphology, necrosis, rather than apoptosis, was considered the likely mechanism of PC12 cell death in this paradigm.

2. In contradistinction to the PC12 cells, exposure to dopamine (1μM) + H_2O_2 (1μM) that engendered marked PC12 cell death in the coculture experiments had little or no effect on the viability of the HO-1 transfected, sham-transfected and non-transfected astroglia themselves. The capacity of stressed astroglia to up-regulate robust antioxidant defenses and cytoprotective heat shock proteins, as well as their ability to adapt to anaerobic metabolism, may account for the relative resistance of these cells to oxidative challenge.

3. Incubation of PC12 cells in astrocyte-conditioned media resulted in similar patterns of PC12 cell death observed in the coculture experiments except on a smaller scale. This finding indicated that, in addition to direct neuronal-glial interactions, hHO-1 transfected astroglia exposed to dopamine + H_2O_2 release neurotoxins (e.g. CO, semiquinone-derived superoxide) to the extracellular space or deprive nearby PC12 cells of glia-derived trophic substances (e.g. GSH).

4. In presence of dopamine (1μM) + H_2O_2 (1μM), the administration of ascorbate to both PC12 cells co-cultured with HO-1 transfected astroglia and PC12 cells exposed to conditioned media from HO-1 transfected astroglia abrogated PC12 cytotoxicity. These findings further implicate oxidative stress as a major factor contributing to PC12 cell killing in these experiments.

5. The addition of iron chelators, DFO or phenanthroline, attenuated DH-induced cell death in PC12 cells co-cultured with HO-1 transfected astrocytes. This finding further supports the contention that the mitochondrial iron deposits resulting from the overexpression of HO-1 behave as a non-enzymatic (or pseudo) peroxidase activity that bioactivate dopamine to neurotoxic intermediates (Figure 1, pathway 2).

6. In conjunction with prior reports from the Schipper lab, the current findings suggest that the sustained induction of ho-1 gene in nigral astroglia may render nearby dopaminergic neurons prone to oxidative and excitotoxic injury in the basal ganglia of PD patients. If confirmed, suppression of glial HO-1 expression in the brains of these patients by pharmacological or other means may have a beneficial effect on dopaminergic neuronal survival and clinical manifestations of this debilitating condition.

REFERENCES

Ambani LM, Van Woert MH, Murphy S. 1975. Brain Peroxidase and catalase in Parkinson's disease. *Arch. Neurol.* 32 pp. 114–118.

Allam MF, Del Castillo AS, Navajas RF. 2005 Parkinson's disease risk factors: genetic, environmental, or both? *Neurol Res.* 27(2):206-8

Aschner M (2000) Neuron-astrocyte interactions: implications for cellular energetics and antioxidant levels. *Neurotoxicology* 21:1101-1107.

Barbeau A. Etiology of Parkinson's disease: A research strategy. *Can. J. Neurol. Sci.* 11 (1984), pp. 24–28.

Beal FM. 1995. In, Mitochondrial Dysfunction and Oxidative Damage in Neurodegenerative Diseases, R. G. Landes, Austin, TX.

Beal FM. 2003. Mitochondria, oxidative damage, and inflammation in Parkinson's disease, *Ann. N. Y. Acad. Sci.* 991 pp. 120–131.

Berns MW. 2000 Gene inactivation by multiphoton-targeted photochemistry. *Proc. Natl. Acad. Sci. USA* 97, 9504

Bowen, B.C., Block, R.E.m Sanchez-Ramos, J., Pattany, P.M.,Lampman, D.A.,Murdock, J.B., Quencer, R.M.,1995. Proton MR spectroscopy of the brain in 14 patients with Parkinson disease. *AJNR Am.J. Neuroradiol.*16, 61-68.

Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC (1997) Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 23:361-366.

Cadet JL, Katz M, Jackson-Lewis V, Fahn S. Vitamin E attenuates the toxic effects of intrastriatal injection of 6-hydroxydopamine (6-OHDA) in rats: Behavioral and biochemical evidence. *Brain Res.* 476 (1989), pp. 10–15.

Castellani R, Smith MA, Richey, PL. Perry G., 1996. Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. *Brain Res.* 737, pp. 195–200.

Chopra VS, Chalifour LE, Schipper HM (1995) Differential effects of cysteamine on heat shock protein induction and cytoplasmic granulation in astrocytes and glioma cells. *Brain Res Mol Brain Res* 31:173-184.

Connor JR, Menzles, S, St. Martin SM, Mufson EJ. 1990. Cellular distribution of trasferrin, ferritin and iron in normal and aged human brain. *J. Neurosci.* 27: 595-611

Dennery PA (2000) Regulation and role of heme oxygenase in oxidative injury. *Curr Top Cell Regul* 36:181-199.

Desagher S, Glowinski J, Premont J. 1996. Astrocytes protect neurons from hydrogen peroxide toxicity. *J. Neurosci.* 16: 2553-2562

DiMonte DA.1998. Parkinson's disease. In Astrocytes in Brain Aging and Neurodegeneration H. M. Schipper, Ed., pp. 111–121. Landes, Austin, TX.

Durante W (2003) Heme oxygenase-1 in growth control and its clinical application to vascular disease. *J. Cell. Physiol.* 195:373-382.

Faucheux BA, Nillesse N, Damier P, G. Spik, A. Mouatt-Prigent, A. Pierce, B. Leveugle, N. Kubis, J.J. Hauw, Agid Y. 1995. Expression of lactoferrin receptors is increased in the mesencephalon of patients with Parkinson disease, *Proc. Natl. Acad. Sci. USA* 92 pp. 9603–9607.

Ferris CD, Jaffrey SR, Sawa A et al. 1999. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol.* 1:152-157

Frankel D, Schipper HM. 1999. Cysteamine pretreatment of the astroglial substratum

(mitochondrial iron sequestration) enhances PC12 cell vulnerability to oxidative injury. *Experimental Neurology* 160:376-385

Frankel D, Mehindate K, Schipper HM (2000) Role of heme oxygenase-1 in the regulation of manganese superoxide dismutase gene expression in oxidatively-challenged astroglia. *J Cell Physiol* 185:80-86.

Fukuda, K.: Richmon,J.D.;Sato, M., Sharp, F.R.;Panter, S.S.;Noble,L.J.Induction of heme oxygenase-1(HO-1) in glia after traumatic brain injury. *Brain Res.* 736:68-75;1996

Ham D, Schipper HM (2000) Heme oxygenase-1 induction and mitochondrial iron sequestration in astroglia exposed to amyloid peptides. *Cell Mol Biol (Noisy-le-grand)* 46:587-596.

Hattori, N.m Tanaka, M., Ozawa, T., Mizuno, Y.,1991. Immunohistocheial studies on complexes I, II, and IV of mitochondria in Parkinson's disease. *Ann. Neurol.* 30, 563-571

Huang FP, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT (2002) Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products. *J Neurosurg* 96:287-293.

Jenner , P., 2003. Oxidative stress in Parkinson's disease. *Ann. Neurol.53 (Suppl3)* S26-S36, discussion S36-S28.

Kadoya C, Domino EF, Yang GY, Stern JD, Betz AL (1995) Preischemic but not postischemic zinc protoporphyrin treatment reduces infarct size and edema accumulation after temporary focal cerebral ischemia in rats. *Stroke* 26:1035-1038.

Koeppen AH, Dickson AC (2002) Tin-protoporphyrin prevents experimental superficial

siderosis in rabbits. *J. Neuropathol. Exp. Neurol.* 61:689-701.

Kontos HA, Wei EP, Ellis EF, Jenkins LW, Povlishock JT, Rowe GT, Hess ML (1985) Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. *Circ. Res.* 57:142-151.

Kok AB. 1997. Ascorbate availability and neurodegneration in amyotrophic lateral sclerosis. *Med. Hypot.* 48: 281-296

Laftah AH, Raja K, Simpson RJ, Peters TJ. 2003 Effect of Tin-mesoporphyrin, an inhibitor of haem catabolism, on intestinal iron absorption. *Br. J. Haematol.* 122(2):298-304

Le WD, Xie WJ, Appel SH (1999) Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. *J. Neurosci. Res.* 56:652-658.

Leveugle B, Faucheux BA, C. Bouras, N. Nillesse, G. Spik, E.C. Hirsch, Y. Agid, Hof PR. 1996. Cellular distribution of the iron-binding protein lactotransferrin in the mesencephalon of Parkinson's disease cases, *Acta Neuropathol. (Berl.)* 91 pp. 566–572.

Liu XM, Chapman GB, Wang H, Durante W (2002) Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. *Circulation* 105:79-84.

Maines MD, Abrahamsson PA (1996) Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. *Urology* 47:727-733.

Maines MD, Polevoda B, Coban T, Johnson K, Stoliar S, Huang TJ, Panahian N, Cory-Slechta DA, McCoubrey WK, Jr. (1998) Neuronal overexpression of heme

oxygenase-1 correlates with an attenuated exploratory behavior and causes an increase in neuronal NADPH diaphorase staining. *J. Neurochem.* 70:2057-2069.

Mawal Y, Berlin D, Kravitz S, Schipper H.M. 2002. Heme oxygenase-1 and Alzheimer disease. In: Abraham, N.G., A.J., Nath, K.A. (Eds.), Heme Oxygenase in Biology and Medicine. Kluwer Academic/Plenum Publishers, New York, pp. 145–155.

Mehindate K, Sahlas DJ, Frankel D, Mawal Y, Liberman A, Corcos J, Dion S, Schipper HM (2001) Proinflammatory cytokines promote glial heme oxygenase-1 expression and mitochondrial iron deposition: implications for multiple sclerosis. *J. Neurochem.* 77:1386-1395.

Mendiratta S, Ou Z, May JM. 1998. Erythrocyte defense against hydrogen peroxide: the role of ascorbic acid. Biochem. Biophys. Acta. 1380:389-395

Morris HR. 2005. Genetics of Parkinson's disease. *Ann. Med.* 37(2):86-96

Nakagami T, Toyomura K, Kinoshita T, Morisawa S (1993) A beneficial role of bile pigments as an endogenous tissue protector: anti-complement effects of biliverdin and conjugated bilirubin. *Biochim. Biophys. Acta.* 1158:189-193.

Ndisang JF Wang R Vannacci A Marzocca C Fantappie C, Mazzanti R, Mannaioni PF, Masini E. 2001 Haeme oxygenase-1 and cardiac anaphylaxis. *Br. J. Pharmacol.* 2001 Dec;134(8):1689-96

Norenberg MD.1998. Astrocyte pathophysiology in disorders of the central nervous system. In Astrocytes in Brain Aging and NeurodegenerationH. M. Schipper, Ed., pp. 41–53. Landes, Austin, TX.

Olanow CW. 1992. magnetic resonance imaging in parkinsonism. In: Neurological clinics Part 2, Vol 10 pp 405-420. Philadelphia: Saunders

Ozawa N, Goda N, Makino N, Yamaguchi T, Yoshimura Y, Suematsu M (2002) Leydig cell-derived heme oxygenase-1 regulates apoptosis of premeiotic germ cells in response to stress. *J. Clin. Invest.* 109:457-467.

Peyton KJ, Reyna SV, Chapman GB, Ensenat D, Liu XM, Wang H, Schafer AI, Durante W (2002) Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. *Blood* 99:4443-4448.

Ponka P, Wilczynska A, Schulman HM (1982) Iron utilization in rabbit reticulocytes. A study using succinylacetone as an inhibitor or heme synthesis. *Biochim. Biophys. Acta.* 720:96-105.

Rogers B, Yakopson V, Teng ZP. 2003. Heme oxygenase-2 knockout neurons are less vulnerable to hemoglobin toxicity. Free Radic. *Biol. Med.* 35:872-881

Ryter SW, Tyrrell RM (2000) The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free. Radic. Biol. Med.* 28:289-309.

Ryter SW, Kvam E, Tyrrell RM (2000) Heme oxygenase activity. Current methods and applications. *Methods. Mol. Biol.* 99:369-391.

Scapagnini G, D'Agata V, Calabrese V, Pascale A, Colombrita C, Alkon D, Cavallaro S (2002) Gene expression profiles of heme oxygenase isoforms in the rat brain. *Brain. Res.* 954:51-59

Schipper HM, Brawer JR, Nelson JF. Felicio LS, Finch CE. 1981. Role of the gonads in the histologic aging of the hypothalamic arcuate nucleus. *Biol. Reprod.* 25: 413-419.

Schipper HM, 1998. Glial iron sequestration and neurodegeneration In: H.M. Schipper,

Editors, *Astrocytes in brain aging and neurodegeneration*, R.G. Landes, Austin (TX). 235–251

Schipper HM, Liberman A, Stopa EG. 1998. Neural heme oxygenase-1 expression in idiopathic Parkinson's disease. *Exp. Neurol.* 150, pp. 60–68.

Schipper HM (1999) Glial HO-1 expression, iron deposition and oxidative stress in neurodegenerative diseases. *Neurotox. Res.* 1:57-70.

Schipper HM (2000) Heme oxygenase-1: role in brain aging and neurodegeneration. *Exp. Gerontol.* 35:821-830.

Schipper HM (2004) Heme oxygenase-1: Transducer of pathological brain iron sequestration under oxidative stress. In: Redox-active Metals in Neurological Disorders (S. LeVine, Connor JR, Schipper HM, eds), pp 84-93. New York: Ann NY Acad Sci.

Schipper HM, Cissé S, Stopa EG (1995) Expression of heme oxygenase-1 in the senescent and Alzheimer-diseased brain. *Ann. Neurol.* 37:758-768.

Schipper HM, Liberman A, Stopa EG (1998) Neural heme oxygenase-1 expression in idiopathic Parkinson's disease. *Exp. Neurol.* 150:60-68.

Schipper HM, Bernier L, Mehindate K, Frankel D (1999) Mitochondrial iron sequestration in dopamine-challenged astroglia: role of heme oxygenase-1 and the permeability transition pore. *J. Neurochem.* 72:1802-1811.

Song W, Su HX, Song SS, Paudel HK, Schipper M. 2005. Over-expression of heme oxygenase-1 promotes oxidative mitochondrial damage in rat astroglia. *J. Cell. Physiol.* In press

Soong, N.W.m Hinton, D.R.m Cortopassi, G.m Arnheim, N.m 1992. Mosaicism for a

specific somatic mitochondrial DNA mutation in adult human brain. *Nat. Genet.* 2,318-323

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN (1987) Bilirubin is an antioxidant of possible physiological importance. *Science* 235:1043-1046.

Suttner DM, Dennery PA. 1999. Reversal of HO-1 related cytopretection with increased expression is due to reactive iron. *FASEB. J.* 13 1800-1809

Takeda A, Perry G, Abraham NG, Dwyer BE, Kutty RK, Laitinen JT, Petersen RB, Smith MA (2000) Overexpression of heme oxygenase in neuronal cells, the possible interaction with Tau. *J. Biol. Chem.* 275:5395-5399.

Vreman HJ, Cipkala DA, Stevenson DK. 1996. Characterization of porphyrin heme oxygenase inhibitors. *Can. J. Physiol. Pharmacol.* 74(3):278-85

Wang X, Managanoaro F, Schipper HM. 1995 A cellular stress model for the sequestration of redox-active glial iron in the aging and degenerating nervous system. *J. Neurochem.* 64:1868-1877

Winterbourn CC, Buss IH (1999) Protein carbonyl measurement by enzyme-linked immunosorbent assay. *Methods. Enzymol.* 300:106-111.

Zhang J, Piantadosi CA (1992) Mitochondrial oxidative stress after carbon monoxide hypoxia in the rat brain. *J. Clin. Invest.* 90:1193-1199.

FIGURE LEGENDS

Figure 1. Enzymatic and non-enzymatic pathways of dopamine oxidation

Enzymatic pathway (pathway1): MAOB catalyzes the oxidation of DA to generate DOPAC, NH_3, and hydrogen peroxide. Nonenzymatic pathway (pathway 2): In the presence of ferrous iron and hydrogen peroxide, dopamine is converted to neurotoxic quinones and ortho-semiquinone radicals.

DOPAMINE OXIDATION

PATHWAY 1

$$DA + O_2 + H_20 \xrightarrow{MAO\ B} DOPAC + NH_3 + H_2O_2$$

PATHWAY 2

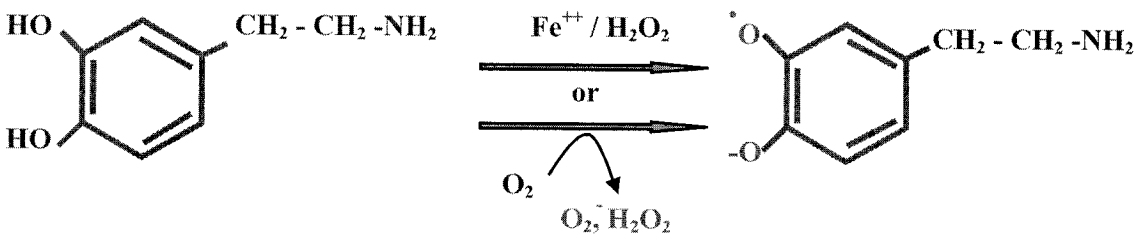


Figure 2. heme oxygenase-mediated heme oxidation (modified after Ryter et al., 2000) HO-1 is the rate-limiting enzyme that catalyzes the oxidative degradation of heme to biliverdin, free ferrous iron and carbon monoxide (CO). Biliverdin is metabolized further to the bile pigment, bilirubin by the action of biliverdin reductase.

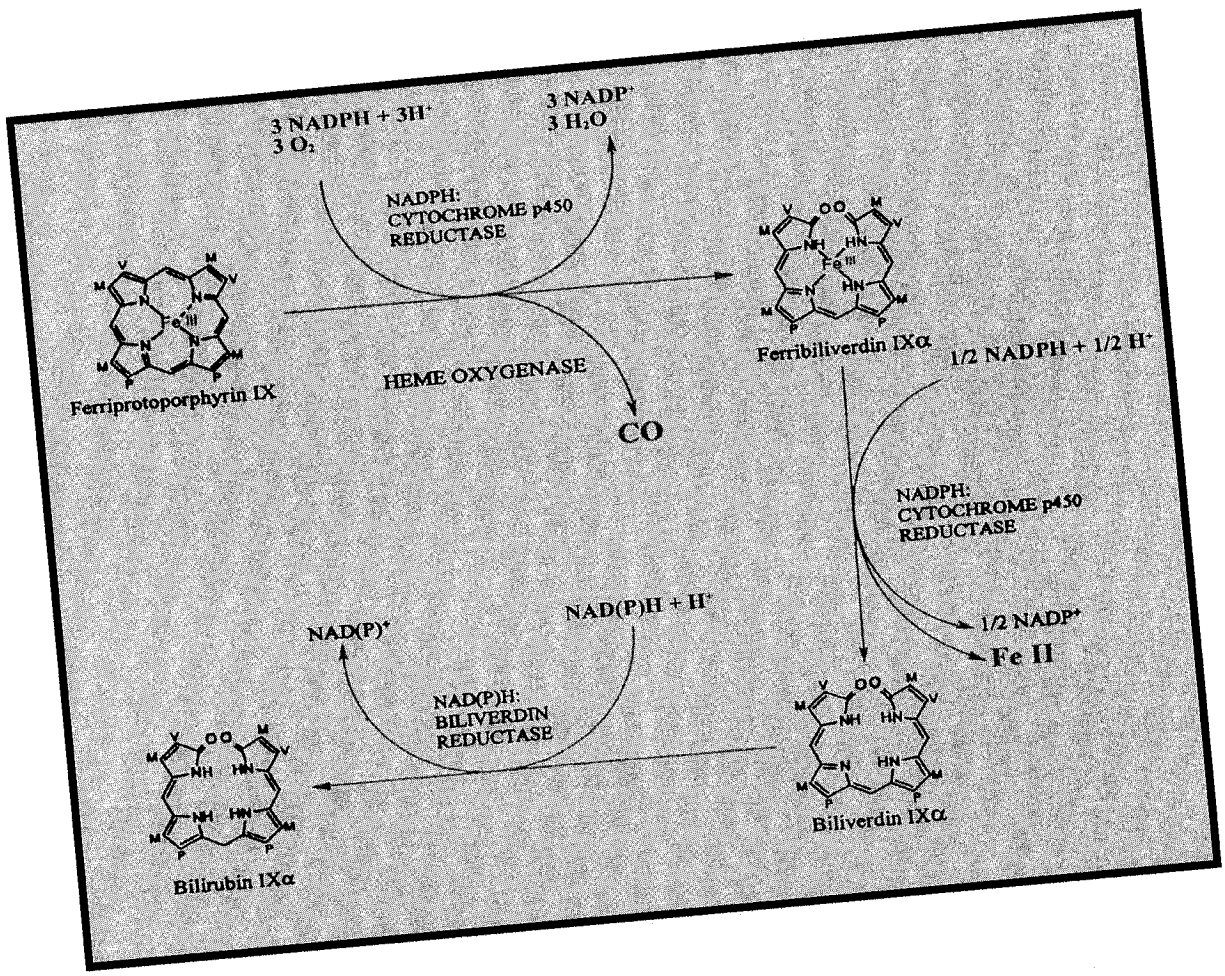
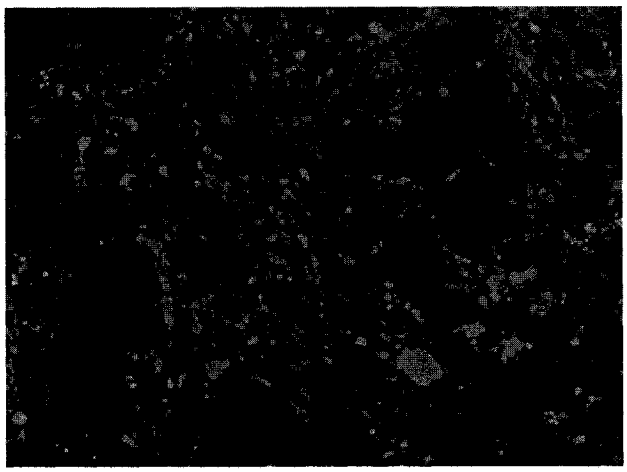

3 NADPH + 3H+
3 O2
3 NADP+
3 H2O
NADPH:
CYTOCHROME p450
REDUCTASE
Ferriprotoporphyrin IX
HEME OXYGENASE
CO
Ferribiliverdin IXα
1/2 NADPH + 1/2 H+
NADPH:
CYTOCHROME p450
REDUCTASE
1/2 NADP+
Fe II
NAD(P)+
NAD(P)H + H+
NAD(P)H:
BILIVERDIN
REDUCTASE
Bilirubin IXα
Biliverdin IXα

Figure 3. Transfection efficiency

Cytoplasmic expression of enhanced green fluorescent protein (EGFP) in primary rat astrocytes co-transfected with pcDNA3.1/Zeo.CMV.Flag.hHO-1 and pEGFP.C1 (A); pcDNA3.1/Zeo.CMV.Flag and pEGFP.C1 (B); or pcDNA3.1/Zeo.CMV.Flag.hHO-1 alone (control; C). All data were collected on post-transfection day 3.

A



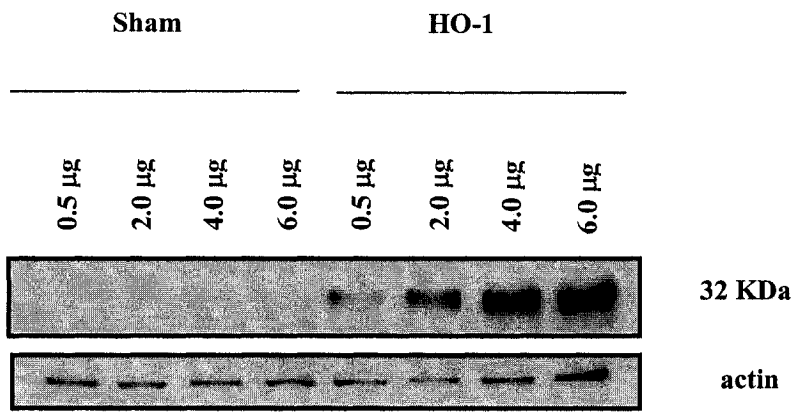
B

C

Figure 4. Western blot analysis for HO-1 expression

A. Anti-Flag Western blotting for the detection of Flag-tagged hHO-1 protein in hHO-1 transfected and control astrocytes. B. Anti-Flag Western blotting for the detection of Flag-tagged hHO-1 protein in hHO-1-transfected. hHO-1 transfected/SnMP (1.5μM)-treated, and sham-transfected astrocytes (4.0μg plasmid DNA per 10^6 cells). All data were collected on post-transfection day 3.

A


Sham
HO-1
0.5 µg
2.0 µg
4.0 µg
6.0 µg
0.5 µg
2.0 µg
4.0 µg
6.0 µg
32 KDa
actin

B

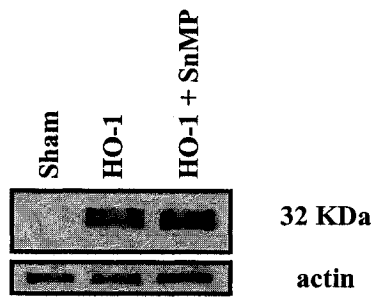

Sham
HO-1
HO-1 + SnMP
32 KDa
actin

Figure 5. Heme oxygenase activity in hHO-1 transfected and control astrocytes in the presence and absence of SnMP (1.5μM).

Heme oxygense activity was calculated as picomoles bilirubin per milgram protein per min (means ± SE). * $P<0.05$ relative to column X, n= 4 per group.

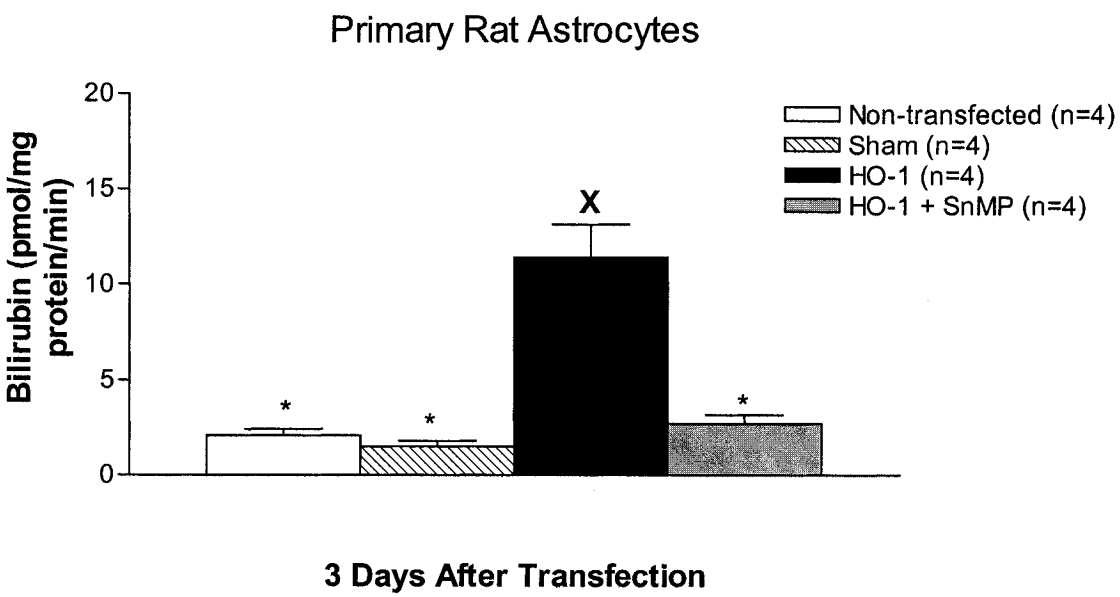

Primary Rat Astrocytes
Bilirubin (pmol/mg protein/min)
20
15
10
5
0
X
*
*
*
Non-transfected (n=4)
Sham (n=4)
HO-1 (n=4)
HO-1 + SnMP (n=4)
3 Days After Transfection

Figure 6. Confocal micrographs of PC12/astrocyte cocultures

Astrocytes were immunostained with anti-GFAP polyclonal antibody and FITC-conjugated secondary antibody (green fluorescence). PC12 cells were immunostained with anti-TH monoclonal antibody and rhodamine-conjugated secondary antibody (red fluorescence). Bar = 50 μm.

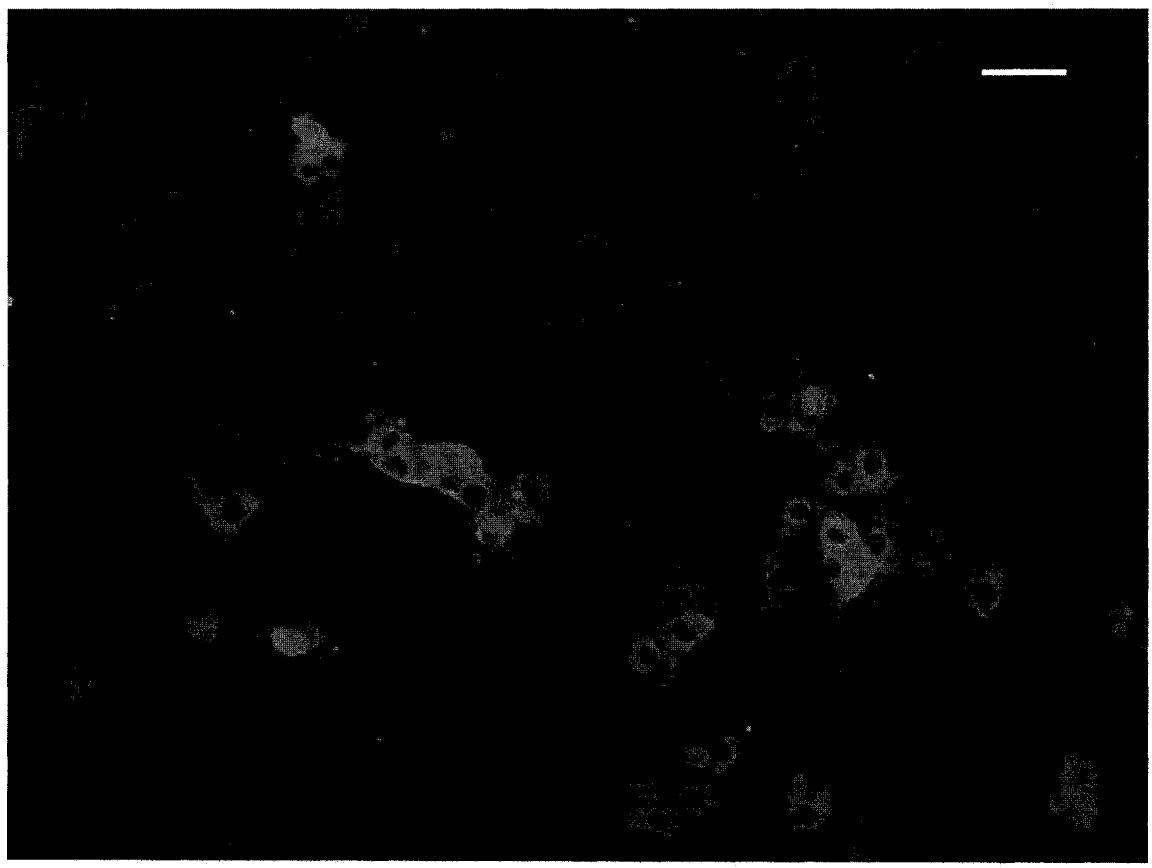


Figure 7. Higher-power confocal micrographs of PC12/astrocyte cocultures Bar = 25 μm

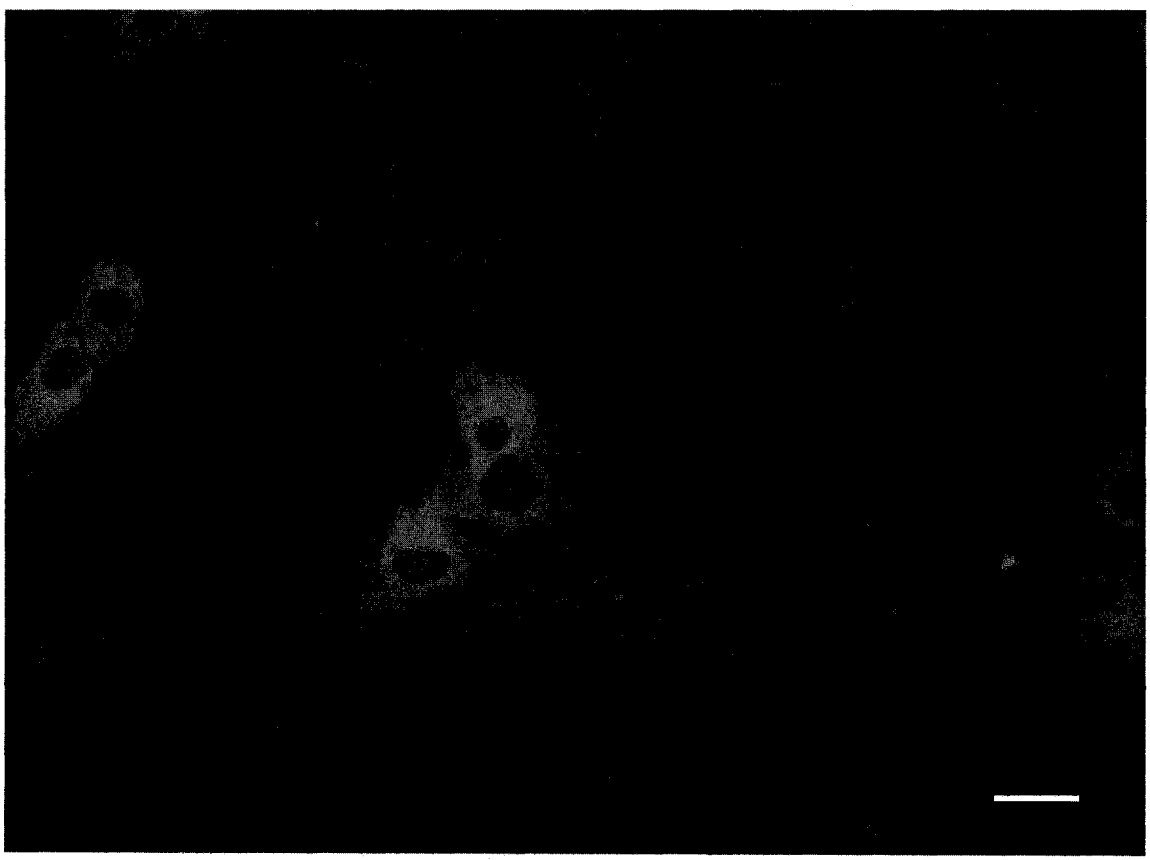


Figure 8. Effects of dopamine/H_2O_2 challenge on cell viability in PC12/astrocyte cocultures

PC12 cells were immunostained with anti-TH monoclonal antibody and FITC-conjugated secondary antibody (green fluorescence), while astrocytes were left unstained. Dead cells are identified by nuclear EMA staining (red fluorescence). Live, EMA-negtive (straight arrows) and dead, EMA-positive (curved arrows) PC12 cells and dead, EMA-positive (arrow heads) astrocytes are illustrated. Bar = 50 μm.

Figure 9. High-power confocal image depicting effects of dopamine/H_2O_2 challenge on cell viability in PC12/astrocyte cocultures. Live, EMA-negtive (arrows) and dead, EMA-positive (arrow heads) PC12 cells are illustrated. Bar = 10 μm.

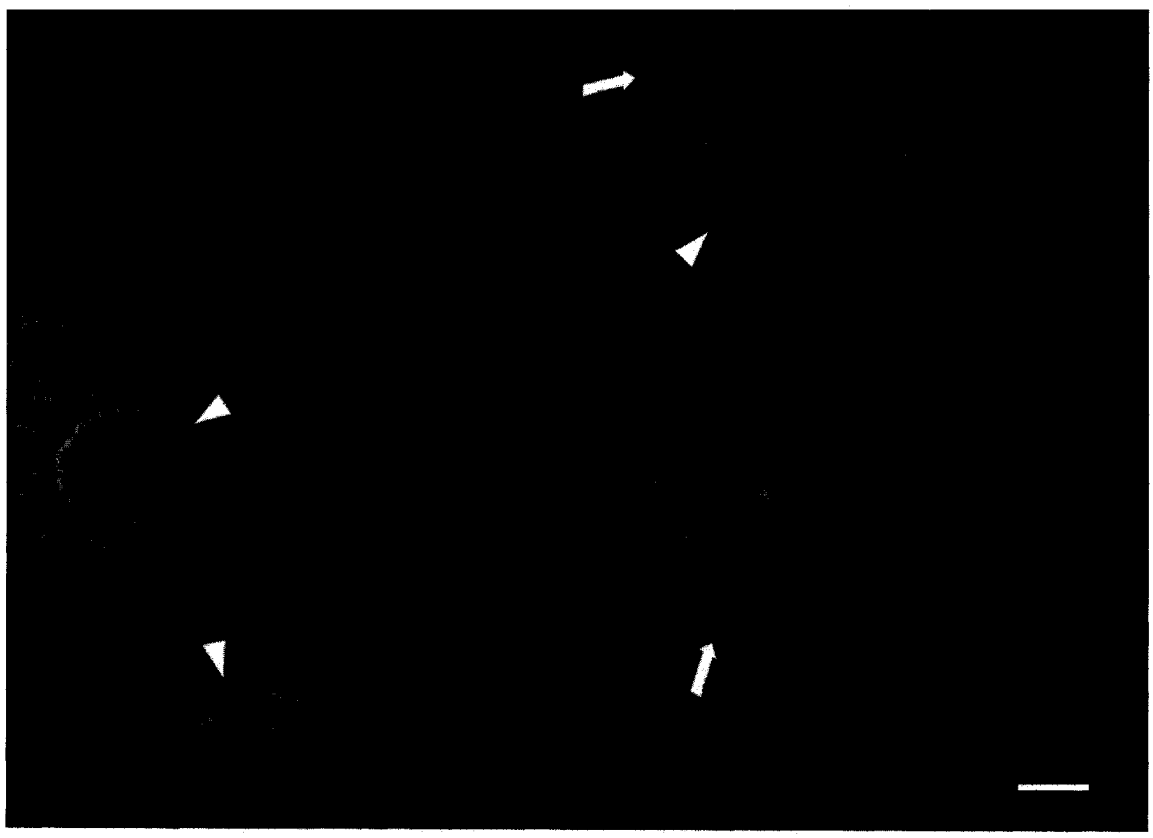


Figure 10. Viability of PC12 cells in PC12/astrocyte cocultures in absence of dopamine/H_2O_2 challenge

Shown are the percentages (means ± SE) of dead PC12 cells in cocultures of PC12 cells with non-transfected, flag-transfected, HO-1 transfected, or HO-1 + SnMP astrocytes without oxidative challenge. * $P<0.05$ relative to column X, n= 6 per group.

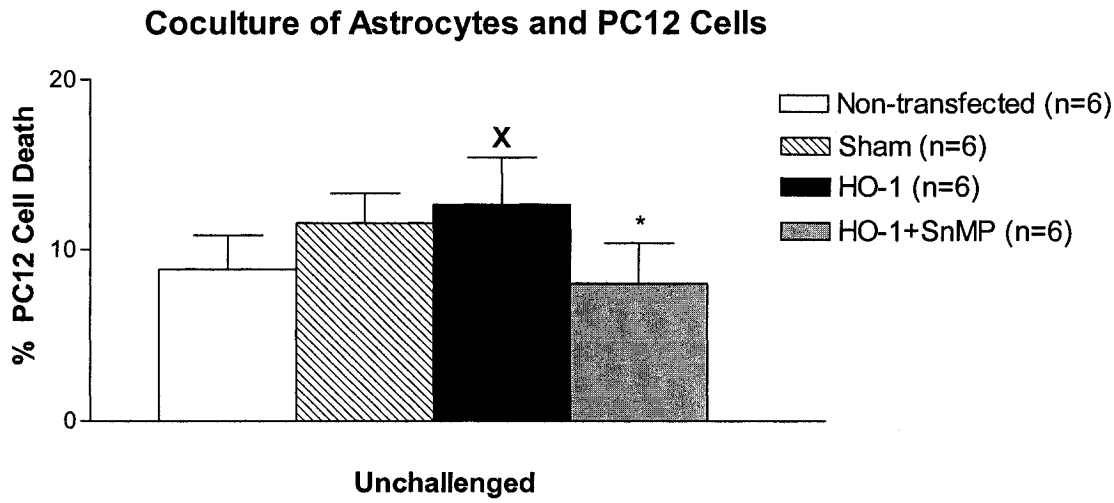

Coculture of Astrocytes and PC12 Cells
% PC12 Cell Death
20
10
0
X
*
Non-transfected (n=6)
Sham (n=6)
HO-1 (n=6)
HO-1+SnMP (n=6)
Unchallenged

Figure 11. Effects of dopamine/H_2O_2 challenge, ascorbate and iron chelators on the viability of PC12 cells in PC12/astrocyte cocultures

Shown are the percentages (means ± SE) of dead PC12 cells in coculture of PC 12 cells with non-transfected, flag-transfected, HO-1 transfected, or HO-1 + SnMP astrocytes under the following experimental conditions: unchallenged; dopamine (1 μM) and H_2O_2 (1 μM) (DH); dopamine (1 μM) alone; H_2O_2 (1 μM) alone; DH with ascorbate (200 μM); DH with deferoxamine (400 μM); and DH with phenanthroline (100 μM). * $P<0.05$ relative to column X, n= 4 per group.

Coculture of Astrocytes and PC12 cells

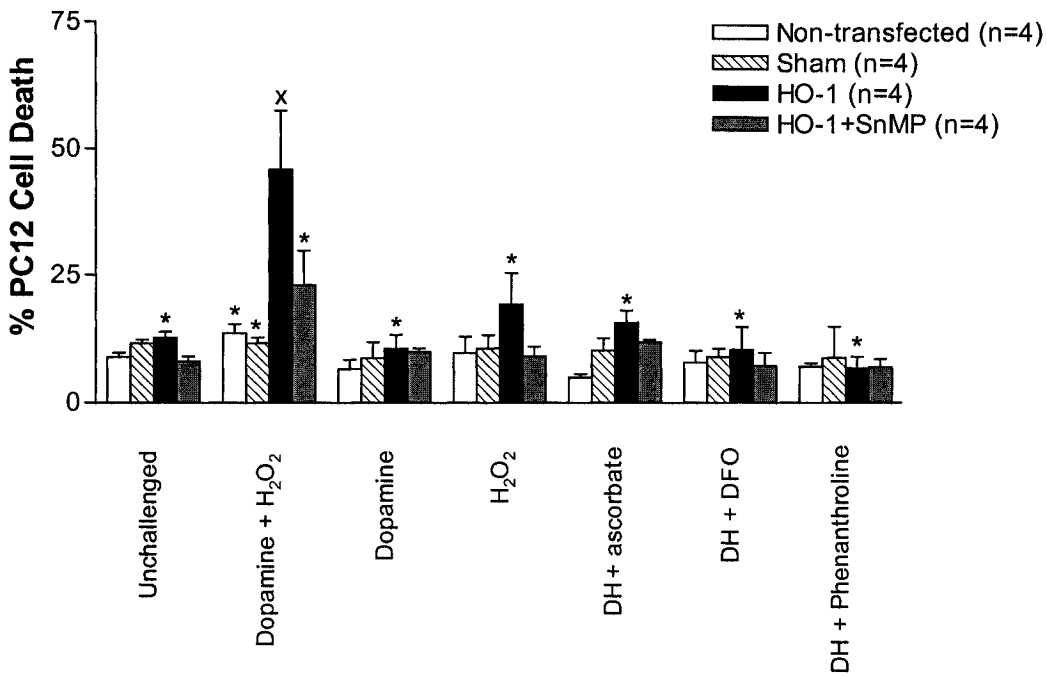


Figure 12. Effects of dopamine/H_2O_2 challenge, ascorbate and iron chelators on the viability of astrocytes

Shown are the percentages (means ± SD) of dead non-transfected, flag-transfected, HO-1 transfected, or HO-1 + SnMP astrocytes under the following experimental conditions: unchallenged; dopamine (1 μM) and H_2O_2 (1 μM) (DH); dopamine (1 μM) alone; H_2O_2 (1 μM) alone; DH with ascorbate (200 μM); DH with deferoxamine (400 μM); and DH with phenanthroline (100 μM). n= 4 per group.

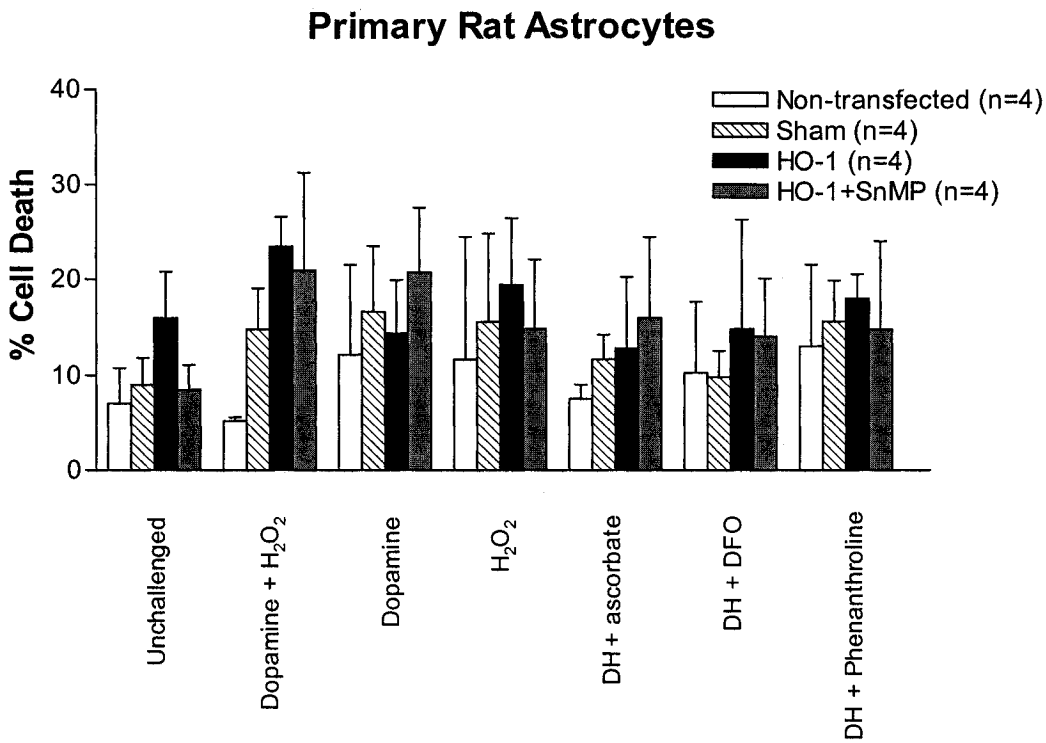

Primary Rat Astrocytes
% Cell Death
40
30
20
10
0
Non-transfected (n=4)
Sham (n=4)
HO-1 (n=4)
HO-1+SnMP (n=4)
Unchallenged
Dopamine + H2O2
Dopamine
H2O2
DH + ascorbate
DH + DFO
DH + Phenanthroline

Figure 13. Effects of exposure to astrocyte-conditioned media on the viability of PC12 cells

Shown are the percentages (means ± SE) of dead PC12 cells in PC12 cells exposed to conditioned media derived from non-transfected, flag-transfected, HO-1 transfected, or HO-1 + SnMP astrocytes under the following experimental conditions: unchallenged; dopamine (1 μM) and H_2O_2 (1 μM) (DH); dopamine (1 μM) alone; H_2O_2 (1 μM) alone; DH with ascorbate (200 μM); DH with deferoxamine (400 μM); and DH with phenanthroline (100 μM). * $P<0.05$ relative to column X, n= 4 per group.

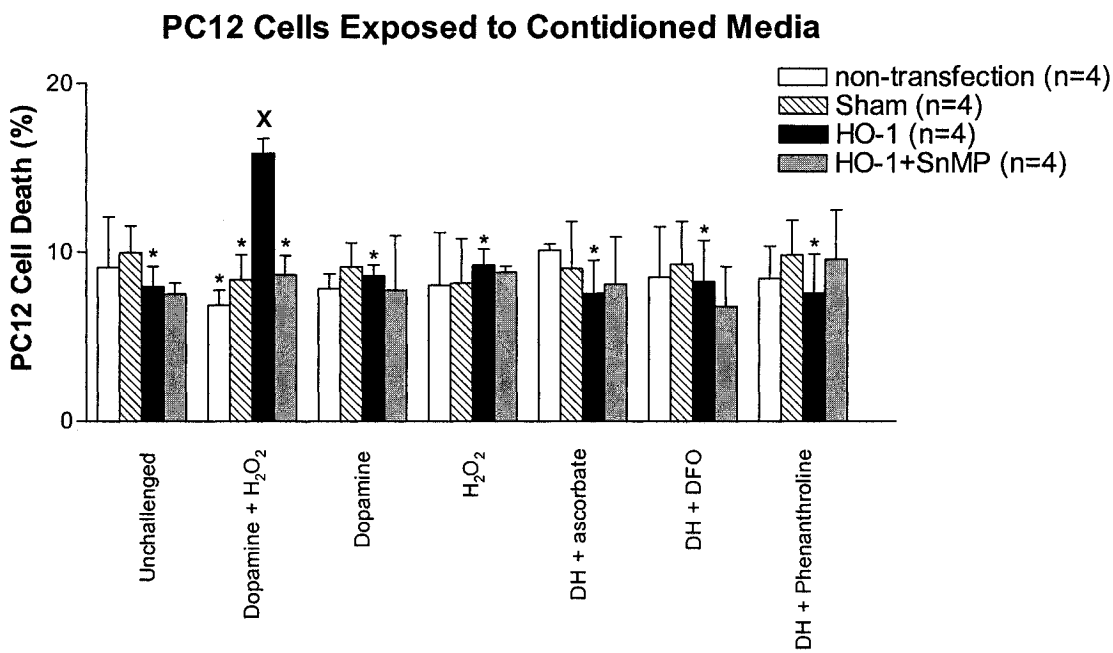
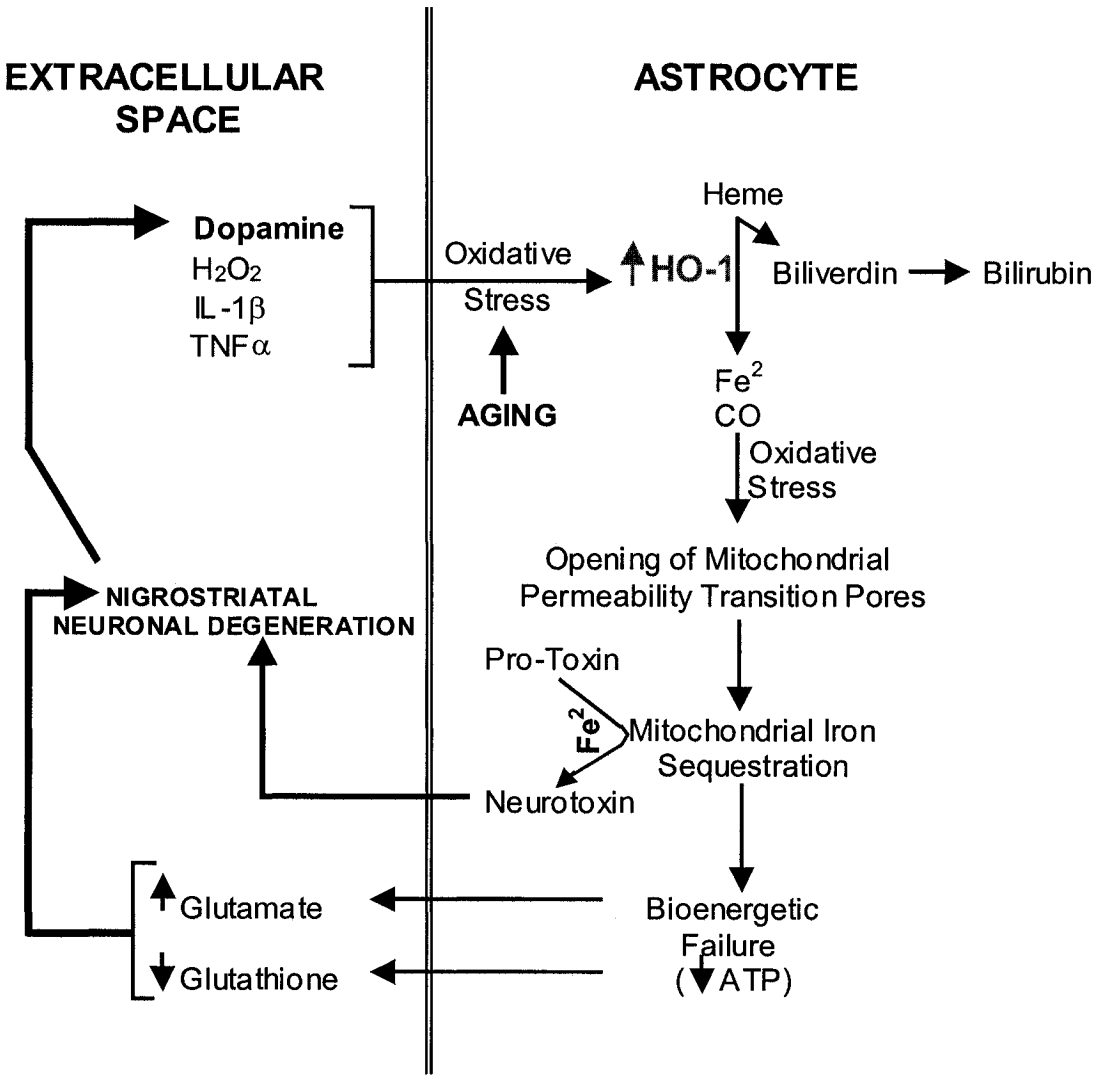

PC12 Cells Exposed to Contidioned Media
non-transfection (n=4)
Sham (n=4)
HO-1 (n=4)
HO-1+SnMP (n=4)
PC12 Cell Death (%)
20
10
0
X
*
Unchallenged
Dopamine + H2O2
Dopamine
H2O2
DH + ascorbate
DH + DFO
DH + Phenanthroline

Figure 14. Putative role of astroglial HO-1 in pathological iron deposition and mitochondrial insufficiency in Parkinson's Disease (modified after Schipper HM 2004).


EXTRACELLULAR SPACE
ASTROCYTE
Dopamine
H_2O_2
IL-1β
TNFα
Oxidative Stress
HO-1
Heme
Biliverdin
Bilirubin
AGING
Fe^2
CO
Oxidative Stress
Opening of Mitochondrial Permeability Transition Pores
NIGROSTRIATAL NEURONAL DEGENERATION
Pro-Toxin
Fe^2
Mitochondrial Iron Sequestration
Neurotoxin
Glutamate
Glutathione
Bioenergetic Failure (ATP)

APPENDIX

6 a) Purpose of Animal Use (Check one):

1. ☐ **Studies of a fundamental nature/basic research**
2. ☒ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/rgo/animal)**

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes, complete SOP # 14
Will the project involve the generation of genetically altered animals? NO ☒ YES ☐ If yes, complete SOP #5
Will the project involve breeding animals? NO ☒ YES ☐ If breeding transgenics/knockouts, complete SOP #4

7. Animal Data

7 a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

Both live animal and cell culture experiments required. In vitro studies permit delineation of key metabolic pathway in uniform cell populations. The relevance of any in vitro changes must be investigated in the intact animal brain.

7 b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

The current project builds on a considerable body of prior data obtained from albino rats

7 c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Rat	Rat				
Supplier/Source	Charles River	Charles River				
Strain	Sprague	Sprague				
Sex	M/F	F				
Age/Wt	3-12 weeks	pregnant				
# To be purchased	72	60				
# Produced by in-house breeding						
# Other (e.g.field studies)						
#needed at one time	12	2				
# per cage	2-3/1* *Rats treated with cysteamine should be kept isolated to prevent scratching (ulceration) at injection sites.	2 pregnant/1 female + pups per cage				
TOTAL# /YEAR	72	60				

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear. ***Space will expand as needed.***

For cell cultures, 2-3 pregnant rats twice monthly (=60/yr). This will provide for approx. 10 million fetal/neonatal brain cells every 2 weeks, sufficient to establish approx. 50 primary chamber slide cultures which will allow testing of 5 drugs at 2 time points (n=5 per group). One concentration curve for each of the 2 stimulatory drugs (2 doses of CSH; 3 doses of alpha-aminoadipic acid) will require a total of 20 rats. One concentration curve for each of 5 heme oxygenase-1 inhibitors and 5 antioxidants/iron chelators will require 20 rats each (total 60 rats/year). In the in vivo expeirments we will test saline + 2 stimulatory drugs (2 doses of CSH; 3 doses of alpha-aminoadipic acid) (with 4 animals/group) so 6x4=24 rats. Second series of rats will pair drug + inhibitor (6x4=24 rats) and a third set will include drugs + chelator (6x4=24) for a total of 72 rats. Hypotheses and in vitro data must be corroborated in whole animal models in order to establish a valid pre-clinical basis for future human drug development. The numbers of animals and cell cultures to be used are the minimum required to achieve statistically meaningful data (by t-test or ANOVA, with $p<0.05$ indicating significance between groups).

The following table may help you explain the animal numbers listed in the 7c table:

Test Agents or Procedures *e.g. 2 Drugs*	# of Animals and Species Per Group *e.g. 6 rats*	Dosage and/or Route of Administration *e.g. .03, .05 mg/kg – IM, IP (4 variables)*	# of endpoints *e.g. 1, 7, 10 days (3 variables)*	Other variables (i.e. sex, weight, genotypes,etc.) *e.g. Male, Female groups (2 variables)*	Total number of animals per year *e.g. 2 x 6 x 4 x 3 x 2 = 288*
same as above					

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ if yes, specify:

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: LDI Room: A.Q.

Indicate area(s) all facilities where animals will be housed:

Building: LDI Room: AQ

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP from must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection UACC#1	☐	Collection of Amphibian Oocytes UACC#9	☐
Anaesthesia in rodents UACC#2	☒	Rodent Survival Surgery UACC#10	☐
Analgesia in rodents UACC#3	☐	Anaesthesia & Analgesia Neonatal Rodents UACC#11	☐
Breeding transgenics/knockouts UACC#4	☐	Stereotaxic Survival Surgery in Rodents UACC#12	☐
Transgenic Generation UACC#5	☐	Euthanasia of Adult & Neonatal Rodents UACC#13	☐

Knockout/in Generation UACC#6	☐	Field Studies Form	☐
Production of Monoclonal Antibodies UACC#7	☐	Phenotype Disclosure Form	☐
Production of Polyclonal Antibodies UACC#8	☐	Other, specify:	☐

10. Description of Procedures

10 a). IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etcAppendix 2 of the Guidelines (www.mcgill.ca/rgo/animal) provides a sample list of points that should be addressed in this section.

A. Brain cell cultures: Prgnant female rats will be anesthetized with Nembutal. Fetuses will be removed from uteri following laparotomy. Mothers will be killed by induced pneumothorax. Fetal brains will be harvested for cell culturing. In some experiments, 2 day old rat pups will be decapitated and brains removed for cell culturing.

B. Whole animal study: Rats aged 3-12 weeks will receive either cysteamine (s.c.) 1-2 x/week for 1day-2 months. After completion of treatment, animals will be anesthetized with Nembutal and perfused intracardically for routine brain histology. In some experiments brains will be removed with prior cardiac perfusion for Northern or Western blotting or tissue iron measurement. The cysteamine-treated animals will be injected with 150-300 mg/kg cysteamine in 0.4 cc normal saline. Some of the treated animals develop small patches of skin ulceration at the injection sites. The animals appear to ignore these lesions when present. However, we will maintain the lattre animals in individual cages (with bedding) to prevent littermates from scratching potential skin lesions. To minimize skin ulceration, the acidic csteamine solution will be neutralized to pH 7.4 prior to injection. Additional animals will be treated with alpha-aminoadipic acid (500-700 mg/kg, s.c., in normal saline) x 1 dose. This glial toxin produces no apparent discomfort to the animals.

10 b) Experimental endpoint – for each experimental group indicate survival time

Cysteamine and aminoadipic acid-treated rats will be sacrificed at varying time points between 1 day and 2 months

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved)

Animals will be euthanized before completion of the study if they exhibit a) >20% weight loss; b) skin ulcers larger than 1 cm

Np special behavioral abnormalities documented with glial toxin

10 d) Specify person(s) who will be responsible for animal monitoring and post-operative care ***(must also be listed in section 4)***

Name: Adrienne Liberman	Phone #: 340-8260 X5278

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. ***Table will expand as needed.***

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Rat	sodium nembutal	50 mg/kg	1 ml	i.p.	X1

10 f) Administration of ALL other substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. ***Table will expand as needed.***

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Rat	cysteamine	150-300 mg/kg	0.4 cc saline	s.c.	1-2X per wk

Rat	alpha-aminoadipic acid	500-700 mg/kg	1 cc saline	s.c.	x 1 dose

10 g) Method of Euthanasia

Specify Species

	☐ **Anaesthetic overdose, list agent/dose/route:**
Rat	☒ **Exsanguination with anaesthesia, list agent/dose/route:** sodium nembutal 50 mg i.p. *trans-cardiac perfusion-fixation
Rat (neonatal)	☒ **Decapitation without anaesthesia *** ☐ **Decapitation with anesthesia, list agent/dose/route:**
	☐ **Cervical dislocation without anaesthesia *** ☐ **Cervical dislocation with anaesthesia, list agent/dose/route:**
	☐ **CO_2 chamber**
Rat	☒ **Other, specify:** perfusion under nembutal anesthesia (adults); induced pneumothorax under nembutal (pregnant), 50 mg/kg ip
	☐ **Not applicable, explain:**

*** For physical method of euthanasia without anaesthesia, please justify:** Neonatal brain cultures will be used forfree radical experiments. Potential redox effects of anesthetics may influence data.

11. Category of Invasiveness: B ☐ C ☒ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.*

Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*

Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review.

A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

12 a) Indicate which of the following will be used in animals:

☐Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			

Survival time after administration			

12 c) After administration the animals will be housed in:

☒ the animal care facility ☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.