**Glutathione depletion induces cell death in human NT2 cells:
Implications for Parkinson's disease**

By

Angèle Byrd, BSc. (Honours), University of Ottawa

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the degree of

Master of Science

Ottawa-Carleton Institute of Biology
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Submitted by

Angèle Byrd, B.Sc.H.

in partial fulfillment of the requirements
for the degree of Masters of Science

Chair, Department of Biology

Thesis Supervisor

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Abstract

Glutathione (GSH) is a major free radical scavenger and antioxidant in the brain. Published literature suggests that a decrease in GSH appears before neuronal loss in presymptomatic Parkinson's disease, which may result in the selective degeneration of dopaminergic neurons. To study the role of GSH depletion in the survival of dopamine neurons, human NT2 cells were treated with the GSH synthesis inhibitor L-buthionine-*S,R*-sulfoximine. The results show that the depletion of GSH triggers a cascade of events, which ultimately result in cell death. Pure cultures of NT2 neurons were more susceptible to the effects of GSH depletion when compared to progenitor cells, mixed cultures of neurons and astrocytes or pure cultures of astrocytes. Cell death occurred with features distinct from classical apoptosis and correlated with the level of intracellular GSH. Our data indicate that depletion of GSH may be a sufficient trigger for cell death and may help elucidate its role in the development of Parkinson's disease.

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Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
BSO	L-buthionine- <i>S,R</i> -sulfoximine
° C	degrees Celsius
CCD	charged coupled device
CDTA	trans-1,2-diaminocyclohexane- <i>N,N,N',N'</i> -tetraacetic acid
CFDA	5-carboxyfluorescein diacetate acetoxymethyl ester
ChAT	choline acetyl transferase
CNS	central nervous system
CO ₂	carbon dioxide
Cu/Zn	copper/zinc
CY3	Cyanine fluorochrome
Cys	cysteine
CysGly	cysteinyglycine dipeptide
DEM	diethylmaleate
DNA	deoxyribonucleic acid
dUTP	deoxy uracil triphosphate
EBSS	Earle's balanced salt solution
EDTA	ethylenediaminetetraacetic acid

EGTA	ethylene glycol-bis[β -aminoethylether]-N,N,N',N'-tetraacetic acid
ETC	electron transport chain
FBS	fetal bovine serum
FIGE	field inversion gel electrophoresis
γ -GCS	gamma-glutamylcysteine synthetase
γ -gluX	gamma-glutamyl amino acid
γ -GT	gamma-glutamyl transpeptidase
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAP 43	growth associated protein 43
GFAP	glial fibrillary acidic protein
Gl	glutaminase
GLAST	glutamate and aspartate transporter
Gln	glutamine
GlnS	glutamine synthetase
GLT-1	glutamate transporter-1
Glu	glutamate
Gly	glycine
GR	glutathione reductase
GS	glutathione synthetase
GSH	reduced glutathione or L-glutamyl-L-cysteinyl-glycine
GSNO	nitrosoglutathione

GSSG	glutathione disulfide or oxidized glutathione
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HG/DMEM	high-glucose Dulbecco's modified Eagle's medium
HMW	high molecular weight
HRP	horseradish peroxidase
HT22	mouse hippocampal neuronal cell line
IgG	immunoglobulin G
ILBD	incidental Lewy body disease
kbp	kilo base pair
KCl	potassium chloride
kDa	kilodalton
LMW	low molecular weight
LOX	lipoxygenase
M	molar
MAO	monoamine oxidase
MAP	microtubule associated protein
Mbp	mega base pair
mg	milligram
mL	millilitre
mM	millimolar
N	normal
NB	nuclear homogenization buffer

NEM	N-ethyl maleimide
NH ₂ OH	hydroxylamine
nm	nanomole
NS20Y	mouse neuroblastoma cells
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotidyl phosphate
NCAM	neural cell adhesion molecule
NO [·]	nitric oxide
NO ⁺	nitrosyl anion
NO ⁻	nitroxyl cation
NT2/D1	NT2 progenitor cell, clone D1
NT2/N	NT2 neuron
NT2/A	NT2 astrocyte
NTera-2 or NT2	human neuronal teratocarcinoma cell line
O ₂ ^{·-}	superoxide radical
OH	alcohol group
OH ⁻	hydroxyl anion
OH [·]	hydroxyl radical
ONOO ⁻	peroxynitrite
OPT	<i>o</i> -phthaldehyde
PBS	phosphate buffered saline

PC12	rat pheochromocytoma cell line-12
pH	-logarithm to the base 10 of the hydrogen anion concentration ($-\log_{10} [\text{H}^+]$)
P _i	inorganic phosphate
PFGE	pulse field gel electrophoresis
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
R-OH	organic alcohol
R-OOH	organic peroxide
ROS	reactive oxygen species
rpm	revolutions per minute
R-SH	protein thiol, where R=protein, SH=thiol group
R-SSG	protein-glutathione mixed disulfide, where R=protein and SSG= glutathione moiety
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	thiol or sulfhydryl group
SOD	superoxide dismutase
SSG	glutathione moiety of mixed disulfides
TBE	Tris borate-EDTA buffer
TBST	Tris buffered saline with 0.05 % Tween-20
TdT	terminal deoxynucleotidyl transferase
TE	Tris-EDTA buffer

TEEN	Tris-EDTA-EGTA-sodium chloride buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick-end labelling
U	units of enzyme activity
μg	microgram
μM	micromolar
μm	micrometer
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
x	times
X	acceptor of γ-glutamyl moiety

1.0 Literature Review

LR 1.1 Oxidative Stress, GSH and Neurodegeneration

Oxidative stress-induced damage has been implicated in normal aging (Bains and Shaw, 1997; Benzi and Moretti, 1995) and in the pathogenesis of various neurodegenerative diseases including Alzheimer's disease (Bains and Shaw, 1997; Halliwell, 1992) and Parkinson's disease (Bains and Shaw, 1997). Reactive oxygen species (ROS) are generally produced during normal processes of mitochondrial cellular respiration. However, the oxidative status of the cell can still be maintained due to the existence of an equilibrium between ROS generation and cellular antioxidant defenses. Oxidative stress can occur when the cellular oxidation-reduction balance is shifted to favor ROS formation. As the brain ages, there is an increase in the formation and release of ROS, likely caused by a decline in antioxidant defenses, and ultimately leads to the disruption of normal cellular activity through biochemical and functional alterations to membranes, increased protein oxidation, lipid peroxidation and DNA damage (Halliwell, 1992). These age-related factors may increase our susceptibility to neurodegenerative diseases. Oxidative stress has been implicated in the pathogenesis of Alzheimer's disease, where it has been shown to enhance the toxicity of β -amyloid found in senile plaques and to lead to the dimerization and polymerization of *tau* protein into filaments called neurofibrillary tangles, both characteristic hallmarks of Alzheimer's disease (Goodman and Mattson, 1994; Mark *et al.*, 1997; Troncoso *et al.*, 1993). Although the pathogenesis of Parkinson's disease may be multifactorial, one hypothesis suggests that ROS formation can shift the cellular redox equilibrium

toward oxidative stress and may eventually damage pigmented neuromelanin-rich dopaminergic neurons within the substantia nigra. The belief of the involvement of oxidative stress stems from post-mortem studies that show evidence of increased lipid peroxidation, impaired glutathione (GSH) system and enhanced superoxide activity in the substantia nigra of Parkinson's disease patients (Dexter *et al.*, 1994; Jenner *et al.*, 1992).

Since oxidative stress occurs when there is an imbalance in ROS formation and cellular antioxidant defenses, it has been postulated that a deficiency in GSH, the brain's primary antioxidant and free radical scavenger, may represent a key factor in the aging process. Several lines of evidence from animal models suggest an age-dependent decline in GSH levels in the human brain (Chen *et al.*, 1989; Hussain *et al.*, 1995; Sohal and Weindruch, 1996) as well as the brain of aged mice (Chen *et al.*, 1989; Hussain *et al.*, 1995), which have approximately 30 % less GSH than younger brains. Because the brain requires extensive ROS detoxification, an age-dependent decline in GSH may lead to damage due to increased oxidative stress and may render the brain more susceptible to damage suggesting a possible link with susceptibility to age-related neurodegenerative diseases. Although alterations in GSH have been shown in a number of neurodegenerative disorders, GSH depletion is most strongly implicated in the pathogenesis of Parkinson's disease. Parkinson's disease is characterized by the selective loss of dopaminergic neurons within the substantia nigra pars compacta, leading to tremor, muscle rigidity, jerky movements, bradykinesia and postural instability. Dopaminergic neurons may be more vulnerable to imbalances in their antioxidant system and oxidative stress for several

reasons. Dopaminergic neurons have a high concentration of neuromelanin, a pigment produced from the auto-oxidative metabolism of dopamine through the enzyme monoamine oxidase (MAO) that can lead to the generation of large amounts of oxygen free radicals (Graham, 1979) and may reduce GSH concentrations, especially under conditions of oxidative stress.

Using human and rodent post-mortem tissues, it has been observed that GSH levels in the substantia nigra are significantly decreased in Parkinson's disease relative to those in the aging brain, while other regions of the brain remain unchanged (Perry *et al.*, 1982; Perry and Yong, 1986; Reiderer *et al.*, 1989; Sofic *et al.*, 1992). It has also been reported that the magnitude of the GSH depletion not only parallels the severity of the disease (Reiderer *et al.*, 1989), but it is also the only common link between presymptomatic Parkinson's disease (also called incidental Lewy body disease (ILBD)) and advanced Parkinson's disease, preceding mitochondrial dysfunction and the loss of dopaminergic neurons (Perry *et al.* 1986; Dexter *et al.*, 1994; Jenner, 1994). In addition, the extent of GSH depletion in incidental Lewy body disease and advanced Parkinson's disease is similar, but the degree of cell death in the substantia nigra is very different between the two stages (Dexter *et al.*, 1994). As a result, alterations in GSH levels in the substantia nigra may be critical in the early stages of the pathogenesis of Parkinson's disease. However, it remains unclear whether GSH depletion causes oxidative stress followed by neurodegeneration or whether mitochondrial dysfunction leads to the generation of excessive ROS, which may lead to the subsequent depletion of GSH and ultimately neurodegeneration (for review see Di Monte *et al.*, 1992). Regardless

of whether GSH depletion is a primary cause or a consequence thereof, a deficient GSH system is somehow involved in neurodegeneration, at least in Parkinson's disease.

LR 1.2 Glutathione and the Brain

The brain is particularly vulnerable to damage from oxidative stress due to its high metabolic rate and high-energy demands, unique lipid composition and minimal cell turnover (Coyle and Puttfarcken, 1993; Sagara *et al.*, 1998). Other characteristics of the brain contribute to its susceptibility to oxidative damage. For example, ROS generation is exacerbated by the high level of iron in the brain, which can lead to ROS generation through Fenton reactions (Gerlach *et al.*, 1994). Also, the high turnover of dopamine and the presence of neuromelanin can generate a large amount of hydrogen peroxide (H_2O_2) and other free radicals (Youdim, 1989). In addition to this, the brain has low levels of free radical detoxification enzymes such as catalase to break down H_2O_2 and superoxide dismutase (SOD) to detoxify the superoxide radical ($\text{O}_2^{\cdot -}$) (Cooper, 1997). As a result, the GSH system has an especially important role as the primary brain antioxidant and in the cellular defense against reactive oxygen species, where it is present at a concentration of approximately 2 mM (Slivka *et al.*, 1987; Ratan and Baraban, 1995). Both neuronal and glial cells have GSH with astrocytes having much higher concentrations than the neurons (Raps *et al.*, 1989; Dringen *et al.*, 2000).

LR 1.3 Functions of Glutathione and its Regulation

Glutathione (GSH; γ -L-glutamyl-L-cysteinyl-glycine) is a ubiquitous tripeptide and one of the most abundant low molecular weight non-protein cellular thiols present in mammalian cells, found at a high physiological concentration ranging from 0.5 mM to 12 mM (Meister, and Andersson, 1983). Concentrations of GSH in various tissues and cell lines with the appropriate references can be found in Appendix I. Glutathione is a unique biomolecule that is protected from aminopeptidase degradation due to the presence of a peptidic γ -linkage rather than the usual α -linkage found in conventional peptides (Meister, and Andersson, 1983). *In vivo*, it is synthesized in the cytosol by two consecutive ATP-consuming enzymatic reactions (**Figure 1.1 A**). The first is a condensation reaction where the enzyme γ -glutamylcysteine synthetase (γ -GCS) uses glutamate and cysteine as substrates to form the dipeptide γ -glutamylcysteine. This is the rate-limiting reaction in GSH synthesis as it is dependent primarily on the availability of cysteine (Dringen *et al.*, 1999; Sagara *et al.*, 1993). The second step synthesizes GSH from γ -glutamylcysteine and glycine and is catalyzed via the action of the enzyme GSH synthetase (GS). The structure of GSH is presented in **Figure 1.1 B**.

Glutathione is a molecule with many important physiological functions. It is involved in the detoxification of xenobiotics by spontaneous non-enzymatic reactions or by the enzymatic formation of GSH conjugates through the action of glutathione-S-transferase (Burke *et al.*, 1983). It is essential for maintaining a stable intracellular redox environment and thiol status required for proper protein synthesis, folding and functioning by preventing oxidation of the sulfhydryl groups

Figure 1.1

- A) The enzymatic synthesis of GSH.** Glutathione is synthesized by two consecutive ATP-consuming reactions. The first is a the rate-limiting condensation reaction generating γ -glutamylcysteine from the precursor amino acids glutamate and cysteine via the enzyme γ -glutamylcysteine synthetase (γ -GCS), followed by the condensation of glycine to the γ -glutamylcysteine via GSH synthetase (GS).
- B) The structure of GSH, γ -glutamylcysteinyl glycine.** The amino-terminal glutamate and cysteine are linked to the carboxyl group of glutamate via a peptidic γ -linkage that is resistant to intracellular peptidases and aids in its stability. The sulfhydryl group is required for GSH's functions, while the glycine moiety at the carboxy-terminal of GSH protects the molecule against cleavage by intracellular γ -glutamylcyclotransferases.

B)

or by reducing disulfide bonds induced during oxidative stress conditions (Meister, 1988). It also provides a continuous source of cysteine (Meister, 1988), an important function since cysteine is highly unstable and is rapidly auto-oxidized to cystine, a non-useable form for many cells, with the formation of toxic oxygen free radicals. Furthermore, it may play a role in the modulation of critical biological processes including DNA synthesis (Meister, 1988), cell death (Ueda *et al.*, 2002 for review), immune function (Furukawa *et al.*, 1987), amino acid transport (Meister and Tate, 1976), as well as cell proliferation and gene expression (Arrigo, 1999 for review).

The most well-studied function of GSH is its protective role as an antioxidant and free radical scavenger. All aerobic organisms are subject to a certain level of oxidative and nitrosative stress resulting from normal aerobic metabolism, which generates free radical intermediates such as O_2^- and H_2O_2 that can in turn lead to DNA, protein and lipid peroxidation, thereby damaging cells. The complex antioxidant and free radical detoxification reactions of GSH are summarized below and in **Figure 1.2**.

A) Glutathione can act as a substrate for the enzyme GSH peroxidase to detoxify hydroperoxides, the predominant mechanism to reduce H_2O_2 , or organic peroxides (ROOH) via the enzyme GSH-S-transferase. During this process, GSH acts as a cofactor for either enzyme and is oxidized to GSSG (GSH disulfide, also known as oxidized GSH) (reaction 1). **B)** Because this results in the consumption of GSH, it can be regenerated from GSSG via the enzymatic action of GSH reductase (GR), at

A schematic of different pathways involved in the cellular detoxification of free radicals and antioxidant functions of GSH.

Hydrogen peroxide (H_2O_2) and organic peroxides (ROOH) are detoxified by GSH peroxidase or GSH-S-transferase, using GSH as a cofactor. GSH can be regenerated from GSSG via GSH reductase. H_2O_2 can also be detoxified by catalase in the peroxisome. Reactive nitrogen species (NO^+ , NO^- and ONOO^-) can be detoxified by the consumption of GSH through the formation of nitrosogluthathione (GSNO), or GSSG.

the expense of NADPH reducing equivalents, thereby forming a redox cycle (reaction 2). Catalase, an enzyme located within the peroxisome, can also eliminate H_2O_2 (reaction 3). Under oxidative conditions, GSH is continually being consumed resulting in the accumulation of GSSG. To maintain the proper GSH/GSSG ratio, excessive GSSG can either be actively exported from the cell or it can react with protein sulfhydryls (R-SH) to form protein-GSH mixed disulfides (RSSG) through a thiol-disulfide exchange in a reaction catalyzed by GSH-S-transferase (reaction 4) (For review, see Klatt and Lamas, 2000). This reaction is fully reversible where GSH and RSSG form GSSG and R-SH via thiol transferase (reaction 5), thus continually shifting between GSH and GSSG to maintain the intracellular thiol status. C) GSH can also react directly with free radicals through nonenzymatic reactions. GSH can detoxify nitric oxide species (nitrosyl cation (NO^+) and nitroxyl anion (NO^-)) to form nitrosoglutathione (GSNO) (reaction 6), a potent antioxidant and nitric oxide donor, or GSSG and hydroxylamine (NH_2OH) (reaction 7), respectively. Nitric oxide (NO^-) can also react with O_2^- to generate peroxynitrite, (ONOO^-) (reaction 8), a potent oxidant that can induce lipid peroxidation, cause DNA damage and oxidize proteins (Torreilles *et al.*, 1999). D) Peroxynitrite can be detoxified by GSH through a two-step reaction (reactions 9 and 10). Superoxide can also react with hydrogen cation to form H_2O_2 through SOD (reaction 11), but the two-step reaction is more favourable and reacts three fold faster than it reacts with SOD (Torreilles *et al.*, 1999). In addition to its regeneration from GSSG via GSH reductase, GSH can be resynthesized from its constituent amino acids by using extracellular GSH and/or GSH conjugates as substrates for the ecto-enzyme γ -

glutamyl transpeptidase (γ -GT). Cellular GSH synthesis and utilization is ultimately regulated through negative feedback inhibition, where GSH itself can regulate the activity of γ -GCS, hence the formation of γ -glutamylcysteine.

LR 1.4 Distribution of GSH

Intracellular GSH is compartmentalized within a cell with approximately 85-90 % of the GSH found within the cytosol and the remaining 10-15 % within the mitochondria (Meredith and Reed, 1982;Griffith and Meister, 1985; Huang and Philbert, 1995). A small percentage (2-5 %) has also been found within the nucleus where it is needed in DNA repair (Jevtovic-Tudorovic and Guenther, 1992) and in the endoplasmic reticulum where it is involved in protein disulfide bond formation (Hwang *et al.*, 1992). Because mitochondria lack the enzymes needed for GSH synthesis, its pool of GSH originates from GSH that is synthesized within the cytosol and subsequently taken up and maintained via an energy-dependent transporter on the mitochondrial membrane (Griffith and Meister, 1985; Kurosawa *et al.*, 1990; Mårtensson *et al.*, 1990). In addition, mitochondria lack catalase and because they are a major intracellular source of ROS, they depend entirely on GSH and SOD to detoxify superoxide radicals that are continually being generated during cellular respiration. As a result, this critical mitochondrial pool of GSH is constantly maintained at 10-15 % of total GSH by the continual influx of cytosolic GSH. It has been shown that during oxidative conditions, the mitochondrial pool of GSH may fall below this critical level and dramatically curtail the ability of cells to compensate for the accumulating oxidative stress and eventually lead to loss of cell

function and cell death (Merad-Boudia *et al.*, 1998; Shan *et al.*, 1993; Seyfried *et al.*, 1999; Wüllner *et al.*, 1999).

LR 1.5 Glutathione Metabolic Interactions Between Astrocytes and Neurons

There is evidence of many important metabolic and protective interactions between astrocytes and neurons including supplying lactate as an energy substrate for ATP production, recycling of neuronal glutamate metabolism, cell-cell communication and neuroprotection, as well as GSH metabolism and defense against ROS (for review, refer to Kirchhoff *et al.*, 2001). It has been shown that astrocytes produce much more GSH than neurons and that astrocytes actually provide neurons with most of the three precursor peptides necessary for GSH synthesis, primarily in the form of glutamine (Gln) and cysteinylglycine dipeptide (CysGly) (Dringen *et al.*, 1999; Dringen *et al.*, 2000; Dringen *et al.*, 2001). In addition, neuronal GSH increases significantly when the neurons are cultured in the presence of astrocytes, compared to being cultured alone (Bolanos *et al.*, 1993). The dipeptide is subsequently hydrolyzed into its individual components, cysteine (Cys) and glycine (Gly), by an aminopeptidase on the extracellular surface of neurons. The amino acids then get transported into the neurons for GSH synthesis (Dringen *et al.*, 2001). While astrocytes can use both cysteine and cystine for GSH synthesis, neurons can only use cysteine and limiting neuronal GSH synthesis (Dringen *et al.*, 1999; Sagara *et al.*, 1993).

A model, representing the likely interactions between astrocytes and neurons regarding GSH synthesis, is presented in **Figure 1.3**. Astrocytes synthesize GSH via

Figure 1.3

Schematic of the proposed metabolic interaction between astrocytes and neurons in GSH metabolism.

GSH is synthesized in astrocytes and subsequently released from the astrocytes and acts as a substrate for the astroglial ecto-enzyme, γ -glutamyl transpeptidase (γ -GT), which transfers an electron from GSH to X, an acceptor of the γ -glutamyl moiety. This generates CysGly, which acts as a precursor for neuronal GSH, and γ -glutamyl-X. The CysGly is further hydrolyzed into its individual amino acids, cysteine (Cys) and glycine (Gly), via an amino ecto-dipeptidase. In the astrocytes, glutamate (Glu) is converted to glutamine (Gln), released from the astrocytes and all three amino acids are readily taken up by neurons to be used for GSH synthesis. In both neurons and astrocytes, mitochondrial GSH is taken up and maintained by the cytosolic pool of GSH through a transporter system, and any GSSG generated from free radical detoxification within the mitochondria can be used to regenerate GSH via GSH reductase or released into the cytosol where it can also be used to regenerate GSH or released from the cell.

This figure is an adaptation of Dringen *et al.*, 2000 and Merad-Boudia *et al.*, 1998.

the two-step reaction previously described (figure 1.1 A) and also provide the three precursor amino acids to neurons. Within astrocytes, glutamate (Glu) is amidated to glutamine (Gln) through the action of the enzyme glutamine synthetase (GlnS), which is present in astrocytes but not neurons (Norenberg and Martinez-Hernandez, 1979). The glutamine is then released and rapidly taken up by neurons where it is hydrolyzed by phosphorylated glutaminase (Gl) to regenerate glutamate (Kvamme *et al.*, 2000). To provide Gly and Cys, GSH itself is released into the extracellular space where γ -GT, found on the extracellular membrane of the astrocytes, hydrolyzes GSH into the precursor CysGly via an acceptor of the γ -glutamyl moiety (X). The CysGly dipeptide is subsequently cleaved into cysteine and glycine by an aminopeptidase ecto-enzyme on the neuronal extracellular membrane. Consequently, the three individual precursors required for GSH synthesis are provided by astrocytes and are available for GSH synthesis within the neurons. Thus, it is clear there is an intimate metabolic and functional relationship regarding GSH metabolism occurring between astrocytes and neurons. This model suggests that a similar relationship may exist in the living brain and that a compromised GSH system within the astrocytes may render both the astrocytes and neurons more susceptible to oxidative damage and ultimately, if the conditions persist, to cell death.

LR 1.6 Experimental Modulation of GSH Levels

Several compounds can be used to lower GSH levels by either reacting with GSH itself to form conjugates or that oxidize GSH to GSSG; however, they act very

quickly, they are not specific to GSH per se and they may cause unrelated biological perturbations. Some of these compounds include ethacrynic acid, diethylmaleate (DEM), N-ethyl maleimide (NEM) and 1-chloro-2,4-dinitrobenzene. Ethacrynic acid is often used in GSH depletion studies because it rapidly depletes both cytosolic and mitochondrial GSH by its electrophilic conjugation to GSH in a reaction catalyzed by GSH-S-transferase (Shen *et al.*, 1995). By far, L-buthionine-(S,R)-sulfoximine (BSO) is most commonly used to deplete GSH. The structure of BSO is presented in **Figure 1.4**. Intracellularly, the sulfoximine site is phosphorylated by ATP and binds irreversibly and specifically to the active site on γ -GCS to effectively inhibit the enzyme required in the rate-limiting step of GSH synthesis (Griffith and Meister, 1979a), preventing the *de novo* synthesis of GSH and reducing the cytosolic pool of GSH (Seyfried *et al.*, 1999). Despite the slower rate of depletion than that achieved by other compounds, total depletion of cytosolic GSH will result with long-term treatment with BSO, which, in turn, results in the loss of mitochondrial GSH levels and the eventual depletion of the mitochondrial pool (Merad-Boudia *et al.*, 1998). There are no documented reports indicating that BSO or the sulfoximine moiety of BSO exert direct toxicity. BSO does not interact with GSH itself, but specifically with an enzyme involved with its synthesis; thus its action is specific to GSH depletion and not due to the formation of toxic conjugates as it occurs with the other GSH-depleting compounds.

Figure 1.4

The structure of L-buthionine-*S,R*-sulfoximine (BSO).

BSO is a potent inhibitor of γ -GCS. The enzyme catalyzes the phosphorylation of BSO allowing the *S*-alkyl moiety of the sulfoximine to be tightly bound to the enzyme at the site that normally binds the acceptor amino acid, thus inhibiting its action.

LR 1.7 Glutathione and the Mode of Neuronal Cell Death:

The mode of cell death in neuronal models following oxidative stress has remained highly controversial, with some groups reporting features of necrosis and some reporting characteristics of apoptosis. Necrosis and apoptosis are two different mechanisms of cell death with very distinct characteristics. Necrosis is a passive mode of cell death, which typically results from excessive toxins or physical trauma and it is characterized by cell swelling, disruption of organelles and mitochondrial swelling, rapid collapse of internal homeostasis and eventually cell lysis, release of cellular contents and activation of an inflammatory response. On the other hand, apoptosis, first described by Kerr *et al.* (1972), is an active program of cell death characterized by distinct morphological and molecular features. Its characteristics are usually cell shrinkage, membrane blebbing, nuclear shrinkage, chromatin condensation, DNA fragmentation into 100-200 bp oligonucleosomal fragments and finally fragmentation of the cell into membrane-bound apoptotic bodies that are subsequently ingested by surrounding phagocytotic cells without inducing an inflammatory response (Kerr *et al.*, 1972). Cellular apoptosis is activated in response to many different signals through a series of cascading events, which most commonly involves a sequential activation of initiator and effector caspases.

Caspases are a family of cysteine proteases that exist as inactive proenzymes whose proteolytic cleavage generates fragments with proteolytic activity which act on a broad range of cellular substrates and commit susceptible cells to apoptotic death. In contrast to necrosis, apoptosis is a normal physiological process during regular development of the nervous system to remove extraneous or damaged cells

and at least 50 % of all neurons die before reaching adulthood (Oppenheim, 1991). However, inappropriate cell death is associated with a number of neurological disorders such as Alzheimer's disease (Su *et al.*, 1994) and Parkinson's disease (Mochizuki *et al.*, 1996). It is thought that cell death in these disorders is linked to oxidative stress as evidenced by the occurrence of excessive lipid and protein oxidation and altered antioxidant systems, particularly the GSH system (For review, refer to Jenner, 1994; Jenner and Olanow, 1996). Neuronal cell death in response to oxidative stress has many characteristics similar to those of apoptosis, including nuclear condensation and chromatin fragmentation (Ratan *et al.*, 1994). However, the mode of cell death induced by GSH depletion is very controversial and the evidence listed below invokes apoptosis, necrosis, a combination of apoptosis and necrosis or even some other uncharacterized mechanism.

Depletion of GSH in thymocytes has been identified as an early event in the apoptotic pathway (Macho *et al.*, 1997) and has been reported in different systems to induce or increase the sensitivity to cell death with the characteristics of apoptosis. For example, the depletion of GSH in the NS20Y neuronal cell line for a period of five days led to increased ROS generation, mitochondrial respiratory chain impairment and a significant decrease in cell number (62 %) with chromatin condensation and DNA fragmentation characteristic of apoptosis (Merad-Boudia *et al.*, 1998). Furthermore, the same group later defined GSH as a mediator of apoptosis in the NS20Y cell line (Nicole *et al.*, 1998). It has also been reported that BSO could induce apoptosis in various human neuroblastoma cell lines as detected by oligonucleosomal DNA laddering, nuclear morphology and TUNEL (Terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick-End Labelling) which all indicated the presence of DNA fragments after 3 days of BSO treatment (Anderson *et al.*, 1999). Additionally, Armstrong *et al.* (2002) showed that a deficiency in GSH by BSO in a human B lymphoma cell line led to the generation of ROS and apoptotic cell death after three days of treatment. GSH depletion in PC12 cells was also found to elicit cell death with nuclear morphology similar to those of apoptosis (Froissard *et al.*, 1997). The sensitivity of some toxins can be enhanced following GSH depletion. Thus, glutamate-induced GSH depletion in embryonic cortical neurons (Ratan *et al.*, 1994) as well as in neuroblastoma and hippocampal neurons (Greenlund *et al.*, 1995) results in oxidative stress and apoptotic cell death. Dopamine-induced toxicity is enhanced when preceded with GSH depletion by BSO causing cell death with apoptotic features including membrane blebbing and oligonucleosomal DNA fragmentation (Stokes *et al.*, 2000). As might be expected, GSH has recently been implicated in protection against the induction of apoptosis and necrosis in a variety of cell types (Fernandes and Cotter, 1994; Ben-Yoseph *et al.*, 1996).

On the other hand, there is also strong evidence that a deficiency in GSH causes cell death through a mechanism that is distinct from classical apoptosis or necrosis. Research by Wüllner and colleagues (1999) show that GSH depletion in cerebellar granule neurons following a 24-36 hour treatment with BSO can lead to cell death. However, the features were atypical of apoptosis. They included distinctive, but previously unknown changes in chromatin structure described as a homogeneous granular pattern. Furthermore, GSH depletion using ethacrynic acid resulted in

rapid cell death with necrotic features including massive mitochondrial swelling, destruction of organelles and rupture of the plasma membrane. In another study, Jurma *et al.* (1997) induced a deficiency in GSH in PC12 cells by transfecting them with antisense γ -GCS oligonucleotides. This led to a significant reduction in GSH levels, increased ROS generation, followed by a disruption of intracellular calcium homeostasis, which in turn led to a marked decrease in dopaminergic cell function and active dopamine uptake, as well as decreased cell viability (50 %). However, cell death did not appear apoptotic since there was no increase in TUNEL staining or oligonucleosomal DNA laddering. It was also found that nitric oxide treatment, following GSH depletion by BSO exposure, triggered a programmed cell death with markers of both apoptosis and necrosis in midbrain primary cultures, changing nitric oxide's neurotrophic role to a neurotoxic role under conditions of low GSH levels (Canals *et al.*, 2001). To date, there are no documented reports on the modes of cell death following GSH depletion in the NTera-2 model studied in this thesis. This system is potentially useful because it is possible to establish defined mixed cultures of astrocytes and neurons. Consequently it may prove to be an exciting study that may help to clarify this highly controversial area of research.

LR. 2.0 The NT2 Model System

NTera-2 cells (NT2/clone D1 or NT2/D1) are a pluripotent clonal embryonal human teratocarcinoma cell line that was derived from a lung metastasis of a 22-year-old Caucasian male with a primary testicular germ cell tumor (Fogh and Trempe, 1978) and established by Andrews and colleagues (1984). When

maintained under standard cell culture conditions, NT2/D1 exhibit characteristics of rapidly proliferating undifferentiated progenitors, however this cell line can be differentiated by a four-week treatment with retinoic acid into a number of morphologically and phenotypically distinct cell types including, primarily, post-mitotic neurons (Andrews, 1984; Lee and Andrews, 1986) and astrocytes (Sandhu *et al.*, 2002; Bani-Yaghoub *et al.*, 1999), which can be maintained as individual pure cultures, mixed cultures or undifferentiated cultures. A representative figure of the NT2 model at various cell culture stages is presented in **Figure 1.5**. Much progress in characterizing this cell line has been achieved over the past two decades.

It was initially thought that the differentiation of human NT2 cells only generated terminally differentiated neurons (NT2/N), however it was recently reported that differentiation by retinoic also gives rise to functional astrocytes (NT2/A) (Sandhu *et al.*, 2002; Bani-Yaghoub *et al.*, 1999), while differentiation by bone morphogenetic protein (BMP-2 and -4) can give rise to epithelial cells (Houldsworth *et al.*, 2001). Because the progenitor cells can be differentiated into neurons and astrocytes, NTera-2 are a valuable model for studying neuronal development and differentiation processes.

In addition to these features, NTera-2 possess numerous characteristics that make them a valuable model for studying the expression of various neurotransmitters such as dopamine and glutamate in both *in vivo* and *in vitro* models of the central nervous system (CNS). NT2/A have been shown to express typical markers of astrocytes such as glial fibrillary acidic protein (GFAP), vimentin and S100 β , and to also contain functional glutamate transporters (GLAST and

Figure 1.5 The NT2 Model at various cell culture stages.

Panel A) A confluent monolayer of undifferentiated NT2/D1 progenitor cells is presented. Incubation of these progenitor cells with 10 μ M retinoic acid for four weeks allows for their differentiation.

Panel B) The resulting multilayered structure of a mixture of differentiating cells at the end of the four-week treatment with retinoic acid. At this time, the cells are replated in the presence of DNA synthesis inhibitors.

Panel C) A representative of a mature NT2-N/A culture. After the four-week retinoic acid treatment, cells are replated at a lower density. Two days following this replating step, mixed cultures of neurons and astrocytes (NT2-N/A) can be generated by harvesting the majority of the neurons and 50 % of the astrocytes. These cultures are left to mature for 9-12 days in the presence of DNA synthesis inhibitors and an additional 9-12 days for a total maturation period of 21 days. An astrocyte (NT2/A) is indicated by the yellow arrow and a neuron (NT2/N) by the white arrow.

Panel D) A representative of mature pure neuronal cultures (NT2/N). After the four-week retinoic acid treatment, cells are replated at a lower density in the presence of DNA synthesis inhibitors. Cells were left to mature for 9-11 days in the presence of DNA synthesis inhibitors. At this time, neuronal cells could be easily identified and harvested. Once harvested, the neurons were left to mature for an additional 9-12 days in conditioned cell culture medium for a total maturation period of 21 days. Details described in section 2.2 of the Materials and Methods. Magnification = 2000X.

GLT-1), as well as functional glutamine synthetase (Sandhu *et al.*, 2002). They have also been shown to possess the astrocyte cytoskeletal marker connexin 43 and to be coupled by gap junctions (Bani-Yaghoub *et al.*, 1999). On the other hand, NT2/N have been very well characterized and shown to possess a number of features that closely resemble human CNS neurons. These include the expression of neuronal cytoskeletal proteins including neurofilament proteins, secretory markers such as synaptophysin and chromogranin, as well as surface markers such as NCAM and GAP43 (Pleasure *et al.*, 1992; Lee and Andrews, 1986). NT2/N also have a similar morphology to rodent primary neuronal cultures, possessing an extensive network of functional dendrites and axons and shown to also express synapsins, MAP1A, MAP1B, MAP2 and *tau* (Leypoldt *et al.*, 2002; Pleasure *et al.*, 1992). Furthermore, they have been shown to produce β -amyloid peptide (Cook *et al.*, 1997; Mantione *et al.*, 1995; Wolf *et al.*, 1995), express mRNA for glutamic acid decarboxylase (GAD) and choline acetyltransferase (ChAT) (Hurlbert *et al.*, 1999), and to express and synthesize a large variety of neurotransmitters including γ -aminobutyric acid (GABA), catecholamine, acetylcholine and serotonin as well as various neuropeptides such as neuropeptide Y, oxytocin, vasopressin and calcitonin (Guillemain *et al.*, 2000).

Since the discovery that transplanted purified NT2/N are capable of surviving, maturing and integrating into the host nervous system (Baker *et al.*, 2000; Kleppner *et al.*, 1995), the NT2 model may be a potential therapeutic candidate for human neurological diseases. NT2/N may eventually be suitable for cell replacement therapy as an alternative to fetal tissue transplantation in the treatment of

neurodegenerative diseases. In fact, there is currently a human clinical trial utilizing NT2/N in cell replacement therapy for stroke patients (Kondziolka *et al.*, 2000). Parkinson's disease may be a candidate for such cell replacement therapy since NT2 cells have been shown to express tyrosine hydroxylase, the first and rate-limiting enzyme involved in the synthesis of catecholamine neurotransmitters such as dopamine (Zigova *et al.*, 2000), express dopamine transporters, produce low levels of dopamine, (Iacovitti *et al.*, 2001; Iacovitti and Stull, 1997), express mRNA for dopamine receptors (Sakolsy and Ashby, 2000; Hurlbert *et al.*, 1999) and possess functional dopamine receptors (Sodja *et al.*, 2002). NT2/N have also been studied in terms of their antioxidant properties and have been reported to exhibit low levels of GSH and α -tocopherol, and reduced activities in many antioxidant enzymes including GSH peroxidase, GSH transferase, GSH reductase, SOD and catalase (Tamagno *et al.*, 2000).

LR. 3.0 Hypotheses and Thesis Objectives:

There is an increasing body of evidence showing that there are numerous important interactions occurring between neurons and astrocytes, both *in vivo* and *in vitro*. Such studies suggest that astrocytes have an extensive supportive role inherent to the survival of neurons by secreting a number of neurotrophic factors and by producing and secreting various antioxidants. In the CNS, as well as in *in vitro* models of the CNS, astrocytes appear to be a major source of reduced GSH, an important intracellular non-protein thiol antioxidant, and appear to supply neurons with most of their GSH (Dringen *et al.*, 2000). Furthermore, a number of *in vivo* and

in vitro studies implicate GSH depletion in neurodegeneration. Based on these findings, there are two major objectives to this thesis. The first is to establish a link between GSH depletion and cell viability, and to investigate the mode of cell death that may be involved. We hypothesize that a decrease in the level of GSH may render neurons highly vulnerable to killing and that the presence of astrocytes may, to some extent, help protect neurons from the effects of GSH depletion. To test this hypothesis, we have used a human teratocarcinoma cell line, NTera-2 (NT2/ clone D1 or NT2/D1) as a cell culture model to study GSH relationships that exists between neurons and astrocytes and the role GSH depletion may play in neurotoxicity and gliotoxicity. The treatment of these progenitor cells with retinoic acid leads to their differentiation into post-mitotic neurons and astrocytes and these cultures can further be maintained as either pure astrocyte, pure neuronal or mixed astrocyte and neuronal cultures. The fact that the NT2 model is a fairly new system, not much is known about interactions that exists between the different cells that arise from differentiation, thus the second objective was to gain more insight into important neuron-astrocyte interactions. Because these cultures can be maintained as pure or mixed cultures, we feel that the NT2 model is a good model to study such interactions.

Currently, NT2 cells are considered to be an excellent *in vitro* representative of neuronal progenitor cells of the CNS and may provide a good experimental model for studying neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. The eventual long-term goal stemming from the results of this thesis is to use this system as a cell culture model of Parkinson's disease, a

progressive neurodegenerative disease characterized by the selective degeneration of dopaminergic neurons in the substantia nigra. The reason why nigral neurons selectively degenerate is only partially understood, however a number of reports suggest that cellular damage mediated by oxidative and/or nitrosative stress may be key to the pathology (Jenner *et al.*, 1992; Serra *et al.*, 2001). In support of this notion, reports show there is a decrease in the level of GSH in the substantia nigra in both presymptomatic and advanced Parkinson's disease (Perry *et al.*, 1986; Dexter *et al.*, 1994; Jenner *et al.*, 1994). Furthermore, it has been previously reported that NT2/N possess many features of a dopaminergic phenotype including the presence of tyrosine hydroxylase, functional dopamine receptors and transporters, and to produce low levels of dopamine (Zigova *et al.*, 2000; Iacovitti and Stull, 1997; Sodja *et al.*, 2002; Iacovitti *et al.*, 2000). Therefore, we believe that human NT2 cells make an excellent model to gain insights into neuron-astrocyte interactions and to help us understand the role of such interactions and GSH depletion may play in the development of Parkinson's disease.

2.0 Materials and Methods

M 2.1 Materials

All cell culture dishes, plates and flasks were purchased from Beckton Dickinson, NJ, U.S.A. Suppliers of all the chemicals used are listed throughout the text.

M 2.2 Cell Culture Conditions

M 2.2.1 Undifferentiated NT2/D1 Cells

All cell culture procedures were carried out under sterile conditions. The human NTera-2 cell line used in this study is a pluripotent clonal embryonal carcinoma cell line (clone # 21) established by Andrews, *et al* (1984). The undifferentiated NT2/D1 cells were purchased from Stratagene (La Jolla, CA). Undifferentiated NT2/D1 cells were seeded at a density of 5×10^6 cells per 75-cm² flask in high glucose Dulbecco's modified Eagle's medium (HG/DMEM; Invitrogen, Burlington, ON) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Wisent Inc., St. Bruno, PQ) and maintained as a 70-80 % confluent monolayer under the strict environmental conditions of 5 % CO₂ and 95 % humidified air at 37°C. The cells were maintained by splitting two to three times per week. To maintain the cultures in this way, the cell culture medium was removed and the cells were washed once with 5 mL of phosphate-buffered saline (PBS). The cells were then trypsinized with 1 mL of 0.15 % (w/v) trypsin dissolved in PBS containing 1mM EDTA (trypsin/EDTA) for 30 seconds to 1 minute and were mechanically dislodged from the substratum. The cells were resuspended in 10 mL of HG/DMEM supplemented with 10 % FBS and

transferred to a 50 mL falcon tube. A 50 μ L aliquot was removed, mixed 1:1 (v:v) with 0.4 % (v/v) trypan blue (Sigma Chemical Co, St. Louis, MO) and counted using a haemocytometer on a Leica DMLS light microscope. Cell density was determined and the cells were seeded at a density of 5×10^6 cells per 75-cm^2 flask and the volume was brought up to 12 mL per flask.

M 2.2.2 Differentiation of NT2/D1

Differentiation of NT2/D1 cells was carried out as described previously by Pleasure, *et al* (1992). Undifferentiated NT2/D1 cells were washed once with 5 mL of PBS, trypsinized using 1 mL of 0.15 % (w/v) trypsin/EDTA and resuspended in 10 mL HG/DMEM with 10 % FBS. Cell numbers were determined using trypan blue as previously described in section 2.2.1, seeded at a cell density of 2.3×10^6 cells per 75-cm^2 flask with a total volume of 12 mL per flask. These cells were maintained in the same flasks for four weeks and treated with 10 μ M all-*trans*-retinoic acid (Sigma Chemical Co., St. Louis, MO). Fresh medium and retinoic acid was added three times per week for four weeks.

M 2.2.3 Preparation of Mixed Differentiated Neuronal and Astrocyte Cultures (NT2-N/A)

Following four weeks of retinoic acid treatment, cells from each flask were washed once with 5 mL of PBS and harvested using 1 mL of 0.15 % (w/v) trypsin/EDTA. They were resuspended in 10 mL of HG/DMEM supplemented with 5 % FBS (without retinoic acid) and the suspension was divided into two

separate 175-cm² flasks (5 mL per flask) and the volume was brought up to 25 mL. After 48 hours in these flasks, the cell culture medium was removed and mixed cultures (NT2-N/A) consisting of approximately 60 % neuronal cells (NT2/N) and 40 % astrocytes (NT2/A) were prepared by mechanically dislodging the majority of the neurons and approximately 50 % of the astrocytes by gently striking the flasks 5-10 times. The cells were resuspended in 10 mL of HG/DMEM with 10 % FBS and the cell suspension was collected and transferred to a 50 mL falcon tube. All sides of the flasks were washed once with 10 mL of PBS. The remaining cells were collected, transferred to the same falcon tube and centrifuged (Labofuge 400) at 800 rpm for 5 minutes at room temperature. The supernatant was removed, 5 mL of fresh HG/DMEM supplemented with 5 % FBS medium was added to the tube and a single cell suspension was generated by triturating the suspension 20 times using a 5 mL-plugged pipette and an additional 10 times with a plugged Pasteur pipette. The cell number was determined and replated at the required cell density for the appropriate size dishes (refer to **Appendix II** for seeding numbers), previously coated with one layer of poly-D-lysine (350, 000 kDa; 10 µg/mL; Sigma Chemical Co., St. Louis, MO) and one coat of Matrigel (Collaborative Research/Becton Dickinson, Bedford, MA) at a working dilution of 1:30 in HG/DMEM without FBS. The procedure for coating was as described below in section 2.2.6. After three days in culture, the NT2/N became securely attached to a healthy underlying monolayer of NT2/A and a mixture of DNA synthesis inhibitors (10 µM 5-fluoro-2'-deoxyuridine, 10 µM uridine and 1 µM cytosine-β-D-arabinofuranoside; all three were purchased from Sigma Chemical Co., St.

Louis, MO) was added to the cell culture medium (HG/DMEM with 5 % FBS) for approximately 10 days to prevent the NT2/A from dividing and taking over the culture. The NT2-N/A were then left to mature for an additional 9-12 days, for a total of 21-23 days of maturation into post-mitotic NT2/N and NT2/A. Fresh HG/DMEM supplemented with 5 % FBS was added every third day during the course of maturation until use.

M 2.2.4 Preparation of Pure Neuronal Cultures (NT2/N)

Purified NT2/N were prepared in a similar manner. Following four weeks of treatment with retinoic acid, all cells were replated in 175-cm² flasks as mentioned in section 2.2.3 and maintained in the same flasks as NT2-N/A cultures for 9 to 11 days in HG/DMEM, 5 % FBS and DNA synthesis inhibitors as previously mentioned. On day 9, 10 or 11, the cell culture medium was removed and rinsed once with 5 mL of PBS. The NT2/N just above the monolayer of NT2/A were collected by incubating the cells with 2 mL of diluted 0.015 % (v/v) trypsin/EDTA (1 mL 0.15 % (w/v) trypsin/EDTA into 9 mL PBS) for 30 seconds to two minutes with a gentle swirling motion and gentle mechanical shaking. The NT2/N were collected in 10 mL of HG/DMEM supplemented with 10 % FBS and transferred to a 50 mL falcon tube. The flasks were not washed in order to minimize the presence of NT2/A in these cultures. The flasks containing the remaining NT2/A were given fresh HG/DMEM containing 5 % FBS and returned to an incubator at 37°C with 5 % CO₂ and 95 % humidified air. The NT2/N were then centrifuged at 800 rpm for 5 minutes at room temperature. The pellet was

loosened by gently flicking the tube with a finger and resuspended in 2 to 3 mL of conditioned medium consisting of 50 % fresh HG/DMEM supplemented with 5 % FBS and 50 % conditioned HG/DMEM with 5 % FBS removed from flasks containing mature (4-8 weeks old), purified NT2/A (see section 2.2.5) and DNA synthesis inhibitors. A single cell suspension was obtained by triturating the cells 5 to 10 times using a plugged Pasteur pipette. The volume was brought up to 10 mL with the same conditioned medium, the cell number was determined and the cells were seeded at the appropriate cell density for the required poly-D-lysine and Matrigel pre-coated dishes (refer to **Appendix II** for seeding numbers). Coating procedure was as described in section 2.2.6. The following day, the cell culture medium was replaced with fresh conditioned medium lacking DNA synthesis inhibitors and allowed to mature into post-mitotic NT2/N for an additional 10-12 days for a total of 21-23 days of maturation. The medium was changed once a week while the cultures were maturing.

M 2.2.5 Preparation of Pure Astrocytes (NT2/A)

Purified NT2/A were generated from the NT2/A that were returned to an incubator following the pure NT2/N harvest, as described above. Once the NT2/A were returned to the incubator, they were left to recover for three days at which time the culture medium was removed and the cells were washed once with 5 mL of PBS. The NT2/A were then trypsinized using 2 mL of diluted 0.015 % (v/v) trypsin (1 mL 0.15 % (w/v) trypsin/EDTA to 9 mL PBS) to remove any remaining neurons. The NT2/A were washed again with 5 mL of PBS, fresh

HG/DMEM with 5 % FBS was added and they were returned to the incubator. One to two days later, 2 mL of normal 0.15 % (w/v) trypsin were added for 3 minutes at 37°C. The NT2/A were resuspended in 10 mL of HG/DMEM with 5 % FBS, collected and transferred to new 175-cm² flasks at a density of 3×10^6 cells per flask. The appropriate volume of HG/DMEM supplemented with 5 % FBS was added to each flask for a total volume of 25 mL per flask. These cells were maintained in HG/DMEM with 5 % FBS, in the absence of DNA synthesis inhibitors. The cells were split once a week for two weeks. Two days before each split, any remaining NT2/N were removed using diluted 0.015 % (v/v) trypsin (1 mL 0.15 % (w/v) trypsin to 9 mL PBS)) as described in section 2.2.4. Fresh HG/DMEM supplemented with 5 % FBS was given once a week for up to three months. NT2/A become cell-cycle arrested after 21-28 days post-retinoic acid treatment. The cell culture medium in these flasks was subsequently used to prepare 50:50 conditioned medium to be used for the maintenance of the NT2/N cultures described earlier in section 2.2.4.

M 2.2.6 Coating of Dishes for Plating of NT2-N/A and NT2/N

The day prior to cell plating, flasks or dishes were coated with high molecular weight poly-D-lysine (350, 000 kDa) as follows. A 1mg/mL stock solution was prepared by dissolving the poly-D-lysine in 0.15 M sodium borate buffer (pH 8.4). The solution was then filter-sterilized through a 0.22 µm filter, divided into 1 mL aliquots and stored at -20°C. The stock solution was diluted to a working concentration of 10 µg/mL in sterile distilled water. The entire surface of the

desired type of flasks or dishes was completely covered with working strength poly-D-lysine (volume depended on the surface area of the dishes) and they were placed at 4°C overnight. The following day, the poly-D-lysine was removed and the flasks or dishes were allowed to dry for one hour. The poly-D-lysine-coated flasks or dishes could then be stored at 4°C for up to one month.

Flasks or dishes were subsequently coated with Matrigel on the same day the cells were plated. Prior to plating, the cell culture flasks or dishes were placed at -20°C for 30 minutes in preparation for Matrigel coating. All steps during coating with Matrigel were carried out under ice-cold conditions to prevent gelling of the Matrigel, including pipettes, pipettor tips and 50 mL flacon tubes. A 1 mL aliquot of freshly thawed Matrigel was diluted in 30 mL of cold HG/DMEM without FBS. Flasks or dishes were coated a few at a time to avoid warming to room temperature and coated with 0.25 mL to 1 mL of working strength Matrigel (volume depended on the surface area of the dishes) using a swirling action. Flasks or dishes were left to coat at room temperature for 30 minutes. After this time, the excess Matrigel solution was removed and the flasks or dishes were left to dry at room temperature for 30 minutes. Flasks or dishes were now ready for plating of cells.

M 2.3 Treatment with the Glutathione-depleting Agent, L-Buthionine-S,R-Sulfoximine (BSO)

Glutathione depletion was carried out on undifferentiated NT2/D1 cells, mixed differentiated cultures (NT2-N/A) and pure neuronal cultures (NT2/N). Two days prior treatment with BSO, undifferentiated NT2/D1 were seeded as described in

section 2.2.1, while the differentiated NT2-N/A were seeded two days post-retinoic acid treatment as described in section 2.2.3 and NT2/N were seeded 9-11 days post-retinoic acid treatment as described in section 2.2.4.

A 25 mM stock solution of BSO (ICN Biomedicals Inc, Costa Mesa, CA) was freshly prepared in deionized, distilled water and filter-sterilized using 0.22 μm syringe-driven filters. From this stock, 50 μM , 100 μM and 250 μM stocks of BSO were prepared in HG/DMEM supplemented with 10% FBS for NT2/D1 and with 5% FBS for NT2-N/A while 50:50 conditioned medium supplemented with 5% FBS was used in the case of NT2/N. The cells were treated with the required concentration of BSO and the desired experiment was carried out at 24, 48, 72, 96 and 120 hours following treatment for NT2/D1 and NT2-N/A, and at 24, 48 and occasionally 72 hours for NT2/N. The culture medium was replaced with fresh medium and BSO on the third day of the experiment.

M 2.4 Measurement of Glutathione Levels

M 2.4.1 Sample Preparation for Glutathione Measurements

Mixed NT2-N/A were set up in 75-cm²-coated flasks two days following retinoic acid treatment at a density of 10×10^6 cells per flask as described in section 2.2.3. Pure NT2-N were set up in 25-cm²-coated flasks at a density of 3.5×10^6 cells per flask 10 days post-retinoic acid treatment as described in section 2.2.4. The NT2/D1 were seeded at a density of 1×10^6 in 75-cm² flasks two days prior the treatment with BSO as described in section 2.2.1. All flasks were set up in duplicate flasks. The cells were treated with varying concentrations of BSO as mentioned in section 2.3. The glutathione content of untreated and BSO-treated

cells was then determined using the *o*-phthaldehyde (OPT) method as previously described by Akerboom and Sies (1982). All steps were carried out at room temperature unless otherwise indicated. Briefly, cells were incubated in the presence of 50 μ M, 100 μ M and 250 μ M BSO for 24, 48 and 72 hours. After 24, 48 and 72 hours, both adherent and non-adherent cells were collected following exposure to 0.15 % (w/v) trypsin/EDTA. The cells were resuspended in 10 mL of HG/DMEM with 10 % FBS and transferred to a 50 mL falcon tube. The flasks were washed once with 10 mL of PBS and the remaining suspension was transferred to the same tube. The cells were centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, the pellet was resuspended in 10 mL PBS and recentrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 1 mL of PBS, transferred to a 1.5 mL eppendorf tube and centrifuged (IEC Micromax microcentrifuge) at 13,000 rpm for 20 seconds. The supernatant was removed and the pellet was resuspended in 100 μ L of ice-cold water with 100 μ L of 2N perchloric acid (Fisher Scientific, Ottawa, ON) and vortexed for 5-10 seconds. The mixture was left on ice for 15 minutes and then centrifuged at 13,000 rpm for two minutes in order to precipitate the proteins. The pellet was reserved for protein quantification and stored at -80°C until use. An 150 μ L aliquot of the supernatant was transferred to a new eppendorf tube, 150 μ L of 1N potassium hydroxide was added, vortexed for 5-10 seconds and left on ice for 15 minutes to precipitate the perchloric acid. This was followed by centrifugation at 13,000 rpm. This supernatant was now the working sample to be analyzed. An aliquot of the sample was transferred to a glass tube

containing 2.3 mL of Buffer A (0.1M potassium phosphate and 1mM EDTA, pH 8.0) and 25 μ L of freshly prepared OPT (Sigma Chemical Co., St. Louis, MO) dissolved in methanol (1 mg OPT /mL methanol). A standard curve was generated in a similar manner using a 100 μ M stock of glutathione (Sigma Chemical Co., St. Louis, MO) dissolved in Buffer A, ranging from 0 to 1000 pmol glutathione. The mixture was left to incubate for 20 minutes in the dark. At this point, 2 mL of the sample was transferred to a cuvette. Fluorescence was measured using an LS5 Perkin Elmer fluorescence spectrophotometer with an excitation wavelength of 350 nm and emission wavelength of 420 nm. Each sample was prepared in duplicate.

M 2.4.2 Protein Quantification

The GSH content data was then corrected for protein content, measured using the fluorescamine assay, as previously described by Udenfriend, *et al*, 1972. Briefly, the protein sample was sonicated using a sonic dismembrator model 300 (Fisher Scientific, Ottawa, ON) at low speeds in 2 % (v/v) sodium dodecyl sulfate (SDS) in water, boiled at 100°C for 5-10 minutes and a 1 μ L aliquot was then transferred to a glass tube containing 1 mL of Solution II (0.3M sodium tetraborate (pH 9.2), 5mM CDTA (trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid) and 0.15 % (v/v) SDS) and 0.5 mL of water. Solution I (0.5 mM fluorescamine (Sigma Chemical Co., St. Louis, MO) dissolved in 100 mL acetone) was quickly added while vortexing simultaneously. The mixture was left to incubate in the dark at room temperature for 5 minutes and 0.5 mL of water

was then added, the mixture quickly vortexed and 1 mL of the mixture was transferred to a 12-well plate. A standard curve was generated in a similar manner using bovine plasma γ -globulin (BioRad Laboratories, Hercules, CA). Fluorescence was read on a cytofluorometer (CytoFluor™ 2300/2350, Millipore, Mississauga, ON) at an excitation of 390nm and an emission of 475nm.

M 2.5 Assessments of Cell Viability and Morphology

M 2.5.1 Determination of Cell Viability Using the CFDA Fluorometric Assay and Nuclear Morphological Analysis Using Hoechst 33258

For these measurements, all cells were grown on glass coverslips in 12-well plates. Two days prior treatment with BSO, NT2/D1 were seeded at a density of 5×10^4 cells per well for the 24 and 48-hour time points, and at 4×10^4 cells per well for the 72, 96 and 120-hour time points to prevent overgrowth as described in section 2.2.1. Differentiated NT2-N/A and NT2/N were seeded on glass coverslips previously coated with poly-D-lysine and Matrigel, as described in section 2.2.6, at a density of 5×10^5 cells per well on day 3 and day 9 post-retinoic acid treatment respectively, as described in sections 2.2.3 and 2.2.4, respectively. The cells were treated with varying concentrations of BSO as described in section 2.3. Cell viability was assessed in purified NT2/N following treatment with BSO for 24 and 48 hours and after 72, 96 and 120 hours of treatment in the case of undifferentiated NT2/D1 and differentiated NT2-N/A using 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA; Molecular Probes, Eugene, Oregon). This is a non-fluorescent, hydrophobic dye that penetrates the cell membrane. Once inside the cell, it is cleaved by non-specific esterases of viable cells to generate a

fluorescent anionic compound, 5(6)-carboxyfluorescein. A CFDA stock was prepared in DMSO (2.5mg/ml DMSO; dimethyl sulfoxide; BDH Chemicals, Toronto, ON) and a final working concentration of 5 μ M CFDA was made up in Earle's balanced salt solution (EBSS; pH 7.4; Sigma Chemical Co., St. Louis, MO) just prior to use. Each concentration was set up in triplicate wells. Prior to incubation with the dye, non-adherent cells were collected as described below in section 2.5.2 for the microscopic observation of nuclear morphology. The cells were washed once with PBS and incubated with 1 mL of 5 μ M CFDA for 30 minutes at 37°C. Fluorescence was read on a cytofluorometer (CytoFluor™ 2300/2350, Millipore, Mississauga, ON) at an excitation wavelength of 480/20 nm and an emission wavelength of 530/25 nm. Fifteen readings were taken from each well, for a total of 45 readings for each concentration. The mean was calculated for each set, normalized to fluorescence values for untreated control cells and expressed as percent control fluorescence. Immediately following reading of fluorescence, the cells were fixed with 1x formalin and stored in the dark at 4°C overnight for microscopic analysis. Fluorescence was viewed with an Olympus BX50 fluorescence microscope with an Olympus 20x N.A. 0.50 or 40x N.A. 0.75 UPlanapo fluorescence objective and equipped with a Sony 3 CCD camera and standard excitation and emission filters. The CFDA green fluorescence was observed using an excitation filter of 470-490 nm and emission filter of 515-550 nm. Nuclear morphology was examined under UV light (excitation filter 330-385 nm; emission filter $\geq$ 420 nm) by the addition of

Hoechst 33258 dye to stain the nuclei. Images were captured using the Northern Exposure software package (Empix Imaging Inc., Mississauga, ON).

M 2.5.2 Determination of Cell Viability by Microscopy

Before the cells were incubated with the CFDA for the assay described in the previous section (2.5.1), the cell culture medium containing non-adherent cells were collected into a 1.5 mL eppendorf tube and centrifuged at 800 rpm for 5 minutes at room temperature. The supernatant was removed, the pellet was resuspended in 1 mL PBS and recentrifuged at 800 rpm for 5 minutes. The pellet was then resuspended in 50 μ L of PBS and 50 μ L of Genofix and left at room temperature for one day. The following day, the cells were centrifuged at 800 rpm for 5 minutes at room temperature and resuspended in 100 μ L PBS. The suspended cells were centrifuged onto microscope slides using a Leica SP1400 cytopsin (Leica Microsystems, Nussloch, Germany) and covered with glass coverslips using DAKO fluorescent mounting medium (DAKO Diagnostics Canada, Inc., Mississauga, ON) spiked with 5 μ g/mL of the nuclear stain Hoechst 33258 (Sigma Chemical Co., St. Louis, MO). Viability cell counts for the non-adherent cells were obtained by counting cells with an altered nuclear morphology under UV light. The coverslips containing the cells that were fixed overnight immediately following the CFDA fluorometric assay (section 2.5.1) were washed twice with water (3 minutes per wash) at room temperature under subdued light and mounted onto microscope slides using DAKO fluorescent mounting medium spiked with 5 μ g/mL Hoechst 33258. Viable and non-viable cells, in both

adherent and non-adherent cell preparations, were counted based on morphology of Hoechst-stained nuclei (non-viable cells) and CFDA labelling cells (viable cells). Approximately 300-600 cells were counted from 5 random fields of view from 3 coverslips for each concentration for both adherent and non-adherent cells. The CFDA green fluorescence was observed using an excitation filter of 470-490 nm and emission filter of 515-550 nm. Morphology was examined by blue Hoechst-stained nuclei under UV light (excitation filter 330-385 nm; emission filter ≥ 420 nm). The data from the coverslips and the supernatant slides was pooled, averaged and normalized to counts obtained from 3 control (untreated cells) coverslips and supernatant slides and results were comparable to those obtained by the fluorometric assay.

Viability was also assessed in BSO-treated NT2/D1 using the trypan blue exclusion assay. Undifferentiated NT2/D1 cells were seeded in 6-cm² petri dishes at a cell density of 5×10^5 for 24 and 48 hours treatments and 3.5×10^5 for the 72, 96 and 120-hour time points as described in section 2.2.1. Non-adherent cells were collected and transferred to a 15 mL tube. The adherent cells were washed once with 1 mL of PBS, trypsinized using 250 μ L of 0.15 % (w/v) trypsin/EDTA and resuspended in 2 mL of HG/DMEM supplemented with 10 % FBS. The suspension was centrifuged at 800 rpm for 5 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 500 μ L of HG/DMEM supplemented with 10 % FBS. Two 50 μ L aliquots for each concentration of BSO was removed and mixed 1:1 (v:v) with trypan blue. Viable and non-viable cells were counted under a Leica DMLS light microscope using a

haemocytometer. Counts were averaged and expressed as percent cell viability. This was not carried out in differentiated cultures due to the presence of cell aggregates, which could lead to false counts due to the rigorous resuspension required to obtain single cell suspensions.

M 2.6 DNA Fragmentation and Pulse Field Gel Electrophoresis (PFGE)

M 2.6.1 Preparation of Samples

The protocol for the preparation of cell samples and embedding in agarose mini-plugs, deproteinization of the embedded cells and gel electrophoresis were as previously described (Walker, *et al*, 1999b). Cells (NT2/D1, NT2-N/A and NT2/N) were set up as described sections 2.2.1, 2.2.3 and 2.2.4, respectively and treated with varying concentrations of BSO as mentioned in section 2.3. Both the adherent and non-adherent cells from each concentration of BSO at each time point were collected, counted and then $2-3 \times 10^6$ cells were reserved for agarose-embedding. The cell suspensions were washed once with 1 mL of nuclear homogenization buffer (NB; 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 1 mM EGTA (ethylene glycol-bis[β -aminoethylether]-N,N,N',N'-tetraacetic acid), 2 mM EDTA (ethylenediaminetetraacetic acid), 0.5 mM spermidine and 0.15 mM spermine) and centrifuged at 800 rpm for 5 minutes at room temperature. The pellet was resuspended and embedded in a 37°C pre-warmed solution of 40 μ L of NB, 40 μ L of melted 1.5 % (w/v) Seakem® LE low melting point agarose (BioWhittaker Molecular Applications, Rockland, ME) and 1 μ L of 20 mg/mL proteinase K (Invitrogen, Burlington, ON) and casted into wells of a mini-plug mould (BioRad Laboratories, Hercules, CA). The moulds were placed at 4°C for

10 minutes to solidify. The plugs were then ejected into an eppendorf tube containing 300 μ L of TEEN digestion buffer (10 mM Tris-HCl, pH 9.5, 25 mM EDTA, 5 mM EGTA, and 10 mM NaCl), 15 μ L of 10 % (v/v) SDS and 2 μ L of 20 mg/mL proteinase K and placed at 37°C in an incubator equipped with a rotator for one and a half hours. The plugs were then rinsed twice in 1 mL of ice-cold TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) at 4°C for 30 minutes per wash and stored dry at 4°C in a sealed microfuge tube until all samples were collected.

M 2.6.2 Sample Analysis by PFGE

Once all plugs were collected, they were loaded in a 0.8% (w/v) agarose gel prepared in 0.5x TBE (45 mM Tris-borate, and 1.25 mM EDTA, pH 8.5). PFGE DNA markers of overlapping size ranges were also loaded on the gels. They included a combination of HindIII digest of lambda DNA (2.0-23.1 kbp), the 123-bp ladder and 1 kbp DNA ladder (10-12.0 kbp; all three from Invitrogen, Burlington, ON), as well as yeast chromosomes (225 kbp-1.2 Mbp), polymerized λ phage DNA ladder (50 kb-1 Mbp) and “low range markers” (0.1-194 kbp; all three purchased from New England Biolabs, Beverly, MA). The wells were sealed using 0.8 % (w/v) low melting point agarose and the entire gel was set at 4°C for 10 minutes. PFGE was then carried out using an Autobase Electrophoresis system (Q-life, Kingston, ON) filled with 3 L of 0.5x TBE running buffer, prechilled to 14°C. The PFGE running parameters were set on a FIGE mapper power supply (BioRad Laboratories, Hercules, CA) as follows: forward direction voltage of 280

V and 90 V for the reverse direction, an initial forward switch time of 0.3 seconds and reverse time of 0.5 seconds, with non-linear ramping to 35 seconds for the forward direction and 35 seconds for the reverse direction for a total run time of 20.5 hours at 14°C. These conditions allowed for an ordered separation of various sizes of DNA fragments ranging from 100 bp to greater than 1 Mbp all on the same gel. After the run was completed, the gel was then stained in 200 µL/L ethidium bromide (10 mg/mL stock) in TBE buffer for 30 minutes, destained in distilled water for 30 minutes to one hour and photographed under UV transillumination using an Ultralum Electronic UV Transilluminator equipped with a Panasonic CCD camera and image acquisition system.

M 2.7 Identification of free 3'-hydroxyl DNA breaks by TUNEL (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labelling):

All cells were grown on glass coverslips in 12 well plates. Two days before treatment with BSO, NT2/D1 were seeded at a density of 5×10^4 cells per well for the 24 and 48-hour time points and at 4×10^4 cells per well for the 72, 96 and 120-hour time points as described in section 2.2.1. Differentiated NT2-N/A and NT2/N were seeded on glass coverslips previously coated with poly-D-lysine and Matrigel as described in section 2.2.6 at a density of 5×10^5 cells per well as described in sections 2.2.3 and 2.2.4, respectively. The cells were treated with varying concentrations of BSO as previously mentioned in section 2.3. Also, for each time point, some wells were prepared as positive controls for 3'-hydroxyl breaks in the DNA by treating NT2/N with 0.1 µM staurosporine (Sigma Chemical Co., St. Louis, MO) for 1 hour and 0.5 µM for 3-4 hours in the NT2/D1

and NT2-N/A. At each time point, cells were washed with 1 mL of PBS and fixed using 1 mL of Genofix™ for 10 minutes at room temperature, followed by two additional 5 minute washes with PBS. In the case of NT2/N cultures, all wash steps were carried out with 1.5 % (w/v) bovine serum albumin (BSA, made in PBS) to minimize loss of cells. All steps were carried out at room temperature unless otherwise specified. The coverslips were turned cell side down over a 25 μ L drop of the reaction mixture consisting of the TdT enzyme (0.3U/ μ L, Invitrogen, Burlington, ON), 10 μ M biotin-16-dUTP (Roche Diagnostics, Laval, Quebec, Canada), and 5x TdT buffer (Invitrogen, Burlington, ON) in a humidified chamber and incubated at 37°C for one hour. PBS and a reaction mixture without TdT were also included as negative controls. Termination of the reaction was achieved by transferring the coverslips, cell side up, into wells containing stop buffer (0.3 M NaCl and 0.03 M sodium citrate) for 15 minutes. This was followed by three washes of 4 minutes each in PBS. The cells were then subjected to a blocking step using 2 % (w/v) BSA for 15 minutes, followed by three washes with PBS. The coverslips were then transferred to a humidified chamber, cell side down, over a 25 μ L drop of Cy3-goat-anti-rabbit conjugated to streptavidin (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, CA) and left to incubate in the dark for 45 minutes. A negative control lacking the secondary antibody was also included. This was followed by an additional three washes with PBS and one wash with distilled water. The coverslips were then mounted onto microscope slides using DAKO fluorescent mounting medium spiked with Hoechst 33258 (5 μ g/mL). Fluorescence was viewed with an

Olympus BX50 fluorescence microscope equipped with a Sony 3 CCD camera. The images were captured using Northern Exposure (Empix Imaging Inc., Mississauga, Ontario, Canada) software. Approximately 200-400 cells were counted and photographed from five random fields of view. Total cell counts were achieved by counting Hoechst-stained nuclei under UV light and TUNEL-positive cell counts by counting those cells visible with an excitation filter of 530-550 nm and an emission > 570 nm.

M 2.8 Western Blot Analysis of the Proteolysis of Poly (ADP-ribose) Polymerase (PARP)

M 2.8.1 Total Protein Extraction Procedure

Mixed NT2-N/A were set up in 75-cm²-coated flasks two days following completion of retinoic acid treatment at a density of 10^7 cells per flask as described in section 2.2.3. Pure NT2/N were set up, in 25-cm²-coated flasks at a density of 3.5×10^6 cells per flask 10 days post-retinoic acid treatment as previously described in section 2.2.4. Undifferentiated NT2/D1 cells were seeded two days before treatment with BSO at a density of 1×10^6 in 75-cm² flasks as described in section 2.2.1. The cells were treated with the desired concentration of BSO as described in section 2.3 and total proteins were extracted at 24, 48, 72, 96 and 120 hours following treatment for NT2/D1 and NT2-N/A, and at 24 and 48 for NT2/N. Additional flasks of NT2/D1 cells were prepared and treated with 1 μ M staurosporine for 3 hours to be used as a positive control for markers of apoptosis. All cells were rinsed with 5 mL of PBS, collected by trypsinization

and washed twice with 1 mL of ice-cold PBS by centrifugation at 1000 rpm at 4°C.

Proteins were extracted from the pellet by homogenization in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % (v/v) Triton X-100, 0.1 % (v/v) SDS, 1 % (v/v) sodium deoxycholate) containing 1 mM PhenylMethylSulfonyl Fluoride (PMSF) and 1x protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada).

M 2.8.2 Protein Quantification

Proteins were quantified using the BioRad standard protein assay. A standard curve ranging from 0 to 20 µg of protein was generated using bovine plasma γ-globulin (1 mg/mL stock; BioRad Laboratories, Hercules, CA). The protein sample (2 µL) was combined with 798 µL of water and 200 µL of BioRad standard protein assay dye reagent concentrate (BioRad Laboratories, Hercules, CA) in disposable polystyrene cuvettes, vortexed for 5 seconds and incubated in the dark for 5 minutes. Absorbance was read at 595 nm on a Beckman Coulter™ DU640B spectrophotometer.

M 2.8.3 Sample Preparation and Western Blotting Procedure

Each sample having a total volume of 35 µL consisted of 50 µg of protein, determined from the standard curve constructed above, was removed from each sample and diluted in water and 10 µL of 5x stock solution of sample loading buffer (2 % (v/v) SDS, 10 % (v/v) glycerol, 1 % (v/v) 2-mercaptoethanol, 0.0005

% (v/v) bromophenol blue, 125 mM MOPS (3-[N-Morpholino]propanesulfonic acid, pH 6.8). The samples were boiled at 100°C for 5 minutes. Thirty μ L of the denatured protein samples (approximately 50 μ g per lane) and 10 μ L of prestained protein molecular weight marker (MBI Fermentas, Burlington, ON) were loaded on the gel. Proteins were resolved on an 8 % resolving gel (30 % (w/v) acrylamide/0.8 % (w/v) Bis-acrylamide, 1.5 mM Tris-HCl, pH 8.8, 10 % (v/v) SDS, 10 % (w/v) ammonium persulfate and TEMED) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1x running buffer (250 mM Tris, 1.92 mM Glycine and 0.1% (v/v) SDS, pH 8.3) at a voltage of 60 V for approximately 90 minutes. The resolved proteins were electrophoretically transferred to a nylon membrane (Immobilon, Millipore, Bedford, MA) in the presence of 1x transfer buffer (250 mM Tris and 1.92 mM Glycine, pH 8.3) at 80 V for one hour at 4°C. The protein separation and transfer were carried out using a BioRad mini-protean II gel apparatus (BioRad Laboratories, Hercules, CA) and a PS 500XT DC power supply (Fisher Scientific, Ottawa, ON). When transfer was complete, successful protein transfer was verified by staining the membranes with 0.1 % (w/v) Ponceau S and 5 % (v/v) acetic acid (Sigma Chemical Co., St. Louis, MO) for 10 minutes at room temperature with gentle shaking. Membranes were rinsed with water until the protein bands could be distinguished and photographed using an Ultralum Electronic Transilluminator under white light and equipped with a Panasonic CCD camera and image acquisition system. Membranes were washed once with TBST (10 mM Tris-HCl, 250 mM NaCl, 0.05 % (v/v) Tween-20

(polyoxyethylenesorbitan monolaurate), pH 8.0) for 5 minutes at room temperature with gentle shaking. Specific antigens were detected by blocking the nylon membranes with 5 % non-fat Carnation® skim milk (Loblaws, Ottawa, ON) in TBST for 2 hours at room temperature with gentle shaking. Membranes were probed with polyclonal anti-PARP primary antibody (1 µg/ml; Upstate Biotechnology, Lake Placid, NY) diluted in 2 % non-fat Carnation® skim milk in TBST, overnight at 4°C with gentle shaking. Membranes were washed with TBST (6 times, 5 minutes per wash) at room temperature and incubated with 1:5000 goat-anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Inc., West Grove, CA) in TBST containing 2 % non-fat Carnation® skim milk for one and a half hours at 25°C with gentle shaking. Peroxidase activity was detected using the ECL plus chemiluminescence kit (Amersham Pharmacia Biotech, Arlington Heights, IL). Detection is based on a combined HRP and peroxide catalyzed oxidation of the lumigen PS-3 Acridan substrate under slightly alkaline conditions generating many acridinium ester intermediates per minute, which produces a sustained, high intensity chemiluminescence with a maximum wavelength of 430 nm resulting in the emission of light that was detected on Kodak Biomax MS scientific imaging film (Amersham Biosciences, Baie d'Urfé, PQ).

M 2.9 Immunocytochemical Labelling of the Apoptosis Markers – 85 kDa Cleaved PARP and Activated 18 kDa Caspase-3 Fragments

All cells were grown in 12-well plates. Two days before treatment with BSO, NT2/D1 were seeded at a density of 5×10^4 cells per well for the 24 and 48-hour

time points, and at 4×10^4 cells per well for the 72, 96 and 120-hour time points as described in section 2.2.1. The cells were treated with varying concentrations of BSO as previously mentioned in section 2.3. Also, for each time point, some wells were treated with 0.5 μ M staurosporine for 4 hours in order to provide a positive control for apoptotic markers. At each time point, the cell culture medium was removed and the cells were rinsed once with PBS. The cells were then trypsinized with 0.5 mL of 0.15 % (w/v) trypsin/EDTA (3 wells for each concentration were pooled as one sample), resuspended in 1 mL of HG/DMEM supplemented with 10 % FBS and transferred to a 15 mL falcon tube. Remaining cells were collected by rinsing the wells with 1 mL of PBS and transferred to the same tube. The cells were centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was removed, the pellet was resuspended in 1 mL of 1 x PBS, and the cells were recentrifuged at 1000 rpm for 5 minutes at room temperature. The pellet was then resuspended and fixed in 250 μ L of PBS and 250 μ L of Genofix™ for 10 minutes at room temperature, followed by two 5 minute washes with 1.5 % (w/v) BSA made up in PBS. The cells were incubated in 1.5 % (w/v) BSA made up in PBS on ice for 15 minutes and centrifuged at 800 rpm for 5 minutes at room temperature. The cells were then resuspended and incubated with the primary antibody of choice (1:100 activated cleaved p85 anti-PARP (Promega, Madison, WI) or 1:50 Cleaved Caspase-3 antibody (Cell Signalling Technology, Mississauga, ON) diluted in 1.5 % (w/v) BSA for one hour on a rotator at 4°C. The cells were gently resuspended every 15 minutes. One mL of 1.5 % (w/v) BSA was placed directly on top of the primary antibody

mixture and centrifuged as mentioned above. The pellet was then resuspended in the secondary antibody, goat-anti-rabbit IgG conjugated to Cy-3 (1:500; Molecular Probes, Inc., Eugene, OR) for 30 minutes on a rotator at 4°C, resuspending the cells every 15 minutes. Again, 1 mL of 1.5 % (w/v) BSA was added on top of the secondary antibody and centrifuged as previously mentioned above. The pellet was resuspended in 25-50 μ L of 1.5 % (w/v) BSA and 5 μ L of this suspension was then mounted on a microscope slide with 5 μ L of DAKO fluorescent mounting medium spiked with 5 μ g/mL of Hoechst 33258. Negative controls lacking the primary or secondary antibodies were also included. Fluorescence was viewed with an Olympus BX50 fluorescence microscope equipped with a Sony 3 CCD camera. Hoechst-stained nuclei were visualized under UV light and the cleaved PARP positive cells were visualized using an excitation filter of 530-550 nm and emission filter > 570 nm. The images were captured using Northern Exposure (Empix Imaging Inc., Mississauga, Ontario, Canada) software.

M 2.10 Statistical Analysis

Data are expressed as mean $\pm$ SEM of 3 independent experiments. Data were analyzed using one-way analysis of variance (ANOVA) followed by *post-hoc* Dunnett's multiple comparison test to identify which treatment condition differed statistically from control or by *post-hoc* Tukey's multiple comparison test to identify differences between treatment concentrations for different days. A value of $p < 0.05$ was considered statistically significant. A $p < 0.01$ or 0.001 was

considered highly statistically significant, respectively. Statistical tests were performed using GraphPad Prism version 3.02 software package.

3.0 Results

R 3.1 Time Course of Glutathione Depletion Induced by BSO in NT2 cells

GSH is a tripeptide that is synthesized in the cytosol in a two-step reaction from glutamate, cysteine and glycine by the consecutive action of γ -glutamylcysteine synthetase (γ -GCS) and GSH synthetase (GS). It is considered to be an important biomolecule in the cell's defense mechanism against free radicals and a decrease in the level of GSH has been shown by others to lead to cell death (Nakamura *et al.*, 2000; Wüllner *et al.*, 1999; Merad-Boudia *et al.*, 1998; Li *et al.*, 1997; Froissard *et al.*, 1997; Grasbon-Frodl *et al.*, 1996). To elucidate the role of GSH as a free radical scavenger and antioxidant in NT2 cells, this tripeptide was selectively depleted from the cells by inhibiting its *de novo* synthesis using BSO, an inhibitor of γ -GCS.

In all NT2 cell types examined for this thesis, BSO treatment led to a progressive decrease in total intracellular GSH content in a time- and dose-dependent manner. The GSH level in untreated NT2/D1 was 10.2 ± 0.7 pmol/ μ g protein (**Figure 3.1**). Upon treatment with BSO for one day, the level of GSH fell to 3.31 ± 0.4 pmol/ μ g protein (depletion of 66.4 %) for 50 μ M BSO ($p < 0.001$), 2.52 ± 0.2 pmol/ μ g protein (depletion of 74.4 %) with 100 μ M BSO ($p < 0.001$) and 2.1 ± 0.4 pmol/ μ g protein (depletion of 78.7 %) for 250 μ M BSO ($p < 0.001$). After three days of BSO exposure, there was less than 10% of the initial GSH content remaining with all concentrations used (0.72 ± 0.04 pmol/ μ g protein, 0.67 ± 0.06 pmol/ μ g protein, and 0.5 ± 0.07 pmol/ μ g protein with 50 μ M, 100 μ M and 250 μ M BSO respectively, $p < 0.001$ for all concentrations vs

Figure 3.1 Depletion of total cellular GSH levels in NT2/D1 following treatment with varying concentrations of BSO over a time period of 3 days.

Glutathione levels were measured by the *o*-phthaldehyde (OPT) method as previously described in Section 2.4 of the Materials and Methods. Both a concentration- and time-dependent decrease in GSH was elicited by BSO. One day of treatment led to a decrease of 66.4-78.7 %, while a treatment for 3 days led to a decrease of 90-93.1% relative to initial untreated control GSH levels of 10.2 ± 0.7 pmol/ μ g protein.

Data are expressed as percentage of initial untreated control values and represent the means $\pm$ SD of two independent experiments, each performed in duplicate. Statistically significant: Between doses: $p < 0.001$ for all concentrations for day 1, 2 and 3 (vs control); Between days: $p < 0.05$ for day 2 vs 3 at 100 μ M and 250 μ M and day 1 vs 2 for 50 μ M.

control). There was no significant difference between day 1 and 2 for all concentrations except for 50 μM ($p < 0.05$), however there was a significant difference between day 2 and 3 for 50 μM ($p < 0.001$) and for 100 μM and 250 μM ($p < 0.05$).

The GSH level in untreated NT2-N/A, where neurons were collected and analyzed separately from astrocytes, was 2.69 ± 0.08 pmol/ μg protein in NT2/N (**Figure 3.2 A**) and 18.9 ± 0.6 pmol/ μg protein in NT2/A (**Figure 3.2 B**). In the NT2/N isolated from NT2-N/A, treatment with 50 μM , 100 μM and 250 μM BSO for 24 hours led to a decrease in GSH content of 78.9 % (1.22 ± 0.1 pmol/ μg protein, $p < 0.05$), 88.9% (0.7 ± 0.01 pmol/ μg protein, $p < 0.05$) and 94.9% (0.35 ± 0.03 pmol/ μg protein, $p < 0.001$), respectively. GSH was completely depleted for all concentrations used following three days of BSO exposure. There was a significant difference between day 1 and 2 for 50 μM and 100 μM ($p < 0.01$) and between day 2 and 3 for 250 μM ($p < 0.001$). In the NT2/A collected from NT2-N/A, exposure to BSO for one day led to a decrease in GSH levels of 75.3 % to a value 4.39 ± 0.5 pmol/ μg protein with 50 μM BSO, of 89.9 % to a value of 1.97 ± 0.2 pmol/ μg protein with 100 μM BSO and of 90.2 % to a value of 1.75 ± 0.2 pmol/ μg protein with 250 μM BSO ($p < 0.001$ for all concentrations vs control). After three days of BSO exposure, there was less than 10% of the initial GSH content remaining with all concentrations tested. Only 6.7 % (1.09 ± 0.3 pmol/ μg protein) remained with 50 μM , 4 % (0.77 ± 0.2 pmol/ μg protein) with 100 μM and 2.6 % (0.5 ± 0.1 pmol/ μg protein) 250 μM BSO ($p < 0.001$ for all concentrations vs control). There was a

Figure 3.2 Depletion of total cellular GSH levels in NT2-N/A following treatment with varying concentrations of BSO over a time period of 3 days.

Glutathione levels were measured by the *o*-phthalaldehyde (OPT) method as previously described in Section 2.4 of the Materials and Methods. NT2/N were analyzed separately from NT2/A. BSO elicited both a concentration- and time-dependent decrease in GSH in both cell types. **(A) Depletion of GSH in NT2/N collected from mixed NT2-N/A.** GSH levels decreased to 56.0-87.0 % after one day of treatment while 3 days led to complete depletion. The initial untreated control GSH level was 2.7 ± 0.08 pmol/ μ g protein. Statistically significant: Between doses: $p < 0.05$ for 50 and 100 μ M for day 1, $p < 0.001$ for 250 μ M for day 1 and all concentration on day 2 and 3 (all vs control); Between days: $p < 0.01$ for day 1 vs day 2 at 50 μ M, 100 μ M ; $p < 0.001$ day 2 vs day 3 at 250 μ M.

(B) Depletion of GSH in NT2/A collected from mixed NT2-N/A. Levels of GSH following 1 day of BSO treatment fell to 75.3-90.2 % and to 94.3-97.4 % of the original level of GSH following 3 days of treatment. The initial untreated control GSH level was 18.9 ± 0.6 pmol/ μ g protein in NT2/A. The extent of depletion was greater in NT2/A than in NT2/N. Statistically significant: Between doses: $p < 0.001$ for all BSO concentrations vs control for day 1, 2 and 3. Between days: $p < 0.05$ for day 1 vs day 2 at 100 μ M and 250 μ M; $p < 0.01$ for day 1 vs day 2 at 50 μ M. Data are expressed as percentage of initial control values and represent the means $\pm$ SD of two independent experiments, each carried out in duplicate.

A **B**

significant difference between day 1 and 2 for 50 μM ($p < 0.01$) and for 100 μM and 250 μM ($p < 0.05$).

Untreated pure NT2/N were found to have an initial level of GSH of 1.42 ± 0.21 pmol/ μg protein and GSH depletion occurred very quickly in these cells. (Figure 3.3 A). After one day of treatment, a decrease of 53.2 % (0.66 ± 0.01 pmol/ μg protein) was measured with 50 μM BSO and 74.4 % (0.36 ± 0.05 pmol/ μg protein) with 100 μM BSO, while 250 μM BSO led to 100% depletion ($p < 0.001$ for all concentrations vs control). All concentrations of BSO led to complete depletion after only two days of treatment. There was a significant difference between day 1 and 2 for 50 μM ($p < 0.01$) and 100 μM ($p < 0.05$).

The initial GSH content in untreated pure NT2/A was determined to be 15.0 ± 0.67 pmol/ μg protein (Figure 3.3 B). A decrease of 64 % (5.03 ± 0.8 pmol/ μg protein) was observed following one day of treatment with 50 μM BSO, while a decrease of 78.6 % (2.99 ± 0.6 pmol/ μg protein) was found with 100 μM BSO and of 90.4 % (1.35 ± 0.3 pmol/ μg protein) with 250 μM BSO ($p < 0.001$ for all concentrations vs control). After three days of GSH depletion by BSO, less than 5 % of the GSH content remained with only 6.5 % (1.06 ± 0.1 pmol/ μg protein), 6.1 % (1.0 ± 0.09 pmol/ μg protein) and 5.2 % (0.8 ± 0.1 pmol/ μg protein) remaining for treatment with 50 μM , 100 μM , and 250 μM BSO respectively ($p < 0.001$ for all concentrations vs control). There was a significant difference between day 1 and 2 for 50 μM ($p < 0.01$) and 100 μM ($p < 0.05$).

Figure 3.3 Depletion of total cellular GSH levels in pure NT2/N (A) and pure NT2/A (B) following treatment with varying concentrations of BSO over a time period of 3 days.

Glutathione levels were measured by the *o*-phthaldehyde (OPT) method as previously described in Section 2.4 of the Materials and Methods. Both a concentration- and time-dependent decrease in GSH was induced by BSO. Depletion of GSH was much more rapid in NT2/N (A) than in NT2/A (B).

(A) GSH depletion in pure NT2/N. There was a decrease in the level of GSH of 53.5-100% following 1 day of treatment with BSO and complete depletion was observed for all concentrations of BSO after only two days. The initial untreated control GSH level was 1.32 ± 0.1 pmol/ μ g protein. Statistically significant: Between doses: $p < 0.001$ for all BSO concentrations vs control for day 1 and 2. Between days: $p < 0.05$ for day 1 vs day 2 (100 μ M); $p < 0.01$ for day 1 vs day 2 (50 μ M).

(B) GSH depletion in pure NT2/A. Treatment for 1 day with BSO lead to a decrease in GSH levels of 64.6-90.5 % while a 93.4-95.1 % decrease was measured following 3 days of treatment. The initial untreated control GSH level was 15.0 ± 0.7 pmol/ μ g protein. Statistically significant: Between doses: $p < 0.001$ for all BSO concentrations vs control for day 1, 2 and 3. Between days: $p < 0.05$ for day 1 vs day 2 at 100 μ M; $p < 0.01$ for day 1 vs day 2 at 50 μ M.

Data are expressed as percentage of initial untreated control values and represent the means $\pm$ SD of two independent experiments, each carried out in duplicate.

R 3.2 Effect of GSH Depletion by BSO on Cell Viability in NT2/D1

The effects of BSO on cell viability in the NT2/D1 were examined by cell counts using trypan blue dye exclusion and the fluorometric CFDA assay with corresponding microscopic cell counts. The trypan blue dye exclusion results from NT2/D1 are shown in **Figure 3.4**. GSH depletion induced by BSO resulted in a time- and dose-dependent reduction in cell viability in the NT2/D1. Cell death in untreated NT2/D1 was insignificant at all time points ($p > 0.05$) and never fell below 95 %. For example, on day 1 of the experiment, cell viability was determined to be 98.2 ± 3.1 while on day 5, viability was measured to be 97.7 ± 7.1 %. There was virtually no effect on cell viability on days 1, 2 or 3 of BSO exposure regardless of concentration, except for 500 μM BSO ($p < 0.05$ vs control for all days). Following 4 days of exposure, cell viability was not drastically reduced for 25 μM ($p > 0.05$), 50 μM and 100 μM BSO ($p < 0.05$), however, it was significantly reduced to 54.4 ± 2.0 % and 50.3 ± 7.7 % with 250 μM ($p < 0.001$) and 500 μM BSO ($p < 0.001$), respectively. By the fifth day, the decrease in cell viability became significant for all concentrations ($p < 0.001$ vs control), excluding the lowest concentration of 25 μM BSO ($p > 0.05$ vs control). The percent viability was reduced to 60.1 ± 7.6 % at 50 μM BSO, 50.4 ± 4.1 % at 100 μM BSO, 31.6 ± 5.1 % at 250 μM BSO and 12.7 ± 4.4 % at 500 μM BSO. The loss in cell viability on day 5 was significantly greater than on day 4 for all concentrations of BSO tested ($p < 0.001$)

Another method to measure cell viability is the CFDA fluorescence assay. CFDA (5-carboxyfluorescein diacetate, acetoxymethyl ester) is a non-fluorescent

Figure 3.4 Quantification of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in NT2/D1 over 5 days.

Cell viability, measured by trypan blue exclusion, in response to BSO exposure was concentration and time dependent. In NT2/D1, the percent viable cells did not decrease significantly until 4 days of treatment while 5 days of exposure to BSO led to a percent viability ranging from 12.7 ± 4.4 % at the highest concentration of 500 μ M BSO to 80.0 ± 3.7 % at a concentration as low as 25 μ M BSO.

Cell counts were carried out at each 24-hour time point. Adherent and non-adherent cells were collected, centrifuged at 1000 rpm for 5 minutes and resuspended in 500 μ L 1x PBS. Two 50 μ L aliquots were removed from each sample and mixed 1:1 with trypan blue just prior to counting. The number of viable (transparent) and dead (blue) cells was counted. The data were averaged and the percent viability was determined for each sample and normalized to the percent viability in untreated control samples for the same time point. Data represent the means $\pm$ SEM of three independent experiments.

Statistically significant - Between doses: $p < 0.05$ for 500 μ M on day 1, 2 and 3, and for 50 μ M and 100 μ M on day 4; $p < 0.001$ for 250 μ M and 500 μ M on day 4 and 50 μ M, 100 μ M, 250 μ M and 500 μ M on day 5 (all vs control). Between days: $p < 0.001$ for day 4 vs 5 for all concentrations.

dye that is taken up by cells where it is cleaved by non-specific esterases in viable cells to generate the fluorescent product, 5-(6) carboxyfluorescein. Cells are incubated with the dye and fluorescence is read on a plate reader to obtain arbitrary fluorescence values. Thus, lower arbitrary fluorescence values represent a lower number of viable cells. The results from this assay in NT2/D1 are presented in **figure 3.5 (A)**. As observed with the trypan blue exclusion assay, there was both a dose- and time-dependent decrease in cell viability and the numbers were also quite similar. Again, loss in cell viability was not highly significant during the first three days of treatment, with the exception of the highest concentration of 250 μM which led to a reduction in cell viability to $48.3 \pm 11.7 \%$ ($p < 0.001$). After 4 days of BSO exposure, 100 μM BSO led to a decrease in percent viability of 69.2 ± 4.3 ($p < 0.05$) and 250 μM led to a further significant decrease to $35.5 \pm 13.2 \%$ ($p < 0.001$). By the fifth day, cell viability was further decreased to $71.4 \pm 4.6 \%$, $40.7 \pm 10.9 \%$ and $13.8 \pm 2.0 \%$ with 50 μM , 100 μM and 250 μM BSO respectively. The loss in cell viability was not highly significantly different between day 2 and 3 for all concentrations, however there was a significant difference for between day 3 and 4 ($p < 0.05$ for 50 μM and 100 μM BSO, $p < 0.001$ for 250 μM BSO), as well as between day 4 and 5 ($p < 0.001$ for all concentrations).

In addition to obtaining fluorescence values, the CFDA assay can also be used to assess cell viability microscopically, at the single cell level. If grown on glass coverslips, the same cells can be fixed, mounted onto microscope slides and examined using fluorescence microscopy. In this way, viable and non-

Figure 3.5 Assessment of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in NT2/D1 cells using (A) 5-carboxy-fluorescein diacetate acetoxymethyl ester (CFDA) fluorometric assay and (B) cell counts based on morphological analysis of nuclei stained with Hoechst 33258.

Cell viability was both dose- and time-dependent for both methods and results were almost identical. Following three days of exposure, the percent viability in cells treated with 50 μ M, 100 μ M and 250 μ M BSO was reduced to 92.8 ± 3.1 %, 82.0 ± 2.0 % and 48.3 ± 11.7 %, respectively. Cell death increased at all concentrations of BSO following 5 days of exposure when the percent viability was reduced to 71.4 ± 4.6 % for 50 μ M, 40.7 ± 10.9 % for 100 μ M, and 13.8 ± 2.0 for 250 μ M BSO.

Cells were grown in 12-well plates and treated with the appropriate concentration of BSO. At each 24-hour time point over 5 days, cells were incubated with CFDA prepared in EBSS (5 μ M) at 37°C for 30 minutes in the dark. Fluorescence was read using a CytoFluor™ 2300/2500 fluorometric plate reader. For each time point, the fluorescence data was averaged and expressed as percent control fluorescence for the CFDA assay results and percent viability relative to the control cells for the cell count data. The data represent means $\pm$ SEM of three independent experiments.

Statistically significant: Between doses: $p < 0.05$ for 100 μ M for day 2, 3 and 4; $p < 0.001$ for 100 μ M on day 5, and for 250 μ M on day 2, 3, 4 and 5 (all vs control). Between days: $p < 0.05$ for day 3 vs day 4 at 50 μ M and 100 μ M; $p < 0.001$ for day 3 vs day 4 at 250 μ M and for day 4 vs 5 at all concentrations.

viable cells can be counted based on the presence or absence of green fluorescence and when used in combination with the nuclear stain, Hoechst 33258, nuclear morphology can also be used as a criterion for determining cell viability. The cell count data from NT2/D1 is presented in the lower graph (B) of **figure 3.5**. As is evident, the results are virtually identical to those obtained from the fluorometric assay, with the numbers being within 5-10 % of each other.

R 3.3 Effect of GSH Depletion by BSO on Cell Viability in NT2-N/A

The effects of BSO on cell viability in NT2-N/A were examined using the fluorometric CFDA assay and corresponding microscopic cell counts. Trypan blue exclusion was not used to determine cell viability because neuronal cells tend to grow in aggregates that require vigorous trituration to generate single cell suspensions, which may damage the cells and result in over-estimation of non-viable cells. The fluorometric data are summarized in **figure 3.6 (A)**. Cell death in untreated NT2-N/A was insignificant at all time points ($> 95 \%$, $p > 0.05$). For example, on day 1 of the experiment, cell viability was determined to be 96.8 ± 1.1 while on day 5, viability was measured to be $96.4 \pm 0.9 \%$. There was a progressive decrease in fluorescence, hence percent viability, with increasing time and concentration of BSO. The time course was similar to that observed in the NT2/D1, with no highly significant cell death occurring until the fourth day of exposure where the percent viability was $86.7 \pm 2.7 \%$ with $50 \mu\text{M}$ BSO ($p < 0.05$), $67.9 \pm 7.4 \%$ with $100 \mu\text{M}$ BSO ($p < 0.05$) and $44.5 \pm 10.9 \%$

Figure 3.6(A) Assessment of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in NT2-N/A using the CFDA assay.

Cell viability was dependent on both BSO exposure time and concentration by both methods and results were almost identical for both assays. Mixed NT2-N/A were slightly more resistant to GSH depletion relative to NT2/D1. The percent viability in NT2/N treated with 50 μ M, 100 μ M and 250 μ M BSO was reduced to 86.7 ± 2.7 , 67.9 ± 7.4 % and 44.5 ± 10.9 %, respectively following 4 days of treatment and after 5 days, it was further reduced to 66.4 ± 9.3 % for 50 μ M, 45.6 ± 9.5 % for 100 μ M, and 24.7 ± 4.9 for 250 μ M BSO. Statistically significant: Between doses: $p < 0.05$ for 100 μ M on day 3 and 4, and 50 μ M on day 5; $p < 0.01$ for 250 μ M on day 3, 4 and 5 and 100 μ M on day 5 (all vs control). Between days: $p < 0.05$ for day 3 vs day 4 at 50 μ M; $p < 0.001$ for day 3 vs day 4 at 100 μ M and 250 μ M and for day 4 vs day 5 at all concentrations on day 5.

Cells were grown in 12-well plates and treated with the appropriate concentration of BSO. At each 24-hour time point over 5 days, the cells were incubated with CFDA prepared in EBSS (5 μ M) at 37°C for 30 minutes in the dark. Fluorescence was read using a CytoFluor™ 2300/2500 fluorometric plate reader. Fluorescence and cell count data were averaged for each time point. The data represent means $\pm$ SEM of four independent experiments.

A

with 250 μM BSO ($p < 0.001$). By the fifth day of treatment, there was a significant decrease in viability with all three concentrations tested. With a BSO concentration of 50 μM BSO, 66.4 ± 9.3 % of the cells were still viable, while 100 μM BSO led to only 45.6 ± 9.5 % viable cells and with 250 μM BSO only a 24.7 ± 4.9 viability was observed ($p < 0.001$ for all concentrations). The difference in cell death was not highly significant between day 2 and 3 for all concentrations, however it was significant between day 3 and 4 ($p < 0.05$ for 50 μM , and $p < 0.001$ for 100 μM and 250 μM BSO), as well as between day 4 and 5 ($p < 0.001$ for all concentrations).

The data from cell counts are presented in **figure 3.6 (B)** (graphs **(A)** and **(B)**). Results were comparable to those from the CFDA fluorometric data, however, upon observation under the microscope, it was noticed that most of the dead or dying cells were NT2/N. Because of this observation, NT2/N and NT2/A were counted and plotted separately with NT2/N counts shown in **(A)** and NT2/A in **(B)**. Neuronal viability was significantly affected after four days of GSH depletion by BSO at concentrations of 100 μM ($p < 0.05$) and 250 μM ($p < 0.001$) resulting in 68.7 ± 7.6 % and 42.7 ± 6.9 % viable NT2/N, respectively. With 50 μM BSO, viability was still 86.8 ± 3.1 % ($p < 0.05$). Neuronal viability was further reduced to 66.4 ± 2.9 %, 43.6 ± 8.2 % and 18.6 ± 7.3 % following five days of GSH depletion with 50, 100 and 250 μM BSO respectively ($p < 0.001$). The difference in the loss of cell viability was not highly significant between day 2 and 3 for all concentrations except for 250 μM BSO ($p < 0.001$), however it was significant for between day 3 and 4 ($p < 0.05$

Figure 3.6 (B) Assessment of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in NT2-N/A using cell counts based on morphological analysis of nuclei stained with Hoechst 33258 in NT2/N from NT2-N/A (A) and in NT2/A from NT2-N/A stained with (B).

(A) Microscopic observation showed that mainly the NT2/N were affected having a percent viability of 85.2 ± 3.1 with 50 μM BSO, of 64.4 ± 7.9 with 100 μM BSO and of 29.7 ± 7.5 with 250 μM BSO following four days of exposure. An additional fifth day of treatment further reduced NT2/N viability to 63.3 ± 1.0 %, 41.8 ± 0.9 % and 13.6 ± 4.5 % with 50 μM , 100 μM and 250 μM BSO, respectively. Statistically significant: Between doses: $p < 0.05$ for 100 μM on day 4; $p < 0.001$ for 250 μM on day 3, 4 and 5, for 50 μM and 100 μM on day 5 (all vs control). Between days: $p < 0.001$ for day 2 vs day 3 at 250 μM , for day 3 vs day 4 at 100 μM and 250 μM and for day 4 vs day 5 at all concentrations; $p < 0.05$ for day 3 vs day 4 at 50 μM . (B) Loss of cell viability in NT2/A from NT2-N/A was minimal after 5 days of exposure to 50 μM , 100 μM and 250 μM BSO with a percent viability of 89.5 ± 1.4 , 84.7 ± 1.5 and 75.3 ± 0.9 , respectively. Statistically significant: Between doses: $p < 0.001$ for 250 μM on day 4, and all concentrations on day 5 (all vs control). Between days: $p < 0.001$ for day 4 and 5 at 250 μM ; $p < 0.05$ for day 4 vs day 5 at 100 μM and 250 μM . Cells were grown in 12-well plates and treated with the appropriate concentration of BSO. At each 24-hour time point over 5 days, the cells were incubated with CFDA prepared in EBSS (5 μM) at 37°C for 30 minutes in the dark. Fluorescence was read using a CytoFluor™ 2300/2500 fluorometric plate reader. Fluorescence and cell count data were averaged for each time point. The data represent means $\pm$ SEM of four independent experiments.

for 50 μM and $p < 0.001$ for 100 μM and 250 μM BSO), as well as between day 4 and 5 ($p < 0.001$ for all concentrations). On the other hand, the effect of GSH depletion on cell viability was minimal in NT2/A grown as mixed NT2-N/A. By the fifth day of depletion, the percent survival was still 91.9 ± 3.9 ($p < 0.05$), 85.9 ± 4.1 ($p < 0.001$) and 73.9 ± 4.7 ($p < 0.001$) using 50 μM , 100 μM and 250 μM BSO respectively. The difference between day 4 and 5 was significant for 50 μM ($p < 0.05$) and 100 μM ($p < 0.05$) and highly significant for 250 μM ($p < 0.001$).

R 3.4 Effect of GSH Depletion by BSO on Cell Viability in pure NT2/N

The effects of BSO on cell viability in NT2/N were examined using the fluorometric CFDA assay and corresponding microscopic cell counts. Trypan blue exclusion was not used to determine cell viability for the same reason as mentioned in the previous section (R 3.3). Pure NT2/N were highly susceptible to GSH depletion by BSO. The CFDA fluorometric data are presented in **figure 3.7 (A)**. Cell death in untreated NT2/N was insignificant at all time points ($> 95\%$, $p > 0.05$). For example, on day 1 of the experiment, cell viability was determined to be 97.4 ± 2.1 while on day 5, viability was measured to be $97.0 \pm 1.9\%$. Cell death was significant after only one day of exposure where 50 μM BSO led to a decrease in percent viability to $76.9 \pm 1.3\%$ ($p < 0.05$), while only $48.0 \pm 10.2\%$ were found to be viable with 100 μM BSO ($p < 0.001$) and $27.4 \pm 8.2\%$ with 250 μM BSO ($p < 0.001$). The percent viability was further reduced to $21.2 \pm 1.8\%$, $18.5 \pm 2.5\%$ and $11.7 \pm 1.7\%$ following only two days of exposure to 50, 100 and 250 μM BSO, respectively ($p < 0.001$ for all

Figure 3.7 Assessment of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in pure NT2/N using (A) CFDA and (B) cell counts based on morphological analysis of nuclei stained with Hoechst 33258.

Cell viability was dependent on both BSO exposure time and concentration by both methods and results were almost identical for both assays. Pure NT2/N were the most sensitive to GSH depletion by BSO following only 24 hours of exposure when the percent viability was reduced to 76.9 ± 1.3 , 48.0 ± 10.2 and 27.4 ± 8.2 in cells treated with 50 μM , 100 μM and 250 μM BSO, respectively. Cell viability was further reduced to 21.2 ± 1.8 % for 50 μM , 18.5 ± 2.5 % for 100 μM and 11.7 ± 1.7 % for 250 μM BSO.

Cells were grown in 12-well plates and treated with the appropriate concentration of BSO. At each 24-hour time point for 5 days, the cells were incubated with CFDA prepared in EBSS (5 μM) at 37°C for 30 minutes in the dark. Fluorescence was read using a CytoFluor™ 2300/2500 fluorometric plate reader. The fluorescence and cell count data were averaged for each time point. The data represent means $\pm$ SEM of three independent experiments.

Statistically significant: Between doses: $p < 0.001$ for 100 μM and 250 μM for day 1 and 2, and 50 μM for day 2 (all vs control). Between days: $p < 0.001$ for treatment time vs day 1 at 100 μM and 250 μM and for day 1 vs day 2 for all concentrations; $p < 0.01$ for treatment time vs day 1 at 50 μM .

A **B**

concentrations vs control). The difference in cell death was highly significant between the initial treatment time and day 1 for 50 μM BSO ($p < 0.01$), 100 μM ($p < 0.001$) and 250 μM BSO ($p < 0.001$), as well as between day 1 and 2 for all concentrations ($p < 0.001$). The data from cell counts are presented in the lower graph (**figure 3.7 (B)**) and were comparable to those found with the fluorometric assay with high susceptibility to GSH depletion after only one day of treatment for all concentrations tested.

R 3.5 Effect of GSH Depletion by BSO on Cell Viability in Jurkat Cells

For comparison purposes, the effect of GSH depletion by BSO on cell viability was also examined in the Jurkat lymphocyte cell lines using the trypan blue exclusion assay (**Figure 3.8**). Cell death in untreated Jurkat cells was minimal throughout the four-day time course of the experiment and never greater than 5 %. For example, on day one of the experiment, cell viability was determined to be 98.2 ± 0.1 % and on the last day (day 4), it was 96.9 ± 0.4 %. A time- and dose-dependent progressive decrease in cell viability was also observed in these cells. Jurkat cells were more sensitive to GSH depletion relative to NT2/D1 and NT2-N/A but less sensitive than pure NT2/N. Significant cell death was observed after only two days of treatment with BSO with a percent viability of 78.3 ± 1.3 ($p < 0.05$), 46.6 ± 2.2 ($p < 0.001$) and 19.5 ± 3.6 ($p < 0.001$) with 50 μM , 100 μM and 250 μM BSO, respectively. After three days of treatment, viability was 54.9 ± 5.2 % with 50 μM BSO, 14.2 ± 2.3 % with 100 μM BSO and negligible with 250 μM BSO with only 2.9 ± 0.1 %

Figure 3.8 Trypan blue exclusion was used for the quantification of cell viability in response to GSH depletion following exposure to varying concentrations of BSO in Jurkat cells over 4 days.

Cell viability in response to BSO exposure was concentration- and time-dependent. The percent viability in Jurkat cells was significantly reduced following only 2 days of treatment and by the fourth of treatment, the percent viability was reduced to 34.4 ± 1.8 % at 50 μ M BSO, 4.5 ± 0.6 % at 100 μ M BSO and only 1.4 ± 0.3 % at 250 μ M BSO. Cell counts were carried out at each 24-hour time point. Cells were collected, centrifuged at 1000 rpm for 5 minutes and resuspended in 500 μ L 1x PBS. Two 50 μ L aliquots were removed from each sample and mixed 1:1 with trypan blue just prior to counting. The number of live (transparent) and dead (blue) cells was counted. The percent viability was determined for each sample and normalized to the percent of live cells in untreated control samples for the same time point. Data represent the means $\pm$ SEM of three independent experiments.

Statistically significant: Between doses: $p < 0.001$ for 250 μ M on day 2, 3 and 4, and for 50 μ M and 100 μ M on day 3 and 4 (all vs control). Between days: $p < 0.001$ for day 1 vs day 2 at 250 μ M and for day 2 vs day 3 and day 3 vs day 4 at all concentrations; $p < 0.05$ for day 1 vs day 2 at 50 μ M and 100 μ M.

viable cells ($p < 0.001$ for all concentrations vs control). By the fourth day of BSO exposure, only 34.4 ± 1.8 % of the cells were viable in the presence of 50 μ M BSO and virtually none in the presence of 100 and 250 μ M BSO with only 4.5 ± 0.6 % and 1.7 ± 0.2 %, respectively ($p < 0.001$ for all concentrations vs control). The loss in cell viability was significant between day 1 and 2 for 50 μ M BSO ($p < 0.05$), 100 μ M ($p < 0.05$) and 250 μ M BSO ($p < 0.001$), and highly significant between day 2 and 3, as well as day 3 and 4 for all concentrations ($p < 0.001$). It was also observed that BSO had a drastic negative affect on cell proliferation in Jurkat cells, which was not observed in the NT2/D1. The untreated cells proliferated quickly, however, the cell numbers in the treated samples did not increase from the original seeded numbers and even decreased as exposure time increased. On the other hand, NT2/D1 cells kept proliferating and became quite confluent until around the fourth day of treatment. Because these are suspension cells, the CFDA assay was not carried out in this cell type.

R 3.6 Morphological Changes Induced by GSH Depletion with BSO in NT2/D1, NT2-N/A, NT2/N and Jurkat Cells

The effects of BSO on cell morphology were studied using fluorescence microscopy to examine the same cells that were used for the CFDA fluorometric assay and/or the trypan blue dye exclusion assay. Immediately following the assay, cells were fixed with 1x formalin and mounted onto microscope slides using DAKO mounting media that was spiked with Hoechst 33258. In this way, the assay could be used to obtain cell viability data at the single cell level and to examine nuclear morphology. Hoechst 33258 is a nuclear stain that intercalates

between the bases of the DNA and can be visualized under UV light. Cells with an altered nuclear morphology can be checked for viability by looking at CFDA fluorescence labelling. **Figure 3.9** is a representative figure showing the morphological changes in NT2/D1 following incubation with 250 μ M BSO for 3 days. The top three panels show untreated control cells. Hoechst's-stained nuclei (**A**) and phase-contrast micrographs (**A''**) indicated the presence of a healthy intact monolayer of cells with no shrunken, condensed or fragmented nuclei and all cells were labelled with CFDA (**A'**). Three days of exposure to 250 μ M BSO resulted in the appearance of a number of morphologically distinct shrunken nuclei with brightly stained chromatin that appeared to be condensed along the margins of the nuclear membrane (**B**). Most of the cells with this distinct altered morphology were not labelled with CFDA (**B'**) indicating loss of viability and phase-contrast photomicrographs showed that the same cells were shrunken, rounded and phase-bright (**B''**). Consistent with the fluorometric assay (**figure 3.5**), the number of morphologically distinct nuclei increased while the intensity of CFDA labelling was reduced with increasing concentration of BSO and time (personal observation, not shown).

Figure 3.10 is a representative figure showing the effects of GSH depletion by BSO on nuclear morphology in NT2-N/A. Panels **A**, **A'** and **A''** show the untreated control samples. All three panels show that healthy and intact smaller neuronal cells and aggregates were interconnected by an intact neurite network and attached to a healthy underlying monolayer of large, flat astrocytes. The number of shrunken or condensed Hoechst-stained nuclei was minimal (**A**) and

Figure 3.9 BSO-induced changes in nuclear morphology in NT2/D1 stained with Hoechst 33258 and identification of CFDA-labelled NT2/D1.

(A), (A') and (A'') are untreated control cells while (B), (B') and (B'') are cells following 3 days of exposure to 250 μ M BSO. (A) and (B) represent Hoechst stained nuclei, (A') and (B') represent CFDA-labelled cells while (A'') and (B'') represent phase contrast photomicrographs.

(B), (B') and (B'') Labelling by CFDA was more intense in the nuclei and very faint in the cytoplasm. Note the number of condensed, brightly stained areas along the margins of the nuclear membrane in (B), which generally correspond to non-CFDA-labelled areas in (B'). There are no such cells in the untreated control panels (A), (A') and (A''). Images are representatives from three independent experiments. Each panel is magnified 400X. The insets are enlargements of the areas indicated by the white arrow (Magnification = 640X)

Undifferentiated NT2/D1 were grown as monolayers on glass coverslips and treated with the appropriate concentration of BSO. At each 24-hour time point, the cells were incubated with 5 μ M CFDA (in EBSS) for 30 minutes in the dark at 37°C, read on a plate reader, immediately fixed with 1x formalin and stored overnight in the dark at 4°C. The coverslips were then mounted onto slides using DAKO mounting media spiked with 5 μ g/mL Hoechst 33258. Fluorescent microscopy was used to examine Hoechst-stained nuclei under UV light and CFDA-labelled cells using an excitation filter of 470–490 nm and emission filter of 515–550 nm.

Figure 3.10 BSO-induced changes in nuclear morphology in NT2-N/A stained with Hoechst 33258 and identification of CFDA-labelled NT2-N/A.

(A), (A') and (A'') are untreated cells, (B), (B') and (B'') are cells following 4 days of exposure to 100 μ M BSO, while (C), (C') and (C'') are cells treated with 50 μ M BSO for 5 days. (A), (B) and (C) represent Hoechst stained nuclei, (A'), (B') and (C') represent CFDA-labelled cells while (A''), (B'') and (C'') represent phase contrast photomicrographs. (A), (A') and (A'') the neurite network is intact and nuclear morphology is normal and all cells are labelled with CFDA. (B), (B') and (B'') some nuclei are condensed and brightly stained (B) and correspond to non-CFDA-labelled cells (B'). Neurites are slightly disrupted and neuronal loss is evident while some NT2/A appear rounded and shrunken (arrowhead) (B' and B''). (C), (C') and (C'') show that most NT2/N have been lost. Some NT2/A appear shrunken and rounded but are still labelled by CFDA (arrowheads). Neurites are completely disintegrated. The yellow arrow indicates the remnant of a neurite. Each panel is magnified 200 X. Insets are enlargements of the areas indicated by the white arrow (Magnification = 640X).

NT2-N/A were grown on glass coverslips and treated with the appropriate concentration of BSO. At each 24-hour time point, the cells were incubated with 5 μ M CFDA (in EBSS) for 30 minutes in the dark at 37°C and immediately fixed with 1x formalin overnight at 4°C. The coverslips were then mounted onto slides using DAKO mounting media spiked with 5 μ g/mL Hoechst 33258. Fluorescent microscopy was used to examine Hoechst-stained nuclei under UV light and CFDA-labelled cells using an excitation filter of 470-490 nm and emission filter of 515-550 nm. Images are representatives from three independent experiments.

all cells were labelled with CFDA (A'). CFDA intensity appeared to be greater in NT2/N and was homogeneous throughout the cell, while in NT2/A, the nuclei appeared more intensely labelled than the cytoplasm indicating greater esterase activity within the nucleus (A'). Treatment with 100 μ M BSO for four days led to the loss of many neurons and the appearance a number of shrunken, condensed and bright Hoechst-stained neuronal nuclei while only a few astrocytes had shrunken nuclei (B). CFDA labelling was reduced or absent in cells with affected nuclei and the neurite network showed signs of disruption (B' and B''). The astrocytes with shrunken nuclei could still hydrolyse the CFDA dye, however, they appeared rounded in the phase contrast micrographs (B''). After the fifth day of BSO exposure, most of the neurons were lost and those that remained were very faintly labelled with CFDA (C') and all neurite extensions were completely disintegrated (C' and C''). The loss of NT2/A was to a lesser extent relative to NT2/N loss, however many had shrunken nuclei that appeared rounded and phase-bright in phase contrast micrographs and those remaining were brightly labelled with CFDA.

The most extreme effects of BSO on nuclear morphology were observed in pure NT2/N and are presented in **figure 3.11**. Panels A, A' and A'' represent the untreated control cells. Nuclei appeared healthy, with no change in size or evidence of chromatin fragmentation (A). All cells were brightly labelled with CFDA (A') and an extensive network of interconnected neurites was evident (A' and A''). Panels B, B' and B'' show that the presence of 50 μ M BSO resulted in shrinkage and condensation of a large proportion of the nuclei (B) with a less

Figure 3.11 BSO-induced changes in nuclear morphology in NT2/N stained with Hoechst 33258 and identification of CFDA-labelled NT2/N.

(A), (A') and (A'') are untreated NT2/N, (B), (B') and (B'') are NT2/N following 1 day of exposure to 100 μ M BSO while (C), (C') and (C'') are NT2/N treated with 50 μ M BSO for 2 days. (A), (B) and (C) represent Hoechst stained nuclei, (A'), (B') and (C') represent CFDA-labelled cells and (A''), (B'') and (C'') represent phase contrast photomicrographs. (A), (A') and (A'') show a nuclear morphology normal, an intact neurite network and bright CFDA-labelled with CFDA cells. (B), (B') and (B'') a number of nuclei appear condensed, shrunken and brightly stained (B). Most NT2/N are labelled by CFDA but with a reduced intensity in both the NT2/N and the neurite network (B'). (C), (C') and (C'') most NT2/N have shrunken, condensed, brightly stained nuclei (C) and CFDA-labelling is minimal and with reduced intensity (C'). (C'') The neurite network is extensively disrupted, many NT2/N have been lost and there is a lot of debris (white arrowhead). Each panel is magnified 200 X. The insets represent a magnification of 800X of the areas indicated by the white arrow.

Pure NT2/N were grown on glass coverslips and treated with the appropriate concentration of BSO. At each 24-hour time point, the cells were incubated with 5 μ M CFDA (in EBSS) for 30 minutes in the dark at 37°C and immediately fixed with 1x formalin overnight at 4°C. The coverslips were then mounted onto slides using DAKO mounting media spiked with 5 μ g/mL Hoechst 33258. Fluorescent microscopy was used to examine Hoechst-stained nuclei under UV light and CFDA-labelled cells using an excitation filter of 470-490 nm and emission filter of 515-550 nm. Images are representatives from three independent experiments.

intense CFDA staining (**B'**) as illustrated by a less visible neuronal network and the loss of viability of many cells (**B'** and **B''**). Exposure to 100 μ M BSO for as little as one day led to drastic changes in the neuronal cells and are shown in panels **C**, **C'** and **C''**. Almost all of the Hoechst-stained nuclei appeared shrunken, condensed and brightly stained, however chromatin fragmentation was not evident (**C**). The majority of the neurons were non-viable as evident by the lack of CFDA labelling, however a select few still had the ability to retain and hydrolyse the dye but the CFDA labelling was quite diffuse and faint in these cells (**C'**). The once extensive interconnection of neurites was completely disintegrated (**C'** and **C''**). After a second day of treatment, almost all neurons were lost with only a few areas having neurons still attached and no CFDA labelling was evident at all three concentrations of BSO tested (personal observation, not shown).

To determine whether these effects of BSO on nuclear morphology were specific to NT2 cells, cell morphology was also examined in GSH-depleted Jurkat cells and a representative figure of Hoechst-stained nuclei is shown in **figure 3.12**. Panel **A** shows untreated control Jurkats, while panel **B**, **C** and **D** show cells treated with 50 μ M, 100 μ M and 250 μ M BSO for two days of exposure, respectively. There were no changes in nuclear morphology after one day of exposure, however, after two days of treatment many cells had an altered nuclear morphology that appeared to be more typical of apoptosis. There were numerous shrunken, condensed and brightly stained nuclei while quite a few nuclei had chromatin fragmented into classical apoptotic bodies at all

Figure 3.12 BSO-induced changes in nuclear morphology in Jurkat cells stained with Hoechst 33258.

(A) represents untreated Jurkat cells, while (B), (C), and (D) represent Jurkats treated with 50, 100 and 250 μ M BSO, respectively. Depletion of GSH by BSO induced typical apoptotic nuclear morphology with chromatin fragmentation into apoptotic bodies as well as several shrunken, condensed and brightly stained nuclei.

Jurkat cells were seeded at a density of 1×10^5 cells per well in 6-well plates and simultaneously treated with varying concentrations of BSO. At each 24-hour time point, the cells were collected and centrifuged at 800 rpm for 5 minutes at room temperature. The pellet was resuspended in 250 μ L of 1x PBS. Two 50 μ L aliquots were removed from each sample for trypan blue exclusion counts. The remaining 150 μ L was fixed with 150 μ L Genofix™, centrifuged onto microscope slides using a Leica cytopsin and allowed to dry for 30 minutes. The area with cells was then covered with a glass coverslip using DAKO mounting medium spiked with 5 μ g/mL Hoechst 33258. Hoechst-stained nuclei were observed under UV light on a fluorescent microscope.

Images are a representative of three independent experiments. Each panel is magnified 200X. The insets show an enlargement of the areas indicated by the white arrow (Magnification = 600X).

concentrations tested. Not only were the nuclear changes different in these cells relative to those in NT2 cells, the time with which the changes became visible was much faster, occurring after only two days in the Jurkat cells. In addition to such changes in morphology, there was also a noticeable decrease in cell number, at both increased exposure time and BSO concentration. Because Jurkats are suspension cells, the CFDA labelling could not be carried out successfully however these observations are consistent with the cell viability data obtained using trypan blue exclusion (**figure 3.8**).

R 3.7 Correlation between GSH Depletion and Loss of Cell Viability in NT2/D1, NT2-N/A and NT2/N

To determine if the loss in cell viability was directly related the degree of GSH depletion, the Spearman rank test was used to analyze the data. This test was chosen because it does not assume a normal Gaussian distribution of the data. These results are presented in **figure 3.13, 3.14 and 3.15**. It should be noted that because cellular GSH levels were measured only for the first three days of depletion, comparisons to cell viability could only be compared using the data obtained up to the third day from the CFDA fluorometric assay, where cell death was not yet significant in most cell types studied. In all NT2 cell types, there was a significant negative correlation between GSH depletion and cell viability where the cell death increased as the level of GSH fell. For NT2/D1, the significance of this correlation was very high with $p < 0.0001$ and $r = 0.9314$. A very significant correlation was found between GSH depletion and cell viability in NT2/N from NT2-N/A ($p < 0.0001$, $r = 0.9611$) and in pure NT2/N

($p = 0.0067$, $r = 0.9063$). No correlation was found in NT2/A from NT2-N/A (p was non-significant (ns) and $r = 0.4718$). The Spearman rank correlation also showed that the loss in cell viability was not highly significant until GSH levels reached a certain critical level. In NT2/D1 (**figure 3.13**), this critical value was found to be near 0.5 pmol GSH/ μ g protein (or 4.9 % of the original level) and this value was reached following three days of depletion for all concentrations tested (refer to **figure 3.1**) where the percent viability was 88.3 ± 0.6 , 82.0 ± 2.0 and 48.3 ± 11.7 with 50, 100 and 250 μ M BSO, respectively (**figure 3.4** and **3.5**). **Figure 3.14** show the correlation results obtained from NT2-N/A. Graph **A** represents the results from the Spearman rank correlation in the NT2/N that were collected and analyzed separately from the NT2-N/A while graph **B** represents the correlation results in NT2/A that were separately collected and analyzed from the NT2-N/A. The isolated NT2/N were found to have a critical value of GSH of less than 0.1 pmol/ μ g protein (or 3.7 % of the original GSH) which was reached somewhere between day 2 and day 3 of BSO treatment. Complete depletion was found on the third day (refer to **figure 3.2**) where the percent viability was still relatively high 91.1 ± 3.4 %, 88.2 ± 3.2 % and 77.6 ± 3.0 % (refer to **figure 3.6 (B)**). Because there was no correlation in NT2/A from NT2-N/A, no critical level was evident since viability was still greater than 95 % at all concentrations of BSO tested despite having less than 5 % of the initial GSH by the third day of treatment. The correlation in NT2/N (**figure 3.15**) was similar to that in NT2/N from NT2-N/A, where the critical GSH value was approximately 0.5 pmol/ μ g protein (27.0 % of the original GSH). However, this

Figure 3.13

Correlation between GSH depletion by BSO and cell viability in NT2/D1 determined using the Spearman rank correlation test.

There was an inverse relationship between GSH depletion for all doses of BSO and cell viability. As the degree of GSH depletion increased, cell viability became further reduced. After 3 days of BSO exposure, cell viability in NT2/D1 began to fall once GSH levels were depleted to less than 0.5 pmol/ μ g protein (approximately 4.5 % GSH remaining) with a percent viability ranging between 48.3 % and 88.3%. A highly significant correlation with a *p* value of < 0.0001 and an *r* value of 0.9314 was determined by the Spearman-rank test.

Figure 3.14

Correlation between GSH depletion by BSO and cell viability in NT2-N/A determined using the Spearman rank correlation test.

(A) Loss of cell viability was highly correlated to GSH depletion by all doses of BSO in NT2/N that were collected and analyzed separately from NT2/A of mixed NT2-N/A. Cell viability only began to fall on the third day of BSO-treatment, once GSH levels fell below 0.1 pmol/μg protein (approximately 3.7 % GSH remaining) and continued to fall as GSH was further depleted. A high level of significance was obtained with a *p* value less than 0.0001 and an *r* value of 0.9611.

(B) There was no correlation found between GSH depletion and cell viability in NT2/A that were separated and analyzed separately from NT2/N from the mixed NT2-N/A. A non-significant *p* value was given with an *r* value of 0.4718.

A

B

Figure 3.15

Correlation between GSH depletion by BSO and cell viability in NT2/N determined using the Spearman rank correlation test.

Cell viability in pure NT2/N was strongly correlated to the level of GSH depletion with all doses of BSO. Once GSH levels reached a value of < 0.35 pmol/ μ g protein (approximately 27% GSH remaining), cell viability was severely reduced, falling to less than 22.0 % viable. A highly significant correlation was found with a p value of 0.0067 and an r value of 0.9063.

critical value was reached and surpassed after only one day of treatment with 100 μ M BSO where it reached a value of 0.36 ± 0.05 pmol/ μ g protein and complete depletion with 250 μ M BSO (**figure 3.3**) with the cell viability reduced to 48.0 ± 10.2 % and 27.4 ± 8.2 %, respectively (**figure 3.7**).

R 3.8 Analysis of DNA Fragmentation by PFGE Following GSH Depletion by BSO in NT2/D1, NT2-N/A, NT2/N and NT2/A

Because the viability experiments demonstrated that simply lowering intracellular GSH levels is sufficient to induce cell death after 3-4 days of BSO treatment in NT2/D1 and NT2-N/A and after only one day in NT2/N, fragmentation of DNA in BSO-treated cells was examined by pulse field gel electrophoresis to attempt to identify the type of cell death involved. **Figure 3.16** shows the 5-day time course of DNA fragmentation in NT2/D1 treated with various concentrations of BSO. For all 5 days, there was no loss of DNA integrity in untreated control cells (lanes 1, 5, 9, 13 & 17), emphasizing that processing of samples did not introduce artifactual breaks in the DNA. No significant DNA degradation was detected over the first three days of treatment at any BSO concentration (lanes 2-4, 6-8 & 10-12). After 4 days in the presence of BSO, some high molecular weight (HMW) degradation became evident at the top half of the gel (lanes 14-16). A clearly resolved fragment of 50-kbp was also detectable. There was also some very faintly staining lower molecular weight (LMW) DNA fragmentation but the oligonucleosomal ladder of 100-200 bp that is typical of apoptosis in some cell types was not evident in NT2 cells. The intensity of staining increased slightly on day 5, which was especially noticeable

Figure 3.16

Analysis of DNA degradation by PFGE in NT2/D1 following GSH depletion induced by BSO over 5 days.

High molecular weight degradation was detected after 4 days of BSO exposure with a clearly resolved 50-kbp fragment. Cells were harvested at each 24-hour time point following GSH depletion, embedded in agarose and processed for PFGE analysis as previously described in section 2.6 of the Materials and Methods. Each block of four lanes represents the indicated day of BSO treatment.

Lanes 1, 5, 9, 13 & 17 are untreated control cells

Lanes 2, 6, 10, 14 & 18 are cells treated with 50 μ M BSO

Lanes 3, 7, 11, 15 & 19 are cells treated with 100 μ M BSO

Lanes 4, 8, 12, 16 & 20 are cells treated with 250 μ M BSO

The remaining five lanes are DNA size markers, as indicated.

in the 50-kbp fragment in the treated samples (lanes 18-20). Thus, BSO induced HMW and low levels of LMW degradation of DNA with a prominent fragment 50-kbp in size in NT2/D1.

The integrity of DNA in NT2-N/A was examined in two types of cultures. Panel A in **figure 3.17** shows DNA from NT2/N collected from mixed NT2-N/A where the culture contained 60% NT2/N and 40% NT2/A while panel B shows DNA from NT2/N collected from mixed NT2-N/A containing 90% NT2/N and only 10% NT2/A. Little DNA damage was detected in the presence of a higher proportion of NT2/A and no significant staining was observed after three days of BSO exposure (**Panel A**; lanes 1-3). The 50-kbp fragment was faintly apparent at 50 μ M and 100 μ M BSO on the fourth day of incubation (lanes 2 & 3) and HMW degradation occurred with 100 μ M BSO on day 4 (lane 6). The intensity in staining of the 50-kb fragment in the 50 μ M and 100 μ M BSO-treated samples increased on day 5 (lane 8 & 9) and HMW fragmentation was evident in the 50 μ M BSO-treated sample (lane 8). Neurons grown in cultures containing only 10% NT2/A were more susceptible to DNA degradation than cultures with a higher proportion of NT2/A (**Panel B**). No significant loss in DNA integrity was apparent after 3 days of treatment with BSO (lanes 2, 3 & 4). However, following four days of treatment, DNA fragments > 600-kbp and the 50-kbp fragment were detected as seen in NT2/D1 (lanes 6-8). By the fifth day, GSH depletion induced by BSO generated a very intense staining continuum of HMW fragments, up to a prominent fragment that was 50-kbp in size (lanes 10-12). A band, 10-kbp in size, which was not observed in the other gels, was evident at all

Figure 3.17 Analysis of DNA degradation in NT2/N grown in the presence of 40 % NT2/A (A) and in the presence of 10 % NT2/A (B) following GSH depletion induced by BSO over 5 days using PFGE.

The NT2/N collected from NT2-N/A containing only 10% astrocytes (B) were much more sensitive to GSH depletion by BSO resulting in much more intensely staining DNA fragments than those collected from mixed cultures containing 40% astrocytes (A). In both cases, only the NT2/N were harvested at each 24-hour time point following GSH depletion, embedded in agarose and processed for PFGE analysis as previously described in section 2.6 of the Materials and Methods.

(A) Little neuronal DNA fragmentation was detected in the presence of 40 % astrocytes. High molecular weight damage was evident with 100 μ M BSO after 4 days and increased in intensity after 5 days. Each block of three represents samples for the indicated 24-hour time point. Lanes 1, 4 & 7 are untreated control cells; Lanes 2, 5 & 8 are cells treated with 50 μ M BSO; Lanes 3, 5, 6 & 9 are cells treated with 100 μ M BSO and the remaining five lanes are the indicated DNA size markers.

(B) Significant HMW degradation was evident with a lower proportion of astrocytes after 4 days of treatment. Following 5 days, the intensity of HMW damage increased and both a 50-kbp and 10-kbp fragment were also detected. Each block of four lanes represents the indicated 24-hour time point of BSO treatment. Lanes 1, 5 & 9 are untreated control samples; Lanes 2, 6 & 10 are 50 μ M BSO samples; Lanes 3, 7 & 11 are 100 μ M BSO samples; Lanes 4, 8 & 12 are 250 μ M BSO, while the last five lanes represent the indicated DNA size markers.

tested concentrations of BSO, however no oligonucleosomal degradation was detectable.

Figure 3.18 shows the PFGE results from pure NT2/N (**Panel A**) and pure NT2/A (**Panel B**). Pure NT2/N were more susceptible to DNA degradation than pure NT2/A, NT2/N grown in the presence of 10 % and 40 % NT2/A and NT2/D1. After one day of treatment with BSO, DNA breaks were minimal (lanes 2-4) but became more detectable after only two days (lanes 6-8). There was some faint HMW (> 50kbp) damage, the common 50-kbp fragment was brightly visible with increasing intensity as the concentration of BSO increased and some degradation between 10 and 50-kbp was also apparent for both 50 and 100 μ M BSO. As for 250 μ M BSO, a continuum of HMW degradation was evident with a very faintly staining 50-kbp band, but no fragments smaller than 50-kbp were detected. Again, the typical oligonucleosomal ladder was not evident in this cell type. No loss in DNA integrity was detected in pure NT2/A after five days of exposure to BSO (lanes 2-4, 6-8 & 10-12).

R 3.9 *In Situ* Staining of 3'-OH DNA Strand Breaks by the TUNEL Technique in NT2/D1, NT2-N/A and NT2/N in Response to GSH Depletion by BSO.

DNA fragmentation is one of the hallmarks of apoptosis. In 1992, Gavrieli and colleagues developed the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling method (TUNEL) that has enabled the *in situ* visualization of DNA fragmentation at the single cell level. In this method, 3'-OH DNA ends generated from endonuclease-induced DNA strand breaks are labelled with biotinylated dUTP via the enzymatic addition by TdT.

Figure 3.18 Analysis of DNA degradation in pure NT2/N (A) and in pure NT2/A (B) in response to BSO-induced GSH depletion using PFGE.

Cells were harvested at each 24-hour time point following GSH depletion, embedded in agarose and processed for PFGE analysis as previously described in section 2.6 of the Materials and Methods. Each block of four lanes represents the indicated day of BSO treatment.

(A) Pure NT2/N were very sensitive to GSH depletion by BSO resulting in significant loss in DNA integrity with the detection of fragments > 50-kbp in size following two days of treatment with BSO. Lanes 1 & 5 are untreated control cells; Lanes 2 & 6 are cells treated with 50 μ M BSO; Lanes 3 & 7 are cells treated with 100 μ M BSO; Lanes 4 & 8 are cells treated with 250 μ M BSO while the remaining five are the indicated DNA size markers.

(B) Pure NT2/A were resistant to DNA degradation following GSH depletion by BSO. Lanes 1, 5, & 9 are untreated control cells; Lanes 2, 6, & 10 cells treated with 50 μ M BSO; Lanes 3, 7 & 11 are cells treated with 100 μ M BSO; Lanes 4, 8 & 12 are the cells treated with 250 μ M BSO.

These labelled fragments can then be visualized using fluorochrome-conjugated streptavidin. To examine the possibility that GSH depletion could lead to cellular death through an apoptotic mechanism, the TUNEL method was used at various time points following exposure to varying concentrations of BSO to identify nuclei of cells undergoing DNA fragmentation through 3'-OH breaks. **Figure 3.19** is a representative of TUNEL staining in NT2/D1. Panels A, A' and A'' are untreated control cells. There was a healthy monolayer of cells with no shrunken, condensed or fragmented nuclei (A and A'') and no TUNEL-positive cells were detected (A'). Panels B, B' and B'' are cells treated with 250 μ M BSO for 3 days. There were brightly staining areas that appeared to be fragments of DNA or free apoptotic bodies (B). Based on the TUNEL results, these brightly staining fragments or apoptotic bodies were all TUNEL-positive indicating the presence of 3'-OH DNA breaks (B'). The inset is an enlargement of the TUNEL-positive elements that resemble free apoptotic bodies. The number of TUNEL-positive cells increased as time progressed and with BSO concentration (**figure 3.20**). The number of TUNEL-positive cells was not significant for day 1 or 2 for all concentration ($p > 0.05$ vs control) but became significant after the third day of treatment with 100 μ M BSO ($p < 0.01$) and 250 μ M ($p < 0.001$). All concentrations resulted in a significant increase in the percent TUNEL-positive cells after 4 and five days of GSH depletion by BSO ($p < 0.05$ for 50 μ M; $p < 0.01$ for 100 μ M; $p < 0.001$ for 250 μ M). There was also a significant increase between day 2 and 3 for 250 μ M ($p < 0.01$), and between day 3 and 4 ($p < 0.001$). In addition, there was a significant difference between day

Figure 3.19 *In situ* detection of 3'-OH DNA breaks in NT2/D1 by the TUNEL technique following GSH depletion by BSO.

The NT2D1 were grown on glass coverslips and treated with the appropriate concentration of BSO. Cells were fixed with Genofix™ at each 24-hour time point and subjected to staining by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (A') and (A'') are untreated control cells while (B), (B') and (B'') are cells following 3 days of exposure to 250 μ M BSO. (A) and (B) represent Hoechst stained nuclei, (A') and (B') represent TUNEL-positive cells while (A'') and (B'') represent phase contrast photomicrographs. There was an increase in the number of TUNEL-positive cells in the presence of BSO relative to untreated control cells. TUNEL positivity appeared to be localized to what may be either highly shrunken and condensed nuclei or chromatin fragments (apoptotic bodies).

Each panel is magnified by 200X and the insets are a magnification of 800X of the areas indicated by the white arrow. Images are representatives from two independent experiments.

Figure 3.20 Quantification of 3'-OH DNA breaks following GSH depletion by BSO in NT2/D1 using the TUNEL technique.

The percent of TUNEL-positive cells progressively increased in a dose- and time- dependent manner in all cell types. Cells were fixed with Genofix™ at each time point and TUNEL-stained as described in section 2.7 of the Materials and Methods. The percent of TUNEL-positive cells in NT2/D1 did not become significant until the fourth day of treatment with BSO and was even more significant after the fifth day with $15.7 \pm 4.6 \%$, $30.6 \pm 6.2 \%$ and $59.2 \pm 13.7 \%$ TUNEL-positive cells with 50, 100 and 250 μM BSO, respectively. Statistically significant: Between doses: $p < 0.001$ for 250 μM on day 3, 4 and 5; $p < 0.01$ for 100 μM on day 3, 4 and 5; and $p < 0.05$ for 50 μM on day 4 and 5 vs control. Between days: $p < 0.001$ for day 4 vs day 5 at all concentrations.

Five fields of view were randomly selected, total cells were counted using Hoechst stained nuclei under UV light and TUNEL-positive cells were counted using an excitation filter of 530-550 nm and emission filter of $> 570 \text{ nm}$. The percent of TUNEL-positive cells was averaged for each time point. Data represents the means $\pm$ SD of two independent experiments.

4 and 5 for all concentrations ($p < 0.001$). **Figure 3.21** is a representative figure for the positive and negative controls. In the top three panels (**A**, **A'**, **A''**), cells were treated with 0.5 μ M staurosporine for 3 hours, which induced chromatin fragmentation in a number of the cells (**A**) and many of the cells with altered chromatin were also TUNEL-positive (**A'**). Phase-contrast micrographs (**A''**) show that the cells were shrunken and rounded, of which many were phase-bright and a lot of debris was evident. Panels **B**, **B'** and **B''** are representatives of the negative control where the TdT enzyme was omitted from the TUNEL reaction mix while panels **C**, **C'**, and **C''** represent the negative control where the secondary antibody was absent. In both cases, shrunken, condensed, brightly stained NT2/N nuclei (**B** and **C**) were not labelled indicating that the method was specific to 3'-OH DNA breaks with no non-specific staining by the antibody.

In NT2-N/A, TUNEL staining was evident only in the NT2/N with shrunken and condensed nuclei (**figure 3.22**). In untreated control cultures, the number of cells with shrunken, condensed or fragmented nuclei (**A** and **A''**) and TUNEL-positive DNA breaks was minimal (**A'**). However, cells depleted of GSH with 100 μ M BSO for 4 days showed shrunken, condensed, bright Hoechst-stained nuclei (**B**), of which many were TUNEL-labelled (**B'**). There was an increase in the number of TUNEL-positive NT2/N over time and with increasing concentrations of BSO (**figure 3.23**) with 12.3 ± 2.5 % of the NT2/N labelled in the presence of 50 μ M BSO ($p > 0.05$), 23.9 ± 0.7 % with 100 μ M BSO ($p < 0.001$) and 36.3 ± 0.5 % with 250 μ M BSO ($p < 0.001$) following four days of exposure and after five days of exposure 18.9 ± 6.9 % ($p > 0.05$), 39.7 ± 0.6 % (p

Figure 3.21 *In situ* detection of 3'-OH DNA breaks in NT2/D1 by the TUNEL technique following a 3-hour exposure to 0.5 μ M staurosporine (A, A', A''), TUNEL labelling in the absence of TdT enzyme (B, B', B'') and TUNEL Labelling in absence of the secondary antibody (C, C', C'').

Undifferentiated NT2-D1 were grown on glass coverslips and treated with the appropriate concentration of BSO. Cells were fixed with Genofix™ at each 24-hour time point and subjected to staining by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (B) and (C) represent Hoechst stained nuclei, (A'), (B') and (C') represent TUNEL-labelled cells, while (A''), (B'') and (C'') represent phase contrast photomicrographs. (A, A' and A'') many cells were TUNEL-positive in the presence of staurosporine relative to untreated control cells. Staurosporine induced classical apoptotic morphology with regard to chromatin fragmentation (A) that corresponded to TUNEL-labelled cells (A'). Cells appeared rounded and phase-bright (A'') relative to the untreated control cells (A''). The insets show an enlargement of the areas indicated the white arrow (Magnification = 800X).

(B, B' and B'') no TUNEL-labelled cells were detected when TdT enzyme was excluded from the TUNEL reaction, indicating that labelling was specific to DNA strand breaks.

(C, C', C'') no TUNEL-labelled cells were detected in the absence of the secondary antibody, indicating the specificity of the secondary antibody for streptavidin.

All panels are magnified 200 X. Images are representatives from two independent experiments.

Figure 3.22 *In situ* detection of 3'-OH DNA breaks in NT2-N/A by the TUNEL technique following GSH depletion by BSO.

Mixed NT2-N/A were grown on glass coverslips and treated with the appropriate concentration of BSO. Cells were fixed with Genofix™ at each 24-hour time point and subjected to staining by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (A') and (A'') are untreated control cells while (B), (B') and (B'') are cells following 4 days of exposure to 100 μ M BSO. (A) and (B) represent Hoechst stained nuclei, (A') and (B') represent TUNEL-positive cells and (A'') and (B'') represent phase contrast photomicrographs. There was an increase in the number of TUNEL-positive cells in the presence of BSO relative to untreated control cells, as well as in the number of shrunken, condensed nuclei (B) that corresponded to TUNEL-labelled NT2/N (B'). The neurite network still remained intact but the neuronal aggregates appeared more phase-bright (B'') relative to the untreated control cells (A''). No TUNEL positive NT2/A were detected.

All panels are magnified by 200X and the insets represent an 800X magnification of the areas indicated by the white arrow. Images are representatives from two independent experiments.

Figure 3.23 Quantification of 3'-OH DNA breaks following GSH depletion by BSO in NT2-N/A using the TUNEL technique.

The percent of TUNEL-positive cells in NT2N/A was minimal during the first three days of treatment. The percent of TUNEL-positive NT2/N became more significant after 4 days and became more significant after 5 days with $18.9 \pm 6.9 \%$ at 50 μM BSO, $39.7 \pm 0.6 \%$ at 100 μM BSO and $59.5 \pm 0.7 \%$ at 250 μM BSO. No TUNEL-positive NT2/A were observed in these cultures over the five-day depletion period. Statistically significant: Between doses: $p < 0.001$ for 250 μM on day 3 and 4, 100 μM on day 4 and 5; $p < 0.05$ for 100 μM on day 3 vs control. Between days: $p < 0.001$ for day 4 vs day 5 at 100 μM and 250 μM , $p < 0.05$ for day 4 vs day 5 at 50 μM .

Five fields of view were randomly selected, total cells were counted using Hoechst stained nuclei under UV light and TUNEL-positive cells were counted using an excitation filter of 530-550 nm and emission filter of > 570 nm. The percent of TUNEL-positive cells was averaged for each time point. Data represents the means $\pm$ SD of two independent experiments.

< 0.001), and $59.5 \pm 0.7 \%$ ($p < 0.001$) of the NT2/N were labelled with 50 μM , 100 μM and 250 μM BSO, respectively. There was a significant difference in the percent of TUNEL-positive cells between day 4 and 5 for all concentrations ($p < 0.05$ for 50 μM ; $p < 0.001$ for 100 μM and 250 μM).

Figure 3.24 is a representative figure of the positive and negative controls for the TUNEL method in NT2-N/A. Panels A, A' and A'' show cells treated with 0.5 μM staurosporine for 3.5 hours. Staurosporine is a bacterial toxin that inhibits protein kinase C and is a known inducer of classical apoptosis. Many of the Hoechst-stained nuclei in both NT2/N and NT2/A had a nuclear morphology that was typical of classical apoptosis with chromatin fragmented into apoptotic bodies (A), of which many were TUNEL-labelled (A'). Panels B, B' and B'' are cells which were subjected to the entire TUNEL reaction in the absence of the TdT enzyme while panels C, C' and C'' are cells that were not incubated with the secondary antibody. As expected, in both cases, there was no TUNEL-labelling (B' and C') in cells with altered nuclear morphologies (B and C).

Evidence of 3'-OH DNA breaks in response to BSO-induced GSH depletion was also detected in NT2/N (**Figure 3.25**). In this representative figure, no shrunken, condensed or fragmented nuclei were evident (A and A'') and no TUNEL-positive DNA breaks were detected in untreated control cells (A'). On the other hand, treatment with 50 μM BSO for one day resulted in many shrunken, condensed and bright Hoechst-stained nuclei (B), which were also TUNEL-positive (B'). There was also a dose- and time-dependent increase in the number of TUNEL-labelled NT2/N (**figure 3.26**). After one day of exposure

Figure 3.24 *In situ* detection of 3'-OH DNA breaks in NT2-N/A by the TUNEL technique following a 3-hour exposure to 0.5 μ M staurosporine (A, A', A''), TUNEL labelling in the absence of TdT Enzyme (B, B', B'') and TUNEL labelling in the absence of the secondary antibody (C, C', C'').

Mixed NT2-N/A were grown on glass coverslips and treated with 0.5 μ M staurosporine for 3 hours. Cells were fixed with Genofix™ at each 24-hour time point and stained by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (B) and (C) represent Hoechst stained nuclei, (A'), (B') and (C') represent TUNEL-positive cells, while (A''), (B'') and (C'') represent phase contrast photomicrographs. (A, A', A'') many neurons were TUNEL-positive in the presence of staurosporine relative to untreated cells. Staurosporine induced chromatin fragmentation that was typical of classical apoptosis morphology (A) that corresponded to TUNEL-labelled NT2/N (A'). Neurons appeared rounded and phase-bright (A'') relative to the untreated cells (A''). No TUNEL-positive NT2/A were detected. The insets show an enlargement of the areas indicated by the white arrows (Magnification = 800X).

(B, B', B'') there were no TUNEL-labelled cells when TdT enzyme was excluded from the TUNEL reaction, indicating that labelling was specific to DNA strand breaks.

(C, C', C'') no TUNEL-labelled cells were detected in the absence of the secondary antibody, indicating the specificity of the secondary antibody for streptavidin

All panels are magnified 200X. Images are representatives from two independent experiments.

Figure 3.25 *In situ* detection of 3'-OH DNA breaks in NT2/N by the TUNEL technique following GSH depletion by BSO.

Pure NT2-N were grown on glass coverslips and treated with the appropriate concentration of BSO. Cells were fixed with Genofix™ at each 24-hour time point and subjected to staining by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (A') and (A'') are untreated control cells while (B), (B') and (B'') are cells following 1 day of exposure to 50 μ M BSO. (A) and (B) represent Hoechst stained nuclei, (A') and (B') represent TUNEL-positive cells and (A'') and (B'') represent phase contrast photomicrographs. There was an increase in the number of TUNEL-positive cells in the presence of BSO relative to untreated control cells and in the number of shrunken, condensed nuclei (B) that labelled by TUNEL (B').

All panels are magnified by 200X while the insets show an enlargement of the areas indicated by the white arrows (Magnification = 800X). Images are representatives from two independent experiments.

Figure 3.26 Quantification of 3'-OH DNA breaks following GSH depletion by BSO in NT2/N using the TUNEL technique.

Depletion of GSH in pure NT2-N generated numerous 3'OH DNA breaks after only one day of treatment with 50, 100 and 250 μ M BSO having 13.2 ± 1.2 %, 38.1 ± 3.8 % and 48.6 ± 2.5 % TUNEL-positive NT2/N, respectively and increased to 42.0 ± 4.3 %, 56.4 ± 3.2 % and 64.8 ± 2.4 % after the second day. Statistically significant: Between doses: $p < 0.001$ for 100 μ M and 250 μ M on day 1 and 2, for 50 μ M on day 2 vs control. Between days: $p < 0.001$ for day 1 vs day 2 at all concentrations.

Five fields of view were randomly selected, total cells were counted using Hoechst stained nuclei under UV light and TUNEL-positive cells were counted using an excitation filter of 530-550 nm and emission filter of > 570 nm. The percent of TUNEL-positive cells was averaged for each time point. Data represents the means $\pm$ SD of two independent experiments.

to BSO, 13.2 ± 1.2 % TUNEL-positive NT2/N were detected with 50 μ M BSO ($p > 0.05$) while 100 μ M BSO resulted in 31.8 ± 3.8 % TUNEL-positive NT2/N ($p < 0.001$) and 250 μ M BSO in 48.6 ± 2.2 % TUNEL-positive NT2/N ($p < 0.001$). Following two days of treatment, 42.0 ± 4.3 %, 56.4 ± 3.2 %, and 64.8 ± 2.4 % TUNEL-positive NT2/N were detected with 50 μ M, 100 μ M and 250 μ M BSO, respectively ($p < 0.001$ for all concentrations vs control). There was also a significant increase in the percent of TUNEL-positive NT2/N between day 1 and 2 ($p < 0.001$ for all concentrations). **Figure 3.27** is a representative of the positive and negative controls for the TUNEL method in NT2/N. Panels **A**, **A'** and **A''** show cells treated with 0.1 μ M staurosporine for 1 hour. Hoechst staining showed shrunken, condensed nuclei (**A**) which were also TUNEL-positive (**A'**). The absence of classical apoptotic nuclear morphology in response to staurosporine may be due to the low dose and short exposure time. These cultures are very sensitive and treatment with higher doses and/or for longer periods were too harsh and resulted in the loss of all NT2/N. Panels **B**, **B'** and **B''** are cells that were subjected to the entire TUNEL reaction in the absence of the TdT enzyme. As expected, there was no TUNEL-labelling (**B'**) in cells with altered nuclear morphologies (**B**). The same results were obtained with the omission of the secondary antibody (**C**, **C'** and **C''**).

R 3.10 Long-Term GSH Depletion by BSO Causes the Proteolysis of Full Length PARP in NT2/D1, NT2-N/A and NT2/N

Activation of various cysteine protease caspases plays a major role the initiation of apoptosis. PARP, or poly (ADP-ribose) polymerase, is a 116-kDA

Figure 3.27 *In situ* detection of 3'-OH DNA breaks in NT2/N by the TUNEL technique following a 1-hour exposure to 0.1 μ M staurosporine (A, A', A''), TUNEL labelling in the absence of TdT enzyme (B, B', B'') and TUNEL labelling in the absence of the secondary antibody (C, C', C'').

Pure NT2-N were grown on glass coverslips and treated with 0.1 μ M staurosporine for 1 hour. Cells were fixed with Genofix™ at each 24-hour time point and subjected to staining by the TUNEL technique as described in section 2.7 of the Materials and Methods.

(A), (B) and (C) represent Hoechst stained nuclei, (A'), (B') and (C') represent TUNEL-labelled cells, while (A''), (B'') and (C'') represent phase contrast photomicrographs. (A, A', A'') many neurons were TUNEL-positive in the presence of staurosporine relative to untreated control cells. Staurosporine induced classical apoptotic morphology with regard to chromatin fragmentation (A) that corresponded to TUNEL-labelled NT2/N (A'). Neurons appear rounded and phase-bright (A'') relative to the untreated control cells (A''). The insets show an enlargement of the areas indicated by the white arrows (Magnification = 400X).

(B, B', B'') there were no TUNEL-labelled cells when TdT enzyme was excluded from the TUNEL reaction, indicating that labelling was specific to DNA strand breaks.

(C, C', C'') no TUNEL-labelled cells were detected in the absence of the secondary antibody, indicating the specificity of the secondary antibody for streptavidin.

All panels are magnified 200X. Images are representatives from two independent experiments.

nuclear DNA-binding protein that exists as an inactive zymogen. It detects DNA strand breaks and functions in base repair (Trucco *et al.*, 1998). Caspase activation results in the cleavage of PARP into a large 85-kDa fragment and a small 21-kDa fragment. Once it is cleaved by activated caspase, it no longer supports its repair function and its cleavage may contribute to the irreversibility of apoptosis. To determine if GSH depletion leads to a loss in cell viability through an apoptotic mode of cell death, the expression of the intact full length PARP and of the larger 85-kDa activated cleaved fragment in total protein extracts were examined. These results are summarized in **figure 3.28**. In the NT2/D1 (Panel A), there was no detectable decrease in the full-length 116-kDa protein after two days of treatment, however the larger cleaved 85-kDa fragment was evident with an increasing intensity as time progressed and with increasing concentrations of BSO. It was not until the fifth day of treatment that a slight decrease in the full-length 116-kDa protein was noticed, however by then there was no longer a detectable 85-kDa fragment. The larger cleaved 85-kDa fragment was also faintly visible in the untreated control cells. Lane 9 represents a sample treated with 0.5 μ M staurosporine for 4 hours. There was both a decrease in the intact PARP protein and the appearance of the larger cleaved activated protein. The triplet bands seen between the arrows marking the location of the 85-kDa and 116-kDa proteins are spurious bands with which the antibody cross-reacts when using RIPA buffer for extraction. Panel B represents the changes in PARP in NT2-N/A. In this case, the 85-kDa fragment became faintly detectable after four days of treatment. Its intensity increased slightly

Figure 3.28 Western blot analysis of the expression of the full-length and the large cleaved fragment of PARP following GSH depletion by BSO in protein extracts from NT2/D1 (A), NT2-N/A (B) and NT2/N (C).

Total cell extracts were prepared at each 24-hour time point. Equivalent amounts of protein (50 µg/lane) were resolved on 8 % SDS-polyacrylamide gels and electro-transferred onto nitrocellulose membranes. Membranes were immunoblotted overnight with polyclonal anti-PARP primary antibody (1 µg/mL 2% skim milk), followed with HRP-conjugated goat-anti-rabbit secondary antibody. Peroxidase activity was detected using the ECL Plus Chemiluminescence kit. Blots are representative of three independent experiments. Each block of four lanes represents samples for the indicated time point. Arrows indicate the estimated size of the observed fragment. (A) & (B) NT2/D1 and NT2-N/A show a progressive decrease in the intensity of the full-length 116-kDa fragment with a subsequent detection in the large 85-kDa cleaved fragment with increasing concentration of BSO and time. The cleaved fragment can no longer be detected after 5 days. Staurosporine-treated cells were used as a positive control for PARP cleavage. Lanes 1, 5, 10 and 14 are untreated samples; Lanes 2, 6, 11 and 15 are samples treated with 50 µM BSO; Lanes 3, 7, 12 and 16 are samples treated with 100 µM BSO; Lanes 4, 8, 13 and 17 are samples treated with 250 µM BSO; Lane 9 is a sample treated with 0.5 µM staurosporine for 3 hours. (C) A dose- and time-dependent decrease in the intensity of the full-length 116-kDa fragment was detected in NT2/N, however cleaved PARP was not detected. Lanes 1 & 5 are the untreated control samples; Lanes 2 & 6 are samples treated with 50 µM BSO; Lanes 3 & 7 are samples treated with 100 µM BSO; Lanes 4 & 8 are samples treated with 250 µM BSO.

with increasing concentrations of BSO. By the fifth day of treatment, a decrease in the intact protein was detectable however the cleaved protein was very faintly visible. Lane 9 represents cells treated with 0.5 μ M staurosporine for 6 hours and shows a very prominent band at the expected location of a fragment 85-kDa in size with a slight decrease in the intact protein. Changes in PARP levels in response to GSH depletion by BSO in NT2/N is shown in panel C. A decrease in the intact PARP protein occurred as early as one day of treatment with BSO and its intensity decreased with the concentration of BSO. There was still a decrease in intact PARP after two days, although the expression appeared to be greater for 50 μ M and 100 μ M BSO and almost completely disintegrated with 250 μ M BSO. The larger 85-kDa cleaved protein was never detectable in these samples.

R 3.11 Immunocytochemical Detection of the 85-kDa Activated Cleaved PARP Fragment and of the 17-kDa Cleaved Caspase-3 Fragment in NT2/D1 Following GSH Depletion by BSO

To clarify the results from the western blot analysis, the expression of the large cleaved 85-kDa PARP fragment and the large 17-kDa cleaved Caspase-3 fragment were examined at the single cell level using immunocytochemistry. **Figure 3.29** shows a representative figure for the immunocytochemical labelling of the 85-kDa PARP fragment. Panel A and A' are the untreated control NT2/D1. The cells in panel A had healthy round nuclei with homogenous chromatin and no activated PARP-positive cells were detected (A'). The middle two panels represent cells treated with 100 μ M BSO for four days. Hoechst staining showed several nuclei with fragmented chromatin and several shrunken,

Figure 3.29 Immunocytochemical staining of the large 85-kDa cleaved PARP fragment in NT2D1 in response to GSH depletion by BSO or 0.5 μ M Staurosporine.

Cells were grown in 12-well plates and treated with varying concentrations of BSO. Cells were harvested at each 24-hour time point, immediately fixed with Genofix™ and subjected to immunolabelling with the polyclonal activated PARP primary antibody (1:100) as described in section 2.9 of the Materials and Methods.

(A) and (A') are untreated control cells, (B) and (B') are cells treated with 100 μ M BSO for 4 days and (C) and (C') are cells treated with 0.5 μ M staurosporine for 4 hours. (A), (B) and (C) represent Hoechst-stained nuclei while (A'), (B') and (C') represent cells positive for the large 85-kDa activated cleaved PARP fragment. (B) Several nuclei had classical apoptosis chromatin fragmentation (white arrowhead) and/or were shrunken with condensed chromatin (yellow arrowhead) but the 85-kDa fragment is not labelled by the antibody in B'. (B') The majority of the nuclei with these types of morphology were also positive for cleaved PARP. The number of cleaved PARP-positive cells was greater in staurosporine-treated cells (C') where the white arrowhead (C) indicates a nucleus with fragmented chromatin that is not labelled with cleaved PARP (C').

All panels are magnified 400 X, while the insets show an enlargement of the areas indicated by the white arrows (Magnification = 800X). Images are representative of two independent experiments.

condensed, brightly stained nuclei (**B**). Some nuclei with clear apoptotic morphology did not get labelled with the antibody. Of all the cells with altered nuclear morphology, three were positive for the cleaved PARP (**B'**). Panel **C** and **C'** shows NT2/D1 treated with 0.5 μ M staurosporine for 3 hours. Nuclei appeared slightly shrunken and condensed (**C**) and many were positive for the cleaved PARP. Note that one nucleus had the characteristic nuclear morphology of an apoptotic cell but was not labelled with the cleaved PARP antibody (white arrowhead). **Figure 3.30** shows a representative figure for the immunocytochemical labelling of the 17-kDa Caspase-3 fragment in NT2/D1. Untreated control cells had healthy, round Hoechst-stained nuclei with homogenous chromatin (**A**) and were negative for cleaved Caspase-3 (**A'**). Following 4 days of treatment with 100 μ M BSO, a number of shrunken and condensed nuclei were evident, while some were shrunken with fragmented chromatin (**B**) and several cleaved Caspase-3 labelled cells were detected (**B'**). Treatment with 0.5 μ M staurosporine for 3 hours resulted in the shrinkage and condensation of a number of nuclei, while several nuclei had fragmented chromatin (white arrowhead) (**C**) and many of them were also positive for cleaved Caspase-3 (**C'**).

Figure 3.30 Immunocytochemical staining of the large 17-kDa cleaved Caspase-3 fragment in NT2/D1 in response to GSH depletion by BSO or 0.5 μ M staurosporine.

Cells were grown in 12-well plates and treated with varying concentrations of BSO. Cells were harvested at each 24-hour time point, immediately fixed with Genofix™ and subjected to immunolabelling with the polyclonal cleaved Caspase-3 primary antibody (1:50) as described in section 2.9 of the Materials and Methods.

(A) and (A') are untreated control cells, (B) and (B') are cells treated with 100 μ M BSO for 4 days and (C) and (C') are cells treated with 0.5 μ M staurosporine for 4 hours. (A), (B) and (C) represent Hoechst-stained nuclei while (A'), (B') and (C') represent cells positive for the large 17-kDa cleaved Caspase-3 fragment. (B) Some nuclei had classical apoptosis chromatin fragmentation (cell at the lower right side of the inset) and/or were shrunken with condensed chromatin (yellow arrowheads), which were also positive for cleaved Caspase-3 (B', not indicated). The cell at the upper left side of the inset shows the cytoplasmic localization of cleaved Caspase-3 (B and B'). (C) Staurosporine resulted in both shrunken and condensed nuclei and in nuclei with fragmented chromatin (C, white arrowhead). (C') The number of cleaved Caspase-3-positive cells was greater in staurosporine-treated cells.

All panels are magnified 400 X while the insets show an enlargement of the areas indicated by the white arrows (Magnification = 800X). Images are representative of two independent experiments.

3.0 Discussion

The complex interrelationship between oxidative stress, GSH antioxidant status and neurodegeneration in Parkinson's disease remains to be resolved. A decrease of 30-40 % in GSH concentration in presymptomatic Parkinson's disease (Sian *et al.*, 1994) suggests that GSH depletion may initiate a cascade of events that ultimately leads to the degeneration of dopaminergic neurons within the substantia nigra. Studying such interactions may provide an insight into the pathogenesis of neurodegenerative disorders that have been associated with decreases in GSH. This study investigated whether GSH depletion in NT2 cells could be a sufficient trigger for cellular death and to elucidate a possible mode of cell death. The NT2 model is a unique experimental system because different cell types can arise from the progenitor cells including neurons, astrocytes and epithelial cells. Thus, one can simultaneously examine at least four different cell types. Because of the novelty of this system, the NT2 model may be especially useful as an *in vitro* representation of the human central nervous system (CNS) since the progenitor cells are considered to be equivalent to stem cells of the CNS that can give rise to both neurons and astrocytes. Thus, an investigation into the role of GSH in cell survival in a unique model may not only be exciting but may prove to be quite insightful. An effective way to examine the role that GSH plays in neuronal cell death is to selectively modulate its intracellular concentration. The most common method to reduce GSH concentrations in both *in vivo* and *in vitro* models is through the inhibition of γ -GCS by BSO. The findings from this study indicate that BSO effectively reduced total intracellular GSH concentrations and that GSH depletion in itself is sufficient to

induce loss of cell viability in NT2/D1, NT2-N/A and NT2/N. On the other hand, results regarding the involvement of a particular mode of cell death are less straightforward showing the presence of a number of typical apoptotic markers with a morphology and DNA fragmentation profile that is atypical of apoptosis.

Treatment with BSO extensively decreased GSH concentrations in a dose- and time-dependent fashion and ultimately led to the loss of cell viability in all NT2 cell types examined. The susceptibility to decreased GSH levels varied with the particular cell type with purified NT2/N having the greatest degree of vulnerability. The difference in susceptibility to changes in GSH content may be attributed to differences in the basal levels of GSH. The initial GSH level in pure NT2/N was very low (1.32 ± 0.1 pmol/ μ g protein) while NT2/N from mixed NT2-N/A had slightly more GSH (2.70 ± 0.08 pmol/ μ g protein, $p = 0.004$ by unpaired student t-test analysis). The fact that NT2/N from mixed cultures were found to have almost twice as much GSH supports the notion that an intimate relationship exists between neurons and astrocytes regarding GSH metabolism by providing neurons with the components required for GSH synthesis (Dringen *et al.*, 1999; Dringen *et al.*, 2000; Dringen *et al.*, 2001). Such basal levels of GSH in pure NT2/N are fairly similar to documented values of ≤ 1.0 pmol/ μ g protein in pure rodent primary neuronal cultures (Raps *et al.*, 1989). There are two known reports of GSH levels in NT2/D1 and NT2/N. Wang *et al.* (1998) documented a GSH content of approximately 2 pmol GSH/ μ g protein in NT2/N while Tamagno *et al.* (2000) reported a much higher value of 13.9 pmol/ μ g protein. Discrepancies in these two values may arise from the presence of a low number of NT2/A in their pure neuronal cultures and is discussed

below in more detail. However, reported values for rodent primary neurons separated from glial cells are quite variable, ranging between 2.2 to 18 pmol GSH/ μ g protein (Ibi *et al.*, 1999; Huang and Philbert, 1995; Sagara *et al.*, 1993). The basal GSH level of 2.70 ± 0.08 pmol/ μ g protein found for NT2/N from mixed cultures was almost exactly the same to that at the lower end of the range (2.2 pmole GSH/ μ g protein), which was reported in rat primary mesencephalic cultures (Ibi *et al.*, 1999). A feasible explanation for such reported discrepancies for basal GSH levels in neurons from mixed cultures could be due to the possibility of astrocyte contamination within the neuronal sample during the separation of neurons from the underlying astrocyte monolayer. Because of the very different basal GSH levels in astrocytes and neurons, it would only take the presence of a few astrocytes in the sample to significantly skew the results. In fact, this was observed during the first replicate for this particular experiment where the presence of NT2/A, albeit few in number, in the separated NT2/N samples produced a reading of 9-10 pmole GSH/ μ g protein. In subsequent experiments, NT2/N separation was carried out with extreme care to ensure the complete absence of NT2/A from the NT2/N sample and the basal level was consistently measured to be similar to the previously mentioned value of 2.7 pmol GSH/ μ g protein. Astrocytes have been shown to supply neurons with the necessary precursors for GSH synthesis in the brain and in mixed cultures (Dringen *et al.*, 1999; Dringen *et al.*, 2000; Dringen *et al.*, 2001). Because of their role in neuronal GSH synthesis, it is expected that astrocytes in mixed cultures would up-regulate their GSH system and resulting in them having a greater amount of GSH than when cultured alone. This was, indeed, what was found as astrocytes from

mixed cultures were observed to have a basal level of 18.9 ± 0.6 pmole GSH/ μ g protein, which was slightly higher than the 15.0 ± 0.70 pmol GSH/ μ g protein ($p = 0.0134$ by unpaired student t-test) measured in pure NT2/A, pointing to the notion of an intimate GSH metabolic relationship between the cell types. There are no known documented values for basal GSH in purified NT2/A or NT2/A from mixed cultures however such basal GSH levels in astrocytes are consistent with those reported in primary cultures (Thorburne and Juurlink, 1996; Sagara *et al.*, 1993; Raps *et al.*, 1989). On the other hand, NT2/D1 had an intermediate basal level of GSH with approximately 10.2 ± 0.7 pmol/ μ g protein. Wang *et al.* (1998) reported that NT2/D1 had a GSH content that was almost 2 fold higher than what was reported in this thesis with approximately 18 pmol GSH/ μ g protein, while the value reported by Tamagno *et al.* (2000) is three fold higher than our measured value at 29 pmole GSH/ μ g protein. In contrast, the basal GSH content in NT2/D1 that was documented in this thesis was consistent with those found by our colleagues (personal communication, J.K. Sandhu and A. Haqqani). Therefore, differences in measured levels of GSH may also arise from various other factors such as cell culture conditions, sample preparation and the method used to measure GSH levels. Nonetheless, during the differentiation process, neurons are produced which have 5-10 times lower levels of GSH than undifferentiated cells.

If a cell's susceptibility is indeed determined by basal GSH levels, it would be expected that GSH depletion in cell types with a lower basal level would be much more rapid and, consequently, affect cell viability much sooner. In fact, pure NT2/N had the lowest basal GSH and it was completely depleted at a time somewhere

between the first and second day time points of BSO exposure and they were the most vulnerable to cell death having a reduced cell viability of 19.6 ± 1.2 %, 16.5 ± 1.7 % and 11.1 ± 0.3 % with 50 μ M, 100 μ M and 250 μ M BSO, respectively, after two days. NT2/N from mixed cultures were slightly more resistant than pure NT2/N. An incubation period of two days resulted in less than 3 % of the initial GSH remaining and complete depletion was observed after three days with a much higher neuronal viability of 89.7 ± 3.6 %, 86.7 ± 4.0 % and 72.4 ± 1.0 % in the presence of 50 μ M, 100 μ M and 250 μ M BSO, respectively. It took an additional day of incubation with BSO for cell viability to become severely reduced. These results are consistent with reports that GSH depletion will ultimately result in a decrease in GSH secretion by astrocytes and hence a reduced level of extracellular precursors available to the neurons (Drukarch *et al.*, 1997; Sagara *et al.*, 1993) further suggesting the existence of an intimate relationship between astrocytes and neurons regarding GSH metabolism. In contrast, GSH was never completely depleted in the NT2/D1, NT2/A from mixed cultures or pure NT2/A over the three-day time course, however the level was very low with less than 10 %, 5 % and 4 % remaining, respectively. These results are consistent with reports that BSO quickly depletes up to 90 % of intracellular GSH level with further depletion occurring at a much slower rate (Griffith and Meister, 1985). The importance of the remaining GSH is reflected in their higher resistance to cell death. In the case of NT2/D1, viability was not severely reduced until the fourth and fifth day of incubation when GSH levels fell by 98 %, whereas astrocytes from mixed cultures did not show signs of death until the fifth day even though only 5 % GSH was remaining. This may

indicate that a only a slightly higher (2-3 %) level of GSH may be sufficient to render cells less susceptible to the effects of GSH depleting agents.

Glutathione appears to be essential to the survival of both NT2 and Jurkat cells as decreasing concentrations of GSH with BSO resulted in a progressive decrease in cell viability in a concentration and dose dependent manner. Pure NT2/N were more vulnerable to GSH depletion than all other cell types examined, followed by Jurkat cells, NT2/N from the NT2-N/A, NT2/D1, NT2/A from NT2-N/A and NT2/A. To our knowledge, there are no documented reports regarding the effects of GSH depletion in the NT2 model, however the detrimental effects of GSH depletion on cell viability and morphology have been previously described in a number of different systems. The cell viability results observed in this study are consistent with a number of previous studies, but differences arise in the time course required to elicit loss of cell viability, which varies greatly with the system being studied. Our viability data are most consistent with those presented by Merad-Boudia *et al.* (1998). The effects of BSO in the NS20Y neuronal cell line were examined for a period of five days and resulted in a similar time-dependent decrease in cell viability after the third day of GSH depletion and by the fifth day of depletion, cell viability was reduced to 37 %. One of the most significant studies implicating GSH deficiency in neurodegeneration and emphasizing the variability in the required time course in different cell systems is an *in vitro* study carried out by Li *et al.* (1997). They found that GSH depletion induced by 30 μ M BSO in immature rat cortical neurons and mouse HT22 hippocampal nerve cells for as little as 20 hours could lead to significant neuronal cell death by triggering the activation of neuronal 12-

lipoxygenase (also known as LOX), which, in turn led to the generation of peroxides and caused lipid peroxidation that could be prevented by the presence of lipoxygenase inhibitors (Li *et al.* 1997). A significant dose-dependent decrease in cell survival (65-94 %) following a 48-hour treatment with 10 to 1000 μ M BSO was also reported in rat embryonic mesencephalic neurons, which could be completely prevented by the presence of Lazaroids, drugs commonly used in the early treatment of Parkinson's disease (Grasbon-Frodl *et al.*, 1996). Furthermore, a BSO concentration as low as 3 μ M led to a significant decrease in cell viability of 60 % in PC12 cells after only 24 hours (Froissard *et al.*, 1997). All of these findings, including those presented in this thesis, provide ample evidence demonstrating that GSH depletion in itself can lead to cell death in neuronal cells.

However, while there are many reports indicating that reduced levels of GSH can trigger cell death, there have also been other results from groups reporting no significant effect on cell viability or morphology (Griffith and Meister, 1979b; Slivka *et al.*, 1998; Anderson *et al.*, 1996; Zeevalk *et al.*, 1997; Nakamura *et al.*, 2000). Such conflicting results may have occurred due to several factors. For example, a number of conflicting reports involve *in vivo* studies and this could be due to the fact that BSO does not easily cross the blood-brain barrier (Griffith and Meister, 1979b; Slivka *et al.*, 1988), thus resulting in insufficient GSH depletion to cause loss of cell viability. For example, *in vivo* studies by Andersen and colleagues (1996) did not observe any biochemical changes in dopamine or dopamine metabolite levels or loss of cell viability in the murine nigrostriatal pathway of adult mice in response to GSH depletion by BSO for a period of up to three days. In this

particular experiment, BSO was administered intraperitoneally and led to a partial depletion in GSH of only 33 % (ie. 67 % of the initial GSH was still remaining) in the substantia nigra and the striatum, relative to control brains after 72 hours of BSO-treatment. This partial depletion may not have been sufficient to cause cell death and may account for the lack of biochemical changes in dopamine activity. This idea is supported by the fact that, although partial depletion was not sufficient to cause cell death, it was enough to cause profound morphological changes in dopaminergic neurons including decreased catecholamine fluorescence per cell, increased levels of lipid peroxidation and increased numbers of dystrophic axons. Zeevalk *et al.*, (1997) also failed to detect any effect from GSH depletion in mouse primary mesencephalic cultures when GSH was partially depleted to 32 % of that of control for an observation period of only 24 hours. This brings up another reason why conflicting results may arise. Inconsistencies may arise when an experiment is ended prematurely, before any effects could be observed. Differences could exist in the time course needed to cause any changes in cell viability or morphology between various cell lines. The occurrence of deleterious effects within 24 hours in one cell system may not apply to another, especially in cultures that contain a larger proportion of astrocytes such as mesencephalic primary cultures, which could be more resistant to GSH depletion due to their intrinsic higher basal GSH levels. Therefore, the study by Zeevalk *et al.* (1997) not only suffered from partial depletion but also an observation period that was too short, resulting in the failure to detect any effect of GSH depletion. Another study emphasizing the importance of the duration of an experiment is one by Nakamura *et al.* (2000). They reported that

GSH depletion by BSO caused a significant decrease in the intracellular level of dopamine and disrupted the function of dopaminergic neurons without affecting dopaminergic neuronal numbers or survival in mouse embryonic mixed neuronal-glial mesencephalic cultures as well as in an *in vitro* reconstituted nigrostriatal system. In this case, nearly complete (> 95 %) depletion was achieved after 48 hours despite exposure to very low concentrations of BSO (2.5 to 10 μ M) but the effects on cell viability or morphology were not examined beyond this time point. The data presented in this thesis showed that GSH was depleted to a similar level after three days in the NT2/D1 (< 10 %), NT2/A from NT2-N/A (< 5 %) and pure NT2/A (< 4 %) with no significant cell death until one or two days beyond this. In fact, in the above study, when treatment was prolonged for up to seven days, GSH depletion did eventually lead to a decrease in the mean total number of dopaminergic neurons per section (Nakamura *et al.*, 2000). These observations, in addition to the viability results presented in this thesis, indicate that GSH depletion may only elicit its detrimental effects once GSH levels fall below a critical value (discussed below).

The Spearman Rank correlation test showed that there was a direct negative correlation between GSH depletion and cell viability in NT2/D1 ($r = 0.9314$, $P < 0.0001$), NT2/N from mixed ($r = 0.9611$, $P < 0.0001$) and pure NT2/N cultures ($r = 0.9063$, $P = 0.0067$), with cell viability progressively decreasing as the concentration of intracellular GSH decreased. Most significantly, this test also showed that there appears to be a critical level of GSH and it is only once the GSH concentration falls below this level that the cellular death becomes significant. This value was less than

0.5 pmol GSH/ μ g protein in NT2/D1, 0.1 pmol GSH/ μ g protein in NT2-N from NT2-N/A and 0.5 pmol GSH/ μ g protein in pure NT2/N. There was no critical level detected after three days of GSH depletion in NT2-A from NT2-N/A or pure NT2/A, as there was no significant loss of viability. In NT2/D1, this value represents approximately 4.9 % of the total GSH and was not reached until the third day of GSH depletion when the cell viability was 92.8 ± 1.8 %, 82.0 ± 1.2 and 48.3 ± 6.8 % with 50, 100 and 250 μ M BSO, respectively. In the NT2/N from mixed cultures, this value was 3.7 % of the GSH remaining and was reached only with the highest BSO concentration where the neuronal cell viability was still 81.1 ± 3.8 % and astrocyte viability was relatively unaffected. Although the critical value in NT2/N was reached very quickly (after 24 hours of treatment with 100 and 250 μ M BSO), it represented a much higher proportion of the initial GSH level at approximately 27 % and still resulted in significant cell death with a neuronal viability of 48.0 ± 10.2 % following treatment with 100 μ M BSO and 27.4 ± 8.2 % following treatment with 250 μ M BSO. This is not surprising because NT2/N, were found to have extremely low basal levels of GSH, thus only slight perturbations may be enough to trigger deleterious effects.

A number of reports suggest that this critical value is due to depletion of the mitochondrial pool of GSH. A single dose of BSO has been shown to deplete GSH to 15-20 % of those of untreated controls in liver and kidney of mice and any further decrease in the level of GSH occurs much more slowly (Griffith and Meister, 1979a). Further studies by Griffith and Meister (1985) showed that GSH is essential for mitochondrial function and because of this requirement the loss of GSH from the

mitochondria is very slow in order to conserve mitochondrial GSH during periods of cytosolic GSH depletion. Further studies infer that only the small pool of GSH within the mitochondria is crucial in the maintenance of cell viability (Meredith and Reed, 1982; Huang and Philbert, 1996; Wüllner *et al.*, 1999; Merad-Boudia *et al.*, 1998; Seyfreid *et al.*, 1999). In fact, Meredith and Reed (1982) documented that the onset of cell injury correlated to the depletion of the mitochondrial pool of GSH, whereas the cytosolic pool could be depleted without inferring cell death. Furthermore, research by Wüllner and colleagues (1999) demonstrated evidence of neuronal degeneration following 24-36 hours of GSH depletion by BSO, when mitochondrial GSH levels fell below 50% of control mitochondrial GSH levels (Wüllner *et al.*, 1999). Seyfried *et al.* (1999) also reported similar findings with no loss in cell viability until GSH was depleted to 5% of the total GSH concentration. These two studies indicate that the critical level of GSH may in fact relate to only a small percentage of the mitochondrial pool of GSH and that cells may be able to prevent the excessive generation of ROS during BSO treatment through the maintenance of a minimal amount of mitochondrial GSH. For this thesis, the cytosolic and mitochondrial fractions were not separated and individually measured for GSH content, thus the actual compartmentalization of GSH remains to be determined. However it has been reported that the mitochondrial fraction makes up between 4 and 15 % of the total intracellular GSH content (Meredith and Reed, 1982; Griffith and Meister, 1985; Huang and Philbert, 1995). Considering that the critical level of GSH was found to be within this range (with the exception of NT2/N, which still had 27 % GSH remaining) it is possible that a similar distribution

exists in NT2 cells. However, whether it relates to the mitochondrial pool of GSH remains to be determined and will most likely be investigated at a later date.

Because mitochondria lack γ -GCS, BSO itself will not directly deplete the mitochondrial pool of GSH, however, once the cytosolic pool of GSH becomes completely depleted, the mitochondrial pool will no longer be maintained and it too will eventually become depleted. This would be expected to lead to the extensive accumulation of ROS, loss of ATP and ultimately to mitochondrial dysfunction, energy impairment and cell death. In support of this, the decline in mitochondrial transmembrane potential and cellular ATP production following extensive depletion of both cytosolic and mitochondrial pools of GSH by ethacrynic acid has been reported to occur prior to the onset of the loss of cell viability in cultured cerebellar astrocytes (Huang and Philbert, 1996). Thus, the loss of mitochondrial GSH may render cells unable to cope with the oxidative load, leading to perturbations in the activities of thiol-dependent enzymes and antioxidant status, mitochondrial dysfunction and energy crisis. The results presented in this thesis document that intracellular GSH must be almost completely depleted before cell viability is lost, indicating that under normal conditions, neurons and astrocytes have sufficient stores of intracellular GSH. However, neuronal cell death similar to that seen in the degeneration of dopaminergic neurons in Parkinson's disease and cortical neurons in Alzheimer's disease may arise from a compromised GSH metabolism.

Although it is clear that GSH depletion is a sufficient trigger to cause cell death in all NT2 cell types, a clear picture did not emerge as to the mode of cell death. Activation of caspases, site-specific proteolysis of PARP, nuclear condensation, 3'-

OH DNA strand breaks and oligonucleosomal DNA fragmentation are characteristic of an apoptotic program of cell death. The presented data show a mixture of typical apoptotic markers including caspase activation, PARP proteolysis and the TUNEL detection of 3'-OH DNA breaks, however nuclear morphology and DNA fragmentation were atypical of apoptosis and necrosis. The activation of caspases has been thought to play a critical role in both the initiation and execution of apoptosis in many cell systems (Nicholson and Thornberry, 1997; Cohen, 1997). The proteolytic cleavage of inactive caspases results in the generation of activated fragments, which in turn can activate other inactive proenzymes, hence resulting in an apoptotic cascade of events. For example, activation of caspase-3 is thought to be an important step in triggering apoptotic DNA degradation in many cells and can result in the site-specific cleavage of poly(ADP-ribose) polymerase (PARP), a substrate for caspase-3. Thus, one way to identify apoptosis is by the detection of activated forms of caspases cleavage of their substrates, such as caspase-3 and PARP. PARP activation was detected by immunoblot analysis and immunocytochemistry. Immunoblotting showed evidence for the proteolysis of the inactive PARP (116-kDa) into activated cleaved fragments, more specifically the 85-kDa fragment, in all NT2 cell types. Although a decrease in the inactive, uncleaved PARP was not detected until the fifth day of incubation with BSO in both NT2/D1 and NT2-N/A, there was an obvious time-dependent increase in the cleaved PARP fragment. A possible explanation for the lack of a decrease in intensity for the uncleaved PARP could be based on the function of the PARP proenzyme. The proenzyme functions in DNA repair and genome stability, thus it is plausible that

under mild conditions of oxidative stress, the messenger RNA for the PARP proenzyme may be up-regulated to repair mild DNA damage induced by free radicals, resulting in an increase in the synthesis of the protein offsetting any observed decrease from its proteolysis. However, once DNA damage becomes so extensive that it becomes beyond the repair capabilities of PARP, cell death may be triggered and a decrease in the level of uncleaved PARP becomes detectable. Despite this lack of decrease in uncleaved PARP, an increase in the cleaved fragment was quite evident indicating PARP proteolysis. The cleavage of PARP preceded the onset of morphological changes and cell death and the degree of cleavage coincided with the BSO incubation period with an increase in the intensity of cleaved PARP as time progressed. In fact, by the fifth day cleaved PARP could no longer be detected suggesting that it has been subsequently degraded. On the other hand, there was an obvious time- and dose-dependent decrease in full length PARP in the NT2/N, however there was no evidence of a coincident increase in the cleaved fragment. Considering the high degree of cell death that was determined by the CFDA assay, it is possible that any cleaved products that were generated were rapidly degraded in this cell type. This notion is supported by immunocytochemical staining of activated PARP and cleaved Caspase-3 in NT2/D1 cells. Activation of both proteins was detected and also correlated with the loss of cell viability and exposure time, however, some cells that clearly had an altered nuclear morphology and/or fragmented chromatin were not labelled. Because all three antibodies recognize the large cleaved fragment (85-kDa for PARP and 17-kDa for Caspase-3), it is possible that the large fragment was cleaved to even smaller fragments that

were not recognized by the antibodies, thus resulting in the absence of its detection. In any case, the Western blot analysis also illustrated that there is an important GSH relationship between NT2/N and NT2/A in that pure NT2/N showed a more significant proteolysis that occurred very rapidly relative to NT2/N from mixed cultures.

Another method commonly used to detect apoptosis is the TUNEL technique. As described earlier, this technique labels free 3'-OH ends on single-stranded DNA breaks and 3' overhangs on double-stranded DNA breaks. It is believed that numerous DNA breaks can accumulate during the apoptotic cascade resulting in the generation of free 3'-OH ends or 3' overhangs of the DNA such that over time there is an increase in the number of the DNA ends that can be visualized by this method. The TUNEL technique demonstrated an increase in the number of TUNEL-positive cells in all NT2 cell types with a positive correlation between both the incubation period and BSO concentration, indicating an increase in the degree of apoptosis through the generation of 3'-OH breaks typical of apoptotic DNA fragmentation. Although 3'-OH DNA breaks are used as typical marker of apoptosis, there are several limitations to its identification by the TUNEL technique. Such 3'-OH ends have also been detected by TUNEL under conditions of necrotic cell death (Charriaut-Marlangue and Ben-Ari, 1995; de Torres *et al.*, 1997), however it has been documented that necrosis generates a higher proportion of 5'-OH ends than 3'-OH in a rat ischemia model (MacManus *et al.*, 1997). Furthermore, the TUNEL technique can also yield false positive results (Jones *et al.*, 1997). For these reasons,

the TUNEL method is not sufficient evidence for the identification of programmed cell death and must be verified by at least one other technique.

Contrary to these characteristics typical of a programmed cell death, classical morphological and oligonucleosomal DNA fragmentation normally seen during apoptosis were not observed in any of the NT2 cell types examined. Although the classical oligonucleosomal ladder was not detected in any NT2 cell type, PFGE illustrated a progressive increase in high molecular weight DNA fragments ranging from 10-kb to > 600-kb in NT2/D1, NT2/N from mixed cultures and pure NT2/N in response to GSH depletion by BSO. Furthermore, this increase in HMW degradation correlated with the increase in cell death determined by the loss of CFDA labelling, which in turn coincided with the degree of GSH depletion. Upon the examination of the time course of events, DNA degradation into fragments 50-kbp in size became apparent after four days of treatment with BSO in NT2/D1 and NT2/N from NT2-N/A and after only one day in pure NT2/N, which appeared to occur concomitantly with the sharp decrease in cell viability on day four and day one, respectively. Purified NT2/A did not show any evidence of DNA fragmentation at any point during the five-day time course. This high molecular weight pattern of DNA fragmentation induced by GSH depletion is consistent with that observed by Higuchi and Matsukawa (1998) who reported large (2-Mbp) and high molecular weight DNA fragmentation (30 to 700-kbp) associated with apoptosis in C6 rat glioma cells in response to GSH depletion. The data from the pure NT2/N and the NT2/N from mixed cultures containing a high and low proportion of NT2/A also support the notion of an intimate relationship between

astrocytes and neurons regarding GSH metabolism. The fact that the degree of DNA degradation in NT2/N was significantly lower in the presence of 40 % astrocytes relative to NT2/N in the presence of 10 % astrocytes and relative to NT2/N in the total absence of astrocytes indicate that astrocytes have an important neuroprotective role that is dramatically increased when the GSH antioxidant status is compromised.

The lack of cleavage into the oligonucleosomal 200-bp ladder in NT2 cells does not necessarily mean that death is occurring from a type of cell death other than apoptosis. It has been documented that DNA fragmentation can proceed through an ordered sequence of stages with the first resulting in high molecular weight degradation initiated by endonucleases that cleave the DNA into 50 to 300-kbp fragments, followed by the generation of 10 to 40-kb fragments and the subsequent internucleosomal DNA cleavage by calcium-magnesium dependent endonucleases (Walker *et al.*, 1994). Therefore, the progressive generation of 300-kbp and 50-kbp DNA fragments in the NT2 cells can still serve as a good indicator of the early stages of apoptosis since these two cleavage steps have been observed in many cell types undergoing apoptosis. Furthermore, a similar pattern of high molecular weight damage has been documented in several cell lines that can undergo normal apoptosis in response to various stimuli without generation of a DNA ladder including the human breast carcinoma MCF7 cells and NT2 cells (Walker *et al.*, 1999a; Oberhammer *et al.*, 1993). The time course of DNA fragmentation relative to the time required for total GSH depletion and the occurrence of significant cell death suggest that GSH must be completely depleted, or at least below some critical level, to result in the induction of apoptosis and DNA fragmentation. It is

conceivable that the induction is brought upon by the generation of excessive ROS, which can no longer be eliminated due to the massive imbalance in GSH antioxidant status. It has been demonstrated that extreme depletion of GSH leads to oxidative stress and favours the induction of apoptosis in different systems (Obrador *et al.*, 1998). For example, GSH depletion in the NS20Y neuronal cell line (Merad-Boudia *et al.*, 1998) and embryonic cortical neurons (Ratan *et al.*, 1994), as well as neuroblastoma and hippocampal neurons (Murphy *et al.*, 1989) resulted in oxidative stress and apoptotic cell death. In addition, reduction of the level of GSH by diethylmaleate (DEM) resulted in the accumulation of ROS and induced apoptotic neuronal cell death (Kane *et al.*, 1993). However, the involvement of ROS as mediators of cell death in response to GSH depletion by BSO in NT2 cells remains to be investigated.

Specific morphological features including chromatin condensation, chromatin reorganization along the nuclear membrane and the formation of apoptotic bodies are also characteristics of apoptosis (Kerr *et al.*, 1972). Chromatin fragmentation in BSO-treated Jurkat cells was typical of classical apoptosis. Hoechst staining identified many shrunken and condensed nuclei with chromatin fragmented into classical apoptotic bodies. This chromatin fragmentation is a hallmark of apoptosis and was not observed in any of the BSO-treated NT2 cell types studied. However, this morphology was observed when NT2 cells were treated with staurosporine, a known inducer of apoptosis. Instead, NT2/D1 became smaller and rounded while the nuclei became morphologically distinct displaying shrunken, condensed and brightly stained crescent-shaped chromatin that appeared to be localized along the

intact nuclear membrane. This was followed by a decrease in cell number relative to control cells and the subsequent detachment of the cell lawn. These morphological changes occurred concomitantly with the observed changes in cell viability, appearing on the third day and increasing in number on the fourth and fifth day.

Neuronal cells from NT2-N/A demonstrated a gradual disruption of the neurite network and some neuronal loss after four days of incubation with BSO and by the fifth day, the majority of the neurons became detached and interconnections between neurites were completely disintegrated suggesting that BSO may be causing synaptic dysfunction. Nuclei of the remaining neurons had a morphology that appeared different from those observed in NT2/D1 with shrunken, condensed, brightly stained nuclei and no evidence of any chromatin fragmentation or marginal nuclear staining. These changes paralleled with the degree of GSH depletion and the time course for the detection of significant cell death, as determined by CFDA labelling. The morphology of astrocytes from NT2-N/A did not become affected until the fifth day of incubation, when some cells became rounded with shrunken nuclei, but did not condense or stain brightly with Hoechst 33258. Pure NT2/N had similar morphological changes to those observed in NT2/N from mixed cultures, however, these changes occurred much sooner, following one to two days of incubation with BSO and were concomitant with the loss in cell viability. Such morphological changes are consistent with those reported in the literature in response to GSH depletion by BSO (Merad-Boudia *et al.*, 1998; Grasbon-Frodl *et al.*, 1996; Froissard *et al.*, 1997; Wüllner *et al.*, 1999). Wüllner and colleagues (1999) found that BSO-induced GSH depletion did not lead to morphological changes typical of apoptosis

where electron micrographs indicated the loss of cell organelles and disruption to the plasma membrane, while the nuclear membrane remained intact and the chromatin structure underwent a distinctive change into homogeneous granular pattern with electron-dense areas of condensation. Preliminary electron microscopy studies in our lab indicate a similar pattern in BSO-treated NT2 cells (unpublished data).

In contrast, ethacrynic acid leads to the rapid depletion of both cytosolic and mitochondrial pools of GSH within only several hours and has been shown to lead to necrotic cell death with massive mitochondrial swelling, destruction of organelles as well as disruption of both the cell and nuclear membranes. However, treatment with ethacrynic acid is very harsh and the effects are not necessarily specific to GSH depletion. Ethacrynic acid causes the depletion of GSH by forming electrophilic conjugates with GSH, which can exert toxic effects on cells that may not be due to GSH depletion but due to the accumulation of these toxic conjugates, hence leading to the observed massive cellular injury. Thus, GSH depletion can lead to a variety of morphological changes in different cell lines and whether these changes are more characteristic of apoptosis or necrosis may not only depend on the degree of GSH depletion but also on how quickly the depletion occurs. A slower depletion would more adequately reflect the slow changes that occur in the diseased brain of Parkinson's patients. Although, depletion by BSO is comparatively rapid to that in Parkinson's disease, it is still a better representation than GSH depletion by ethacrynic acid. In fact, the morphological changes observed in differentiated NT2 cultures resemble those observed in post-mortem brain tissue from patients with Parkinson's disease where many researchers have observed some degree of

apoptotic-like changes, but not classical apoptosis in nigral neurons in the Parkinsonian brains (Graeber *et al.*, 1999; Kösel *et al.*, 1997; Tompkins *et al.*, 1997; Banati *et al.*, 1998).

The issue of the mode of cell death induced by GSH depletion in neurons and astrocytes remains unresolved and the results presented in this thesis reflect the confusion in this field of research. It is possible that the mode of cell death may actually be a combination of apoptosis and necrosis or possibly even some other uncharacterized process. It may even be plausible that there is more than one form of apoptosis that can vary between cell types and in response to different stimuli. Perhaps the features originally described by Kerr *et al.* (1972) only apply in some situations meaning that apoptosis may proceed through different pathways with each having similar but slightly different features. If this is so, then the term apoptosis may need to be redefined and updated. In fact, Graeber and colleagues (1999) are attempting to do so and have described a new term, “apoptosis”, the actual meaning of which is “withering”, and it closely resembles the neurodegeneration in Parkinson’s disease. The authors refer to apoptosis as ‘a slow, non-developmental, non-physiological and non-apoptotic death of degenerating neurons’ and they suggest that Parkinson’s disease may involve mitochondria dysfunction and may be caspase-independent since the classical morphological characteristics of apoptosis relies on the activation of caspase-related cysteine proteases.

The effects of GSH in NT2/D1, NT2-N/A and pure NT2/N have now been described and discussed in detail. It is clear that BSO can induce complete depletion of intracellular GSH, resulting in significant cellular death through an unusual

mechanism that is distinctly different from both apoptosis and necrosis. One may question the relevance of examining these effects in pure NT2/N cultures since these are not a realistic representation of the brain. The purpose of such studies was to gain an understanding into the relationship existing between NT2/N and NT2/A based on the hypothetical model of GSH metabolism described in the introduction (section I 1.3 and Figure 1.1). Although we did not examine the specifics such as cysteine uptake, GSH breakdown by γ -GCS or CysGly breakdown by aminopeptidases, we can conclude from the results presented that there is an intimate relationship between NT2/A and NT2/N in mixed cultures. This is supported by the fact that pure NT2/N were extremely sensitive to the effects of GSH depletion and were more sensitive than NT2/N from mixed cultures, while NT2/A were relatively resistant to GSH depletion by BSO determined by various parameters such as cell viability, DNA fragmentation, PARP proteolysis, TUNEL labelling and nuclear morphology. In the absence of astrocytes, neurons became depleted of GSH within 24-48 hours and cell death was imminent. An additional day was required in NT2/N from mixed cultures. It is likely that once GSH is depleted from the pure NT2/N and there is no other GSH source, it will lead to the accumulation of ROS, a decline in ATP levels followed by death of the cell. On the other hand, NT2/N from mixed cultures can maintain their GSH levels above the certain critical level due to the supply of GSH precursors stored in the astrocyte. However, eventually a critical level of GSH within the NT2/A will be reached, triggering the complete depletion of GSH in the neurons followed by the degeneration of neuronal cells. The NT2/A themselves eventually also become

completely depleted of GSH and likely die soon after, as observed on the fifth day of depletion.

In summary, the findings from this study demonstrate that GSH is essential for the survival of NT2 cells as its depletion triggered significant cell death, with a differential susceptibility that may be attributed to differences in basal levels of GSH and offer exciting insights in the area of neuronal cell death. There is increasing evidence for the involvement of GSH depletion and oxidative stress in normal aging and in the pathogenesis of various neurodegenerative diseases. The examination of purified NT2/N and mixed NT2-N/A reveal the existence of an intimate relationship between NT2/N and NT2/A regarding GSH metabolism that was consistent with reported models. It supports the notion that astrocytes, considering their abundant basal GSH, likely play a crucial role in neuroprotection and cellular defenses against free radicals in NT2-N/A. The fact that intracellular GSH must be completely depleted before cell viability is lost suggests that there are sufficient stores of GSH to prevent the excessive accumulation of ROS under normal conditions and that only under a situation where GSH is severely compromised will cells be rendered vulnerable. Although the results do not answer the 'cause-and-effect' question regarding GSH depletion and the neurodegeneration of dopaminergic neurons in Parkinson's disease and the specific mechanism and mode of cell death remain to be resolved, these results show that GSH plays a critical role in the survival of NT2 cells and that a compromised antioxidant system can lead to deleterious effects. Furthermore, the existence of an intimate relationship concerning GSH metabolism between NT2/N and NT2/A, proves that the NT2 model is a very good system that

may be comparable to GSH metabolism in the *in vivo* central nervous system, and therefore may be a useful model to study the pathogenesis of neurodegenerative disorders associated a compromised GSH antioxidant status such as Parkinson's disease. A better understanding is necessary to fully understand the degeneration of dopaminergic neurons in Parkinson's disease. Further studies should be aimed at determining the role of mitochondrial GSH, the generation of oxidative and nitrative stress and clarifying the mode of cell death.

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Appendix I Basal GSH levels in various cell lines and tissues.

<i>In vitro</i> Cell type	Region (if available)	Basal GSH (nmol/mg protein)	References
primary rat brain cells	whole brain: -neurons -glia -total -astrocytes - oligodendrocytes cerebellar astrocytes cerebellar granule neurons mesencephalon	7.1 ± 4.2 - 18.8 ± 1.8 23.6 ± 0.5 - 31.5 ± 1.0 16 (no error given) 5 (no error given) 14.8 ± 0.61 - 19.0 ± 3.5 9.6 ± 0.51 – 29 ± 1 8.4 ± 2.4	Sagara <i>et al.</i> , 1993 Sagara <i>et al.</i> , 1993 Thornburne and Juurlink, 1996 Huang and Philbert, 1995 and 1996; Wüllner <i>et al.</i> , 1999 Grasbon-Frodl <i>et al.</i> , 1996
mouse HT22	hippocampus	21.8 ± 1.4	Li <i>et al.</i> , 1997
PC12	not applicable	1.53 ± 0.56 - 12.6 ± 14.06	Froissard <i>et al.</i> , 1997; Seyfried <i>et al.</i> , 1999
human SK-N-SH neuroblastoma	not applicable	$0.5 - 42.3 \pm 10.5$	Anderson <i>et al.</i> , 1999; Stokes <i>et al.</i> , 2000
human B lymphoma cell line (PW cells)	not applicable	57 (no error given)	Armstrong <i>et al.</i> , 2002
rat	isolated hepatocytes - mitochondria	1.6 ± 0.2	Shan <i>et al.</i> , 1993
<i>In vivo</i> Tissue	Region (if available)	Basal GSH (varies)	References
Adult Human Normal	various	($\mu\text{g/g}$ tissue) 56.86 ± 12.95 (or $0.8 - 3.1$ mM)	Riederer <i>et al.</i> , 1989 (Slivka <i>et al.</i> , 1987)
Parkinson's brain	various	6.29 ± 2.70 – 29.66 ± 12.21	Riederer <i>et al.</i> , 1989
Adult Mouse	liver whole brain substantia nigra striatum	38 ± 5.5 nmol/mg protein 1.2 - 2.3 mM 1.5 ± 0.1 nmol/mg protein 2.15 ± 0.1 nmol/mg protein	Griffith and Meister, 1985 Slivka <i>et al.</i> , 1988 Andersen <i>et al.</i> , 1996
Adult rat	whole brain	1.5 - 3.3 mM	Pileblad and Magnusson, 1989
Adult monkey (<i>Macaca fascicularis</i>)	various	1.8 - 2.6 mM	Slivka <i>et al.</i> , 1987

Appendix II Seeding numbers for each NT2 cell type according to the surface area of the cell culture dish or flask. Note that seeding numbers for NT2/D1 varies depending on the day of the experiment in order to prevent overgrowth. All other NT2 cell types are post-mitotic and could be seeded at the same number for each day of the experiment.

Cell Type	Size of Dish/Flask	Plating Density (cell #)
NT2/D1	12 well plate	Day1, 2 and 3: 5×10^4 Day 4 and 5: 4×10^4
	25 cm ² flask	$1-2 \times 10^6$
	75 cm ² flask	5×10^6
NT2-N/A	12 well plate	5×10^5
	75 cm ² flask	$9-12 \times 10^6$
NT2/N	12 well plate	5×10^5
	25 cm ² flask	$1.5-2 \times 10^6$