2013

DIFFERENTIATION-INDUCED CHANGES IN ANTIOXIDANT CAPACITY AND ANTIOXIDANT RESPONSE IN SH-SY5Y CELLS: ROLE OF NRF2

Akua Afriyie Karikari
The University of Montana

DIFFERENTIATION-INDUCED CHANGES IN ANTIOXIDANT CAPACITY AND ANTIOXIDANT RESPONSE IN SH-SY5Y CELLS: ROLE OF NRF2

By

Akua Afriyie Karikari

BSc. Human Biology (University of Cape Coast, Cape Coast, Ghana)

Thesis presented in partial fulfillment of the requirements for the degree of

Master of Science

in

Neuroscience

The University of Montana

Missoula, Montana

Summer 2013

Approved by:

Dr. Fernando Cardozo-Pelaez, Chair

Department of Biomedical and Pharmaceutical Sciences

Dr. Elizabeth Putnam

Department of Biomedical and Pharmaceutical Sciences

Dr. Darrel Jackson

Department of Biomedical and Pharmaceutical Sciences

Dr. Mark Grimes

Department of Biological Science

Akua Afriyie Karikari M.S., July 2013 Neuroscience

DIFFERENTIATION-INDUCED CHANGES IN ANTIOXIDANT CAPACITY AND ANTIOXIDANT RESPONSE IN SH-SY5Y CELLS: ROLE OF NRF2

Chairperson: Dr. Fernando Cardozo-Pelaez

Organisms are exposed to reactive oxygen species from internal metabolism and environmental toxicant exposure, which have been linked to the initiation and progression of many neuronal diseases such as Alzheimer's and Parkinson's diseases. Exposure to reactive oxygen species are counterbalanced by antioxidant defense systems such as the Nrf2-ARE pathway, the primary regulator of endogenous antioxidant response. Whereas neuronal cells are post-mitotic and are particularly susceptible to oxidative stress because of high oxygen consumption, astrocytes are mitotic and rely more on glycolytic metabolism. In this study, the difference in antioxidant response and capacity in mitotic and post-mitotic cells were investigated using undifferentiated and differentiated SH-SY5Y neuroblastoma cells. The investigations of this study focused on the induction of antioxidant enzymes in SH-SY5Y cells by Nrf2 using tert-Butylhydroquinone (tBHQ). The levels of GSH, Mn-SOD, HO and OGG1 which are all induced by Nrf2 were determined. The results of the experiment showed that differentiated (post-mitotic) and undifferentiated (mitotic) cells responded similarly to Nrf2 induction as was observed in the higher levels of GSH, HO, Mn-SOD and OGG1 compared to the control group. As compared to undifferentiated cells, differentiated cells had relatively lower antioxidant levels except in the case of Mn-SOD. This suggests that post-mitotic and mitotic cells respond to antioxidant induction similarly but differ in their antioxidant capacities. The study also compared DTNB and HPLC methods in determining GSH levels in these cells. The outcome of the experiments showed that HPLC offers a selective method in determining GSH levels.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to the Almighty God for the strength, blessings and protection.

I am very grateful to my advisor, Dr. Fernando Cardozo-Pelaez, for his patience, guidance, criticisms, and support to make this work a reality.

I indebted to my family; indeed you are the best gift I have. God bless you for all the advice, love and for being there for me.

I am grateful to the Foreign Fulbright Program and the Delta Kappa Gamma Society for the sponsorship they provided for my study.

I would also like to acknowledge the Centre for Structural and Functional Neurosciences and the Department of Biomedical and Pharmaceutical Sciences for providing me the educational opportunity and resources to complete this degree.

I wish to further thank the members of my thesis committee for their input and efforts: Drs. Putnam, Grimes and Jackson.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

Introduction and Background

Oxidative stress

The survival of all cells and organisms depend on the ability to perform basic biochemical processes to provide energy, which ultimately leads to growth. The maintenance of homeostasis is essential for these biochemical processes to be undertaken. Homeostasis can be defined as the regulation of the cell's internal environment within normal temperature, acid-base and redox balance that is needed for the survival of the cell. When the normal physiological environment is not maintained, cellular functions are affected and can be detrimental to the cell. The imbalance of redox state resulting in the formation of more oxidants than antioxidants causes oxidative stress.

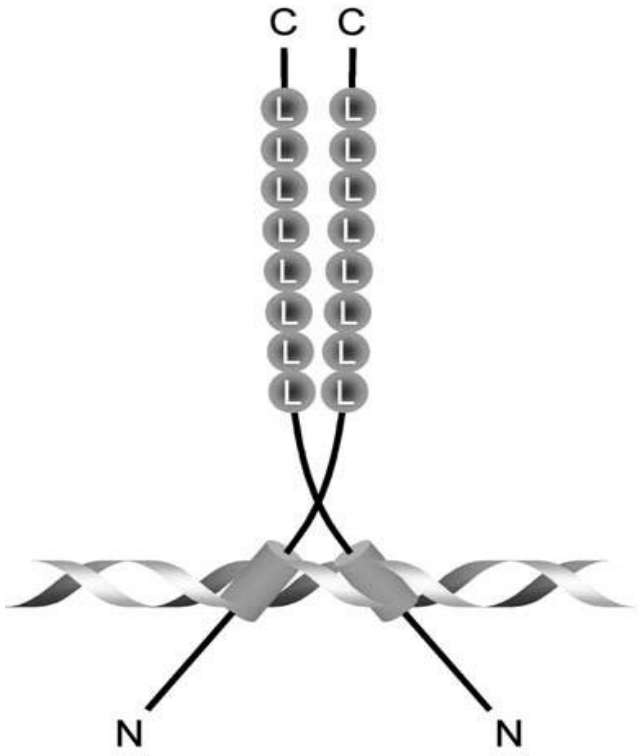
Oxidative stress represents an imbalance between the production and degradation of reactive oxygen species (ROS), such as $\bullet O_2^-$ and H_2O_2 (Sayre et al., 2008). The generation of ROS may cause oxidation of lipids, proteins, sugars and/or polynucleotides, with evidence of this type of oxidation found in several neurological diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Sayre et al., 2008). Oxidative stress can occur as a consequence of various cellular mechanisms including ATP production, inflammatory response, metabolism and reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite ($ONOO^-$). It can also be attributed to the exposure to toxic environments like ionizing radiation (Comhair and Erzurum, 2002). Though ROS and RNS differ with regard to their stability, reactivity and molecular targets, a common denominator is that when their generation exceed the antioxidant capacity of the cell, the net result is damage and oxidation of lipids, proteins, and/or nucleic acids, as well as of several other biomolecules (Mohora et al., 2007).

Cellular processes resulting in Oxidative Stress

Reactive oxygen species occur as a result of biological reactions. ROS generation occurs during mitochondria, cytochrome P-450, peroxisomes and other cellular processes (Bedard and Krause, 2007). Cells use oxygen to undergo respiration through the mitochondria to generate energy in the form of ATP. During normal oxidative phosphorylation to produce ATP, small amounts of oxygen (between 0.4 and 4% of all oxygen consumed) are reduced to the superoxide anion ($\bullet O_2^-$) by the mitochondrial electron transport chain (Mohora et al., 2007). Under normal conditions, $\bullet O_2^-$ is acted upon by mitochondrial enzyme manganese superoxide dismutase (Mn-SOD) or by copper and zinc superoxide dismutase (Cu/Zn-SOD), which are found in the cytosol (Mohora et al., 2007) where $\bullet O_2^-$ will be metabolized to H_2O_2. Subsequently, H_2O_2 will be removed by the action of either catalase or glutathione peroxidase, both enzymes found in the mitochondria. Thus, mitochondria antioxidant enzymes play an important role in protecting the cell against damage by ROS and RNS. Though mitochondria antioxidant enzymes play an integral role in protecting cells against oxidative damage, ROS are also continuously generated as a result of mitochondria respiration. The mitochondria are known to produce approximately 85 to 90% of cellular ROS, with the main sources of the ROS produced generated from mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX4), α-ketoglutarate dehydrogenase, monoamine oxidase and glycerol-3-phosphate dehydrogenase (Aon et al., 2012).

Nuclear Erythroid 2- Related Factor 2 (Nrf2)

Organisms designed to depend on oxygen to sustain life are continually exposed to damaging oxidizing agents. As a mechanism of defense, organisms which are dependent on oxygen have developed a highly evolutionarily conserved mechanism for preventing oxidative stress. The nuclear factor erythroid 2-related factor 2 (Nrf2), is the primary regulator of endogenous antioxidant response (McMahon et al., 2004, Lindl and Jordan-Sciutto, 2008). Nrf2 was cloned by Kan and coworkers in 1996, as a transcription factor that binds to the NF-E2 repeat of the β-globin gene promoter (Zhang, 2006). It belongs to the "cap 'n' collar" (CNC) subfamily of the basic leucine zipper transcription factors (Figure 1). Nrf2 is composed of six highly conserved homologue domains known as the Nrf2-ECH homology (Neh) domains, designated as Neh 1–6. The Neh1 domain consists of the CNC-bZIP domain that mediates the heterodimerization of Nrf2 with small Maf proteins (Motohashi and Yamamoto 2004). The N-terminal Neh2 domain allows for binding of Nrf2 to the Kelch domain of Keap1, which serves as a cytosolic repressor of Nrf2 (Zhang et al., 2004). The Neh2 domain also contains seven lysine residues for ubiquitin conjugation, conferring a negative regulation of Nrf2 activity through proteasome-mediated degradation (Zhang et al., 2004). The C-terminal Neh3 is involved in the transcriptional activity of Nrf2 by recruiting chromodomain-helicase-DNA-binding protein 6 (CHD6), (Zhang, 2006). The Neh4 and Neh5 are two independent transactivation domains that are rich in acidic residues and cooperatively recruit CREB-binding protein (CBP) to antioxidant response elements (ARE) regulated genes (Katoh et al., 2001 and Zhang, 2006). The Neh6 domain is essential for the degradation of Nrf2 and is known to be rich in serine residues (McMahon et al., 2004).



Adapted from (Hinoi et al., 2002)

Figure 1. A schematic representation of the basic leucine-zipper (b-ZIP): the leucine-zipper is made up of leucine residues at every seventh position which directs toward to one side of an α-helix and it mediates dimerization of proteins.

Keap1 regulation of Nrf2

In the cytoplasm, Nrf2 is bound to its cytoplasmic regulator known as the Kelch-like ECH-associated protein 1 (Keap1), which negatively regulates its activity (Zhang, 2006). Keap1, is found in the cytoplasm bound to actin protein. It is homologous to the Drosophila actin-binding protein, Kelch (Baird and Dinkova-Kostova, 2011). Keap1 contains three major domains which include; an N-terminal BTB (broad complex, tramtrack, and bric-a brac) domain, an intervening region (IVR), and a C-terminal Kelch domain, which consists of double glycine repeat (DGR) (Zhang, 2006 and Kobayashi et al., 2004). The BTB domain is involved in the homodimerization of Keap1. The intervening region is a cysteine-rich domain that has been proven to be indispensable for the activity of Keap1 and acts as an oxidative and electrophilic stress sensor (Zhang, 2006 and Kobayashi et al., 2004). The C-terminus contains six conserved Kelch repeat sequences and binds to the Neh2 domain of Nrf2 (Zhang, 2006). Under normal cellular conditions, Nrf2 is largely bound in the cytoplasm to Keap1. It has been established that Keap1 does not only prevent the nuclear translocation of Nrf2 in the absence of oxidative stress or inducers, but it also targets Nfr2 for ubiquitination and proteasomal degradation (Kobayashi et al., 2004).

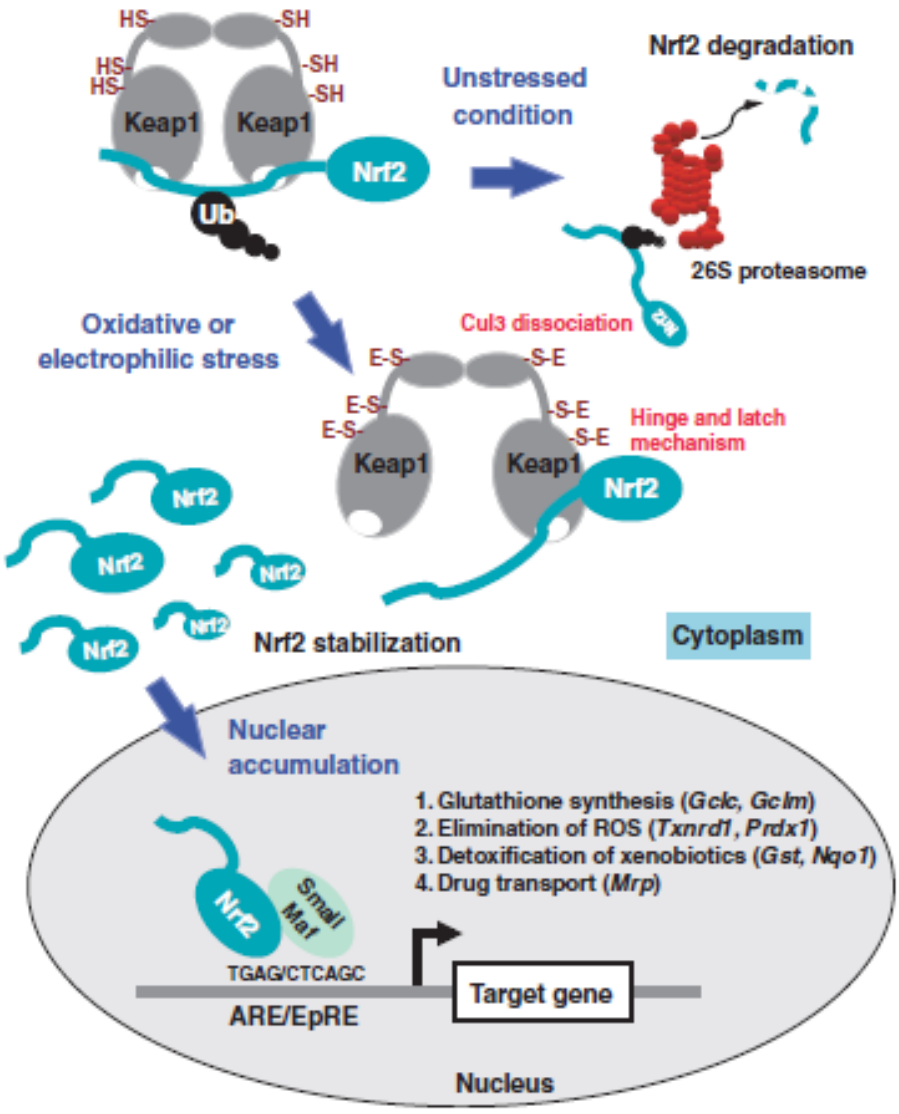
Disruption of Keap1 and Nrf2 Pathway

In the N-terminus of Keap1 including the BTB domain, Cullin-3 (Cul-3) is known to associate with Keap1. Thus, Keap1 serves as a substrate-specific adaptor for Cul-3 based ubiquitin E3 ligase and mediates polyubiquitination of Nrf2 (Taguchi et al., 2011 and Kobayashi et al., 2004). Under unstressed conditions, Nrf2 is constantly degraded with a half-life of less than 20 minutes (Taguchi et al., 2011). However, in the presence of electrophiles or ROS, the degradation of Nrf2 ceases and there is a disruption of the Keap1 and Nrf2 pathway (Figure 2). The dissociation of Nrf2 from

Keap1 is due to the cellular redox sensing mechanism of the cysteine residues found in the intervening region (IVR) of Keap1. There are about 27 cysteine residues in the human Keap1 protein which act as sensors for changes in the cellular redox environment and the residues at positions C257, C273, C288, C297, are known to be the most reactive (Zhang, 2006). The cysteine residues in Keap1 can undergo modifications such as forming disulfide bridges during oxidative stress, a reaction of an inducer (such as tert-Butylhydroquinone (tBHQ)) with two cysteine molecules in spatial proximity and an irreversible alkylation of the cysteine molecules, leading to the dissociation of the Keap1 and Nrf2 complex (Figure 2) (Dinkova-Kostova et al., 2002). The stabilized Nrf2 now accumulates in nucleus, heterodimerizes with small Maf proteins and activates target genes for cytoprotection through ARE or the electrophile response elements (EpRE) (Taguchi et al., 2011). The ARE are cis-acting transcriptional regulatory elements involved in the activation of genes coding for a number of antioxidant proteins and phase II detoxifying enzymes which include: NADPH quinone oxidoreductase (NQO1), glutathione peroxidase (GPx), ferritin, and hemeoxygenase (HO), xCT, (the core subunit of the cystine/glutamate membrane transporter, which is responsible for the uptake of cystine that in turn is rapidly reduced to cysteine and used for the synthesis of glutathione) (Chen et al., 2006 and Baird and Dinkova-Kostova, 2011).

In addition to its role in regulating antioxidant proteins and detoxifying enzymes, several recent studies using Nrf2-deficient mice have demonstrated that the Nrf2-ARE pathway is involved in immune and inflammatory processes. In one of such experiments, Nrf2-deficient mice developed a lupus-like nephritis (Chen et al., 2006 and Zhang, 2006). Moreover, increasing evidence points to a protective role of Nrf2 against many human pathological conditions, such as Alzheimer's and Parkinson's diseases, ischemia, aging and cardiovascular diseases (Chen et al., 2006 and Zhang, 2006). These findings suggest that Nrf2 deficiency and the resulting impaired antioxidant activity

are important in the determination of susceptibility to autoimmune and inflammatory diseases as well as other pathological conditions (Chen et al., 2006).



Adapted from (Taguchi et al., 2011)

Figure 2. Relationship between Nrf2 and Keap1: under quiescent conditions, Nrf2 undergoes ubiquitin-mediated degradation. However, the Keap1 and Nrf2 pathway can be disrupted through the following mechanisms;

(1) Reaction of a single inducer molecule such as phenylarsine oxide, with two cysteine residues that are in spatial proximity.

(2) Direct oxidation of the cysteine residues in the IVR of Keap1 to form a disulfide bridge in the protein

(3) An irreversible reaction with an inducer including, Dexamethasone-21-mesylate leading to cysteine alkylation (Dinkova-Kostova et al., 2002).

Cellular Antioxidant Defense

Glutathione (GSH)

To reduce oxidative damage to cellular structures and molecules, there are endogenous antioxidants that are capable of reacting with ROS and RNS to prevent or ameloriate the deleterious effects of these free radical compounds. One of such endogenous antioxidants is glutathione (GSH). Glutathione is a tripeptide consisting of L-glutamate, L-cysteine and L-glycine which exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) states, with GSH being the predominant form present in most cells (Lu, 2009). The concentration of GSSG is less than 1% of GSH and the ratio of GSH/GSSG is a good measure of oxidative stress in an organism (Mohora et al., 2007). Glutathione is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments (Mohora et al., 2007). Thus, GSH is synthesized in the cytosol via two ATP dependent processes as shown below;

(a) L-glutamate + L-cysteine + ATP → γ-glutamyl-L-cysteine + ADP + Pi

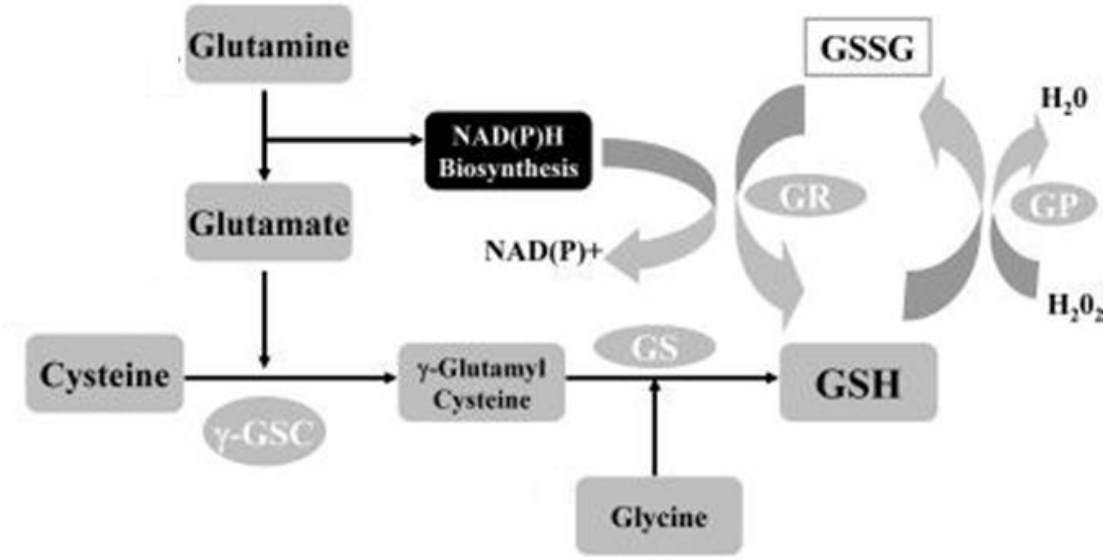
(b) γ-glutamyl-L-cysteine + L-glycine + ATP → GSH + ADP + Pi (Yan and Meister, 1990).

The first step in the synthesis of GSH involves the ligation of glutamate to cysteine catalyzed by glutamate cysteine ligase (GCL) to form γ-glutamylcysteine (γ-GC) (Figure 3). GCL consist of a heavy or catalytic unit (GCLC, Mr ~ 73,000) and a light or modifying unit (GCLM, Mr ~ 30,000), which are encoded by different genes in many species (Lu, 2009; Yan and Meister, 1990). GCL is regulated via a feedback inhibition by GSH binding competitively to glutamate and another site on the enzyme and also by the availability of its precursor (L-cysteine) (Lu, 2009). GCL is considered as the rate limiting enzyme in GSH synthesis because its product γ-glutamylcysteine,

is present at very low concentrations when GSH synthetase (GSS) is present (Grant et al., 1997). The overexpression of GSS fails to increase GSH levels whereas overexpression of GCL increases GSH levels (Grant et al., 1997). The second process involves the coupling of γ-GC with glycine, catalyzed by GSS (Lavoie et al., 2009). There is no feedback inhibition of GSS by GSH and this enzyme is not as important in GSH synthesis as GCL (Lu, 2009). However, GSS deficiency in humans is known to cause lower GSH levels and dramatic metabolic consequences because, the accumulated γ-glutamylcysteine is converted to 5-oxoproline, which can cause severe metabolic acidosis, hemolytic anemia, and central nervous system damage (Shi et al., 1996).

GSH-system is essential to neutralize ROS produced by the intense metabolism and oxygen consumption that occur physiologically. The major functions of GSH include; (i) glutathione is a cofactor of several enzymes that prevent oxidative stress, among which are glutathione peroxidase (GPx), glutathione reductase, glyoxalases and enzymes involved in leukotriene synthesis (ii) scavenging hydroxyl radical and oxygen molecules and directly detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase (iii) maintaining essential thiol status of proteins (iv) providing a reservoir for cysteine (v) modulating critical cellular processes such as DNA synthesis, microtubule related processes, and immune function (vi) it is also capable of recycling other antioxidants such as vitamins C and E, to their active forms (Mohora et al., 2007; Lu, 2009). Dehydroascorbic acid (DHA) and α-tocopheroxyl are oxidized forms of vitamins C and E respectively, which are reduced by GSH into their active forms to maintain a reduced milieu in cells (Li et al., 2001, Siow et al., 1998) (vii) GSH is also involved in maintaining nitric oxide (NO) homeostasis. S-nitrosoglutathione (GSNO) is formed by the binding together of GSH and NO (Hudson, 2001). Without the presence of GSH, the half-life of NO is ~ 3 to 7 seconds (Chapman, 2005), while the half-life of GSNO is approximately 5.5 hours. Thus, GSNO serves as

a reserve for NO and is a critical component in the availability of NO because it is the vehicle by which NO is transported within in the body (Kowaluk and Fung, 1990, Mathews and Kerr, 1993).



(Morris et al., 2008)

Figure 3. This diagram shows the synthesis of GSH and the regeneration of GSH and from GSSG.

(1) During NADH and NADPH biosynthesis, glutamine is converted to glutamate. Though this process is independent of GSH synthesis, the byproduct of the reaction can be used as a substrate for GSH synthesis.

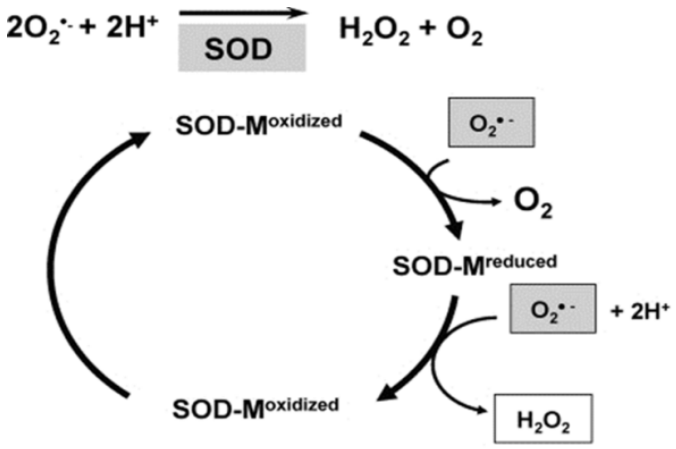
(2) Glutamate and cysteine are catalyzed by γ-GSC to γ-glutamyl-cysteine, which is metabolized to GSH through the actions of GSS.

(3) GSH is oxidized to GSSG during the scavenging of free radicals. It can also be enzymatically oxidized by glutathione peroxidase (GPx) during the reduction of hydrogen peroxide and other peroxides.

(4) GSSG is then reduced and recycled back to GSH by the NADPH-dependent glutathione reductase (GR).

Super oxide dismutase, SOD

Another antioxidant that is employed by cells in combating oxidative stress is the superoxide dismutase (SOD). SOD is an enzyme that catalyzes the dismutation of the superoxide anion to O_2 and H_2O_2, which in turn, can be removed by GSH peroxidase (GPx) and/or catalase (CAT) (Kankofer, 2002). There are three varieties of the SOD isoenzymes that have been identified. These include the Cu/Zn-SOD found in the cytoplasm and nucleus, Mn-SOD which is localized in the mitochondria and EC-SOD present mainly in extracellular spaces (Figure 4) (Huang et al., 2012). The Cu/Zn-SOD (SOD-1) is a homodimer with a molecular weight of about 32 kDa. It is susceptible to cyanide and hydrogen peroxide inhibition and found in the cytoplasm (Kankofer, 2002). The Mn-SOD form also known as SOD-2 is a tetramer with a molecular weight of about 80 kDa. The extracellular form (EC-SOD or SOD-3) is a tetramer with Cu/Zn and sugar residues (Kankofer, 2002). The mechanism of dismutation of $\bullet O_2^-$ to H_2O_2 by SOD involves alternate reduction and reoxidation of a redox active transition metal, such as copper (Cu) in SOD-1 and SOD-3 and manganese (Mn) in SOD-2 at the active site of the enzyme (Figure 3), which demonstrates that SOD activity involves a catalytic metal (Fukai and Ushio-Fukai, 2011). The enzymatic function of SOD-1 and SOD-3 hinges on the presence of Cu and Zn. Zn participates in proper protein folding and stability of the enzyme while the SOD activity is dependent on the amount of Cu bound in the native Cu site (Fukai and Ushio-Fukai, 2011). For proper functioning of the enzyme, Cu is not replaceable with another metal, whereas Zn can be replaced with cobalt and its absence does not alter the activity of the enzyme at low pH (Fukai and Ushio-Fukai, 2011).



(Fukai and Ushio-Fukai, 2011)

Figure 4. Mechanism of scavenging $^{\bullet}O_2^-$ by SOD. The enzymatic activity of SOD involves alternate reduction and reoxidation of catalytic metal (Cu or Mn) at the active site of the enzyme, represented by $M^{reduced/oxidized}$ (Fukai and Ushio-Fukai, 2011).

Among the members of the SOD family, Mn-SOD is the only enzyme that is essential for the survival of life in the aerobic environment under physiological conditions and this critical function may be due to the strategic location of Mn-SOD in the mitochondria (Dhar and Clair, 2012). This is because the mitochondria continuously generate potentially damaging ROS in the process of oxidative phosphorylation and when these ROS are not continuously detoxified by cellular antioxidants and antioxidant defense enzymes, it causes cellular damage which can drive cellular senescence (Velarde et al., 2012).

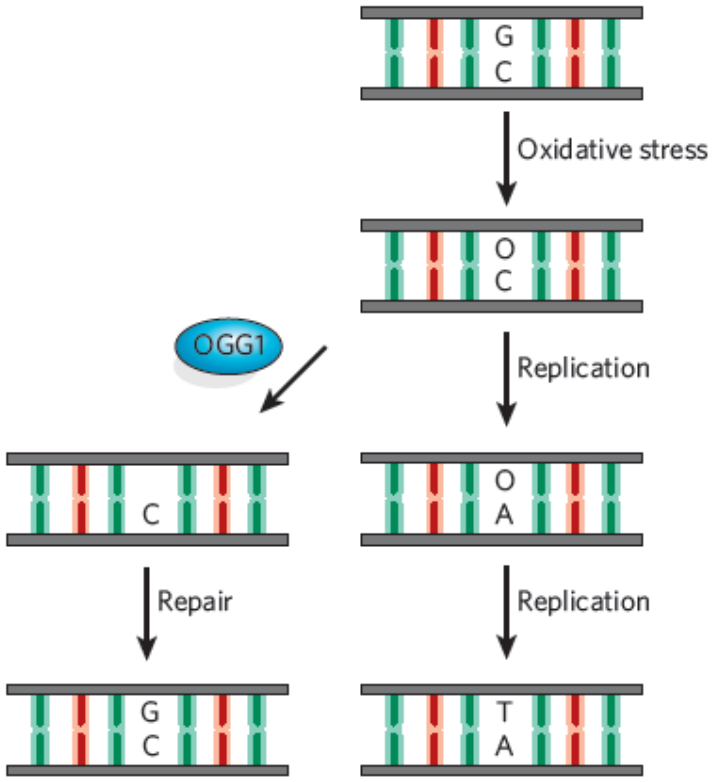
Antioxidant defense may also come from dietary sources such as vitamin C (L-ascorbic acid) and vitamin E (α-tocopherol). Ascorbic acid and α-tocopherol are known to prevent the oxidation of lipid and oxidative modification of low density lipoproteins (LDL) by scavenging reactive oxygen species in the aqueous environment (Villacorta et al., 2007).

OGG1 and DNA repair

Production of ROS and hence oxidative stress can result in a plethora of DNA lesions, including oxidized bases, abasic (AP) sites and strand breaks. If unrepaired, these DNA damages can compromise cell viability by blocking essential processes such as transcription or replication (Amouroux et al., 2010). Among oxidative lesions, 8-oxoguanine (8-oxoG) is one of the most common base lesions formed after oxidative attack to DNA (Lee, 2003). The low redox potential of guanine (G) makes this base particularly vulnerable and leads to a plethora of oxidized guanine products, one common example being the 7,8-dihydro-8-oxoguanine (David et al., 2007). The 7,8-dihydro-8-oxoguanine lesion is detrimental because it has the ability to mimic the function of thymine (T) and if not removed before replication can result in G-to-T transversion mutations (David et al., 2007). This DNA lesion has been implicated in carcinogenesis, ageing and several

age-related degenerative diseases (Habib, 2009). To prevent the mutagenic effects of 8-oxoG, there are DNA glycosylases that recognize 8-oxoG:C and catalyze the excision of 8-oxoG. In mammalian cells, the basic enzyme against the mutagenic effects of 8-oxoG in cellular DNA is 8-oxoguanine-DNA glycosylase (OGG1), which is not structurally related but functionally similar to formamidopyrimidine glycosylase (Fpg) in *Escherichia coli* (Lee, 2003).

The human OGG1 (*hOGG1*) gene is found on chromosome 3p26.2 and allelic deletion of this region occurs in a variety of human cancers (Lee, 2003). In human tissues, the expression of OGG1is relatively higher in the thymus, testis, kidney, intestine, brain and the germinal center of B cells as compared to other tissues (Lee, 2003). During base excision repair (BER), OGG1 excises 8-oxoG from the 8-oxoG:C base pair and it has an apurinic or apyrimidinic site (AP) lyase activity. After removing the base, OGG1 cleaves the DNA strand at the AP site resulting in a single-stranded break with blocked ends (David et al., 2007, Hegde et al., 2010) (Figure 5). The resulting nucleotide gap in the damaged strand is then filled by a DNA polymerase, typically DNA polymerase β (Pol β) and the final nick sealing is carried out by DNA ligase IIIα (LigIII α) (Hegde et al., 2010). The promoter region of hOGG1 has a putative binding site for the transcriptional factor Nrf2, which promotes the transcription of OGG1 (Piao et al., 2011). Consequently, OGG1 can be activated under oxidative stress as part of the cellular defense mechanism to combat ROS. A deficiency in BER can result in reduced stability of the cellular chromosomes which in turn can lead to mutagenesis, cellular dysfunction and abnormal phenotypes (Jeppesen et al., 2011). Genomic instability because of a deficiency in BER can increase the risk of cancer and neurological diseases. (Jeppesen et al., 2011).



(Adapted from David et al., 2007)

Figure 5. The process of DNA repair: The presence of 8-oxoG (O) in DNA can result in G-to-T transversions, as illustrated above. OGG1 removes 8-oxoG from 8-oxoG:C base pairs and creating an AP site. The actions of DNA polymerase and DNA ligase are summarized as 'repair' in the diagram.

SH-SY5Y Neuroblastoma Cells

Neuroblastoma is defined as an immature (blast) tumour (oma) of the nervous system and is one of the embryonic malignancies of childhood (Fisher and Tweddle, 2012). The tumors are derived from primordial neural crest cells that finally develop into sympathetic ganglia and the adrenal medulla. Neuroblastoma can develop anywhere along the sympathetic chain from the neck to the groin (Fisher and Tweddle, 2012).

SH-SY5Y is a human catecholaminergic neuroblastoma cell line that is a third clone from SK-N-SH cells. The SK-N-SH cells were originally obtained from a bone marrow biopsy of a neuroblastoma patient with a sympathetic adrenergic ganglial origin in the early 1970's (Xie et al., 2010, Tieu et al., 1999). The SK-N-SH cell line contains cells with three different phenotypes, which are the (i) neuronal (N type), (ii) Schwannian (S type), and (iii) intermediary (I type). The SH-SY5Y cell line mainly consists of neuroblast-like cell line (N type) (Xie et al., 2010). These cells have been proposed as a useful in vitro model for studying normal neurons as they possess many biochemical and functional properties of neurons (Xie et al., 2010). SH-SY5Y cells provide some benefits over the use of primary neurons, some of which include;

(i) this neuronal cell line is obtained from human origin, a source from which primary neurons are difficult to obtain.

(ii) the cell population is relatively homogenous, which hypothetically translates into reproducibility.

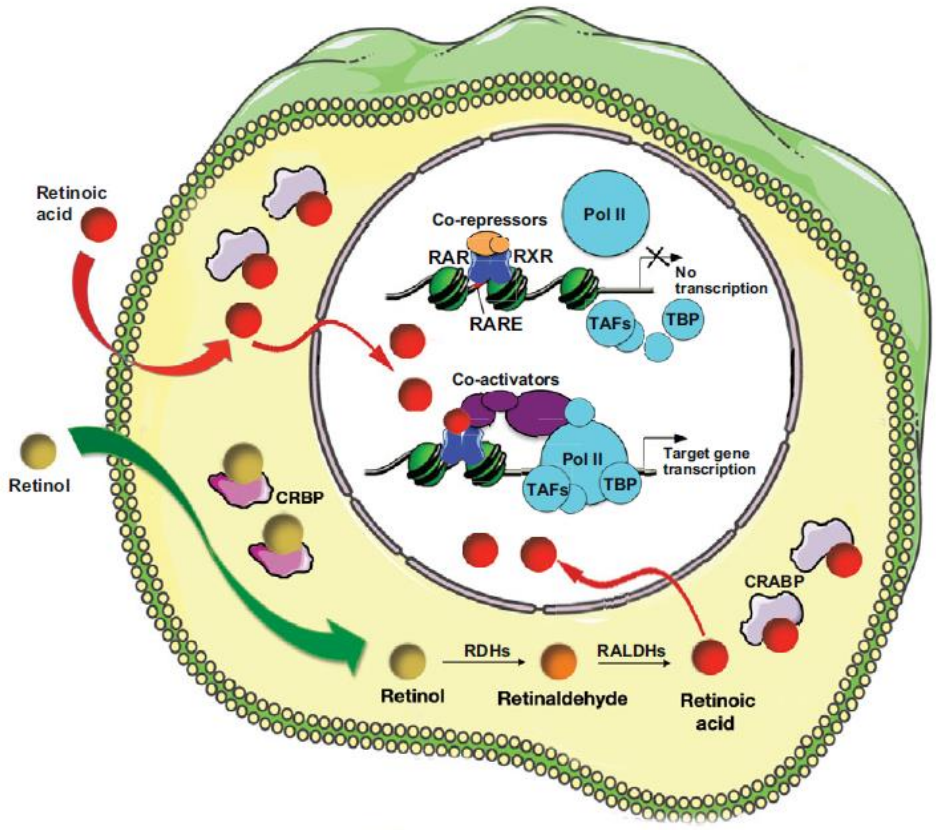
(iii) moreover, because these cells are oncogenic, they are mitotic and thus propagation to yield a sufficient quantity for investigations can be achieved (Tieu et al., 1999).

The SH-SY5Y cell line exhibits neuronal marker enzyme activity (tyrosine and dopamine-β-hydroxylases), specific uptake of norepinephrine (NE) and express neurofilament proteins. They also express opioid, muscarinic, and nerve growth factor receptors (Ciccarone et al., 1989). SH-SY5Y cells can be induced to differentiate upon treatment with various agents such as retinoic acid (RA), the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), brain derived neurotrophic factor (BDNF), dibutyryl cyclic AMP (dBcAMP), purine, or staurosporine (Xie et al., 2010). A number of neurological studies consisting of analysis of neuronal differentiation, metabolism, and function related to neurodegenerative and neuroadaptive processes, neurotoxicity, and neuroprotection involved the use of SH-SY5Y cell line (Xie et al., 2010). Most of these studies either used differentiated or undifferentiated cells, especially as a cell model of Parkinson's disease (PD). However, use of undifferentiated SH-SY5Y cells as PD cell model involves some disadvantages, which is discussed in chapter 2.

Retinoic Acid Differentiation of SH-SY5Y Cells

Retinoic acid (RA) is derived from the liposoluble vitamin A (retinol). Vitamin A is known to be indispensable for vision, as its derivative retinaldehyde acts as a light sensitive molecule, the isomerization of which triggers the phototransduction process in photoreceptor cells of the retina (Rhinn and Dollé, 2012). The source of retinoids in most animals is derived from diet, because these compounds are not synthesized de novo. The main circulating retinoid in mammals is retinol, which is bound to a carrier protein known as the retinol-binding protein 4 (RBP4). Typically, in mammals RBP4 is taken up by target tissues and this uptake is facilitated in some tissues by a transmembrane protein that is the product of the RA-inducible gene STRA6 (stimulated by retinoic acid 6) (Rhinn and Dollé, 2012).

The synthesis of RA begins with the oxidation of retinol to retinaldehyde by two enzymes which include the various cytosolic alcohol dehydrogenases (ADHs) and microsomal dehydrogenases/reductases (retinol dehydrogenases, RDHs) (Rhinn and Dollé, 2012, Duester, 1998). ADH5 (previously called ADH3) is ubiquitously expressed in the embryo and adult, whereas ADH1 and ADH7 (previously called ADH4) are tissue restricted (Rhinn and Dollé, 2012, Duester, 1998). The following step in RA synthesis is the oxidation of retinaldehyde to RA which is carried out by three retinaldehyde dehydrogenases (RALDHs) namely; RALDH1, RALDH2 and RALDH3 (Rhinn and Dollé, 2012). The two classes of receptors that mediate the effects of RA are nuclear receptors which involve retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Kim et al., 2000). In the absence of a ligand, the presence of co-repressors prevents the interaction of RAR/RXR with promoter regions of target genes (Figure 6). Upon RA binding, a conformational change in the helicoidal structure of the RAR ligand-binding domain changes its protein-protein interaction properties. Subsequently, there is a release of the co-repressors and recruitment of co-activator complexes that destabilize the nucleosomes and/or facilitate assembly of the transcription pre-initiation complex (Rhinn and Dollé, 2012). The transcriptional initiation complex consist of; RNA polymerase II (Pol II), TATA-binding protein (TBP) and TBP associated factors (Rhinn and Dollé, 2012). Some of the target genes of RA include nuclear receptors such as retinoic acid receptor α, β and ɣ (Rarb α, Rarb β, and Rarb ɣ) and members of the homeodomain (Hox) transcription factors including; pancreatic and duodenal homeobox 1 (Pdx1), paired-like homeodomain transcription factor 2 (Pitx2) and T-cell leukemia homeobox 2 (Tlx2) (Balmer and Blomhoff, 2002). RA initiates differentiation by regulating the transcription of neurotrophin receptor genes, the Wnt signaling pathway and pathways involving type II protein kinase A (Clagett-Dame et al., 2006, Kim et al., 2000).



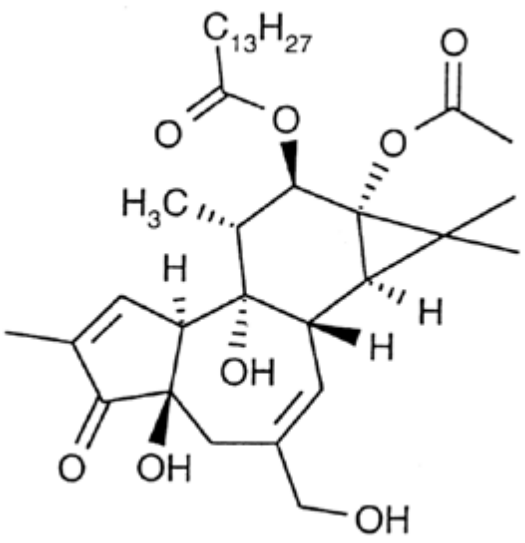
(Adapted from Rhinn and Dollé, 2012)

Figure 6. Action of retinoic acid: RA is synthesized intracellularly from circulating retinol or diffuses from an adjacent cell (red arrow) and finally reaches the nucleus. The cellular retinol-binding proteins (CRBPs) help present retinol to retinol dehydrogenases (RDHs) for RA synthesis. Cellular retinoic acid-binding proteins (CRABPs) are proposed to be involved in the transfer of RA into the nucleus. The dimers of RA receptors (RARs) and retinoid X receptors (RXRs), collectively known as RAR/RXR, then bind to RA-response elements (RAREs) in their target genes. In the absence of a ligand, the presence of co-repressors prevents the binding of these receptors to their target genes.

Previous studies have demonstrated that the phenotype of RA-treated SH-SY5Y is that of matured cholinergic phenotype and that there is a higher expression of vesicular monoamine transporter (VMAT) and an increase in choline acetyl transferase (ChAT) activity (Xie et al., 2010). Treatment of SH-SY5Y cells with RA alone induces a cholinergic phenotype, however, there are reports that a combined treatment of RA and phorbol esters produce a dopaminergic neuronal phenotype (Xie et al., 2010).

Phorbol Esters (TPA) as Differentiating Agents in SH-SY5Y Cells

The term phorbol is a description of a family of naturally occurring compounds that can be referred to as tigliane diterpenes. Phorbol esters are poly-cyclic compounds in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids (Goel et al., 2007). The hydroxylation of this basic structure at different positions and the bonding of esters to the various acid moieties results in the formation of varieties of phorbol ester compounds (Goel et al., 2007).The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Figure 7) is the active component of croton oil and its known to be a potent tumor promoter (Kolb and Davis, 2004). TPA imitates the action of diacyl glycerol (DAG) and activates protein kinase C (PKC), which in turn regulates different signal transduction pathways and other cellular metabolic activities (Kolb and Davis, 2004, Huang et al., 1995). The activation of PKC by TPA leads to the regulation of many transcription factors, such as AP1, NF-KB, p62TCF and other proteins, to alter the expression of cellular genes. The induction of immediate early genes which are fos, myc, and jun are observed in many different cell types but the overall biological response to TPA can be divergent (Huang et al., 1995). Though phorbol esters were originally identified as promoters of papilloma development in mice, TPA has also been reported to inhibit the growth and suppress the tumorigenicity of human HT29 colon cancer cells that overexpress the β-1 isoform of PKC.



(Modified from Nakagawa, 2012)

Figure 7. The chemical structure of 12-O-tetradecanoylphorbol 13-acetate (TPA): TPA is one of the most potent phorbol esters and at nanomolar concentration induces an increase in the concentrations of noradrenalin and neuron-specific enolase (Mattsson et al., 1986).

In some cases such as in hematopoietic cells, TPA could stimulate the proliferation of peripheral blood lymphocytes but promotes the differentiation and growth arrest of many leukemia cell lines, such as HL60 and K562 (Huang et al., 1995).

Thus, depending on the cell type, the response to TPA could differ. In SH-SH5Y cells, TPA causes cell growth and differentiation in nanomolar concentrations (Xie et al., 2010). TPA treated SH-SY5Y cells have low ornithine decarboxylase (ODC) activity and they are partially growth inhibited. The activity of ODC cannot be stimulated even after changing to fresh medium (Xie et al., 2010). ODC is the rate-limiting enzyme in the biosynthesis of polyamines, and during mitogenic stimulation of cultured cells including SH-SY5Y cells, ODC activity is rapidly induced (Mattsson et al., 1986). The induction of ODC is, however, abolished by TPA treatment.

Studies have revealed that both IGF-I-and II promote growth of SH-SY5Y cells and that the mitogenic effect of these factors in TPA-differentiated cells is essentially lost. Moreover, the binding sites for IGF-II, present in undifferentiated cells, disappear almost completely after TPA treatment (Mattsson et al., 1986). This might explain the low activity of ODC, which is stimulated by IGF-1 and IGF-II.

As stated earlier, the differentiating effects of phorbol esters are primarily mediated by protein kinase C (PKC) isoforms that might enhance norepinephrine (NE) release in SH-SY5Y cells by enhancing Ca^{2+} channel activity (Mattsson et al., 1986, Xie et al., 2010). Morphologically, TPA-treated cells acquire long cell processes and an increased amount of neurosecretory granula (Mattsson et al., 1986). TPA treatment also leads to increased resting membrane potential and the cells are depolarized by high concentrations of potassium. TPA at an optimal concentration also induces a 200 fold increase in NE whereas only a 4 fold increase is observed with RA treatment

(Mattsson et al., 1986, Xie et al., 2010). Therefore, treatment of SH-SY5Y cells with TPA induces an adrenergic phenotype compared to the phenotype seen after treatment with RA.

There are a number of neurological studies involving the use of SH-SY5Y cell line. Most of these studies however, either used differentiated or undifferentiated cells as models for studying neurotoxicity, neurodegeneration, metabolism and neuroprotection. None of these investigations involved the use of both differentiated and undifferentiated cells in the same study in the determination of antioxidant response and capacity, neuroprotection and neurodegeneration.

CHAPTER TWO

The differences between Differentiated and Undifferentiated SH-SY5Y Cells

Differentiation induces neuronal phenotype in SH-SY5Y Cells

Some investigations in the field of neuroscience are restricted by the lack of appropriate in vitro models resembling functionally mature neurons that express human proteins. In the case of Parkinson's disease, the development of a reliable dopaminergic neuronal cell model is specifically important for studying the pathogenesis of PD and developing therapeutic strategies. Ideally, an in-vitro PD cell model should be established in post-mitotic human dopaminergic neuronal cells susceptible to neurotoxins produced during PD so as to address questions regarding the selective loss of dopaminergic neurons in the substantia nigra (Xie et al., 2010). Using primary mesencephalic neurons are a good representation of dopaminergic neurons. However, human primary neurons are extremely difficult to obtain, culture, and maintain (Xie et al., 2010). There are also ethical challenges in obtaining sufficient and appropriate human primary neurons which also limit the use of this cell source in experiments. Using of rodent cells, including primary or immortalized lines, such as PC12 cells, face the problem of slight but relevant metabolic differences between rodents and humans (Herman, 2002). The SH-SY5Y cell line provides an unlimited supply of cells of human origin with similar biochemical characteristics to human dopaminergic neurons (Xie et al., 2010). These characteristics of SH-SY5Y cells make them suitable for studies involving neurodegeneration, toxicity and differentiation. Though SH-SY5Y cells have the ability to proliferate in vitro, they stop proliferating, show extensive neurite outgrowth as well as become a more stable population after differentiation. This means that there can be two phenotypes to this cell line, depending on whether they are differentiated or not. Both differentiated and undifferentiated cells have however been used in experiments. For example,

undifferentiated SH-SY5Y cells have been extensively used as model cell line for many neuronal pathological conditions such as PD (Cheung et al., 2009). The disadvantages in using undifferentiated cells include;

(i) Undifferentiated SH-SY5Y cells in cultures are unsynchronized and may not exhibit the typical markers of mature neurons, which leads to uncertainty in experiments.

(ii) The cells continuously undergo division, making it difficult to predict the effect of toxins or protective agents.

(iii) These cells are relatively deficient in dihydroxyphenylalanine (DOPA) decarboxylase activity thus, having lower synthesis of dopamine and norepinephrine.

(iv) Even though undifferentiated SH-SY5Y cells express dopamine-β-hydroxylase and tyrosine hydroxylase (TH) activity, dopamine and norepinephrine synthesis may be limited by a deficiency of dihydroxyphenylalanine decarboxylase activity (Xie et al., 2010).

These suggest that although undifferentiated SH-SY5Y cells offer a good model for neuronal pathological research, these weaknesses mean that the cell line is not an ideal model.

Similarly, there are many lines of evidence that indicate that differentiation of SH-SY5Y cells result in a functionally mature neuronal phenotype. The characteristics of differentiated cells are;

(i) Cells stop proliferating, become a stable population, and show extensive neurite outgrowth with morphological similarity to living neurons.

(ii) Differentiated SH-SY5Y cells are more excitable and their membrane potential increase relative to undifferentiated cells.

(iii) These cells have high levels of dopamine-β-hydroxylase, TH and dopamine transporter (DAT) activity.

(iv) Evidence shows that protein levels of G-protein coupled D2 and D3 dopamine receptors are elevated in differentiated SH-SY5Y cells (Ciccarone et al., 1989, Xie et al., 2010).

Despite the differences in the phenotypes of differentiated and undifferentiated cells, there are several studies that have involved either of the two for neurological studies. Since the brain consists largely of non-proliferative (post-mitotic) neuronal cells and proliferative (mitotic) glial cells, it might be appropriate for experiments conducted with SH-SY5Y cells to be done in both undifferentiated and differentiated cells at the same time (Cheung et al., 2009).

Hypothesis and Specific Aims

ROS production is higher in post-mitotic cells as compared to mitotic cells, due to the fact that oxygen consumption in post mitotic cells is greater than in mitotic cells (Chen et al., 2009). Post-mitotic and proliferating cells will have differences in antioxidant response and capacity. Therefore, this study was conducted to test the hypothesis that due to the process of differentiation there will be differences in antioxidant response and/or capacity between differentiated (post-mitotic) and undifferentiated (proliferating) SH-SY5Ycells. To test this hypothesis, the main objective was;

1) To determine antioxidant levels in differentiated and undifferentiated SH-SY5Y cells; mainly looking at the response to Nrf2 by treating cells with tBHQ, an Nrf2 inducer.
2) To determine the differences between DTNB and HPLC in measuring GSH levels in SH-SY5Y cells.

To determine the response to Nrf2 in SH-SY5Y cells, tBHQ a synthetic phenolic antioxidant (a metabolite of butylated hydroxyanisole (BHA)) used as a model inducing agent for ARE-driven transcription via the Nrf2-Keap1 pathway was used. The levels of GSH, HO, OGG1 and SOD were determined in differentiated and undifferentiated cells after treatment with tBHQ.

Materials and Methods

Cell culture

SH-SY5Y human cells were grown to confluence in T-75 flasks with 1X Dulbecco's Modification of Eagle's Medium (ATCC #30-2006) supplemented with 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate, and 1200 mg/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 1X non-essential amino acids, 1000 international units penicillin, 1mg/mL streptomycin, and incubated at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed twice a week and cells were passed at about 90% confluence.

Differentiation and treatments

For differentiation, unless stated otherwise, cells were first treated with 10 μM of RA (Påhlman et al. 1984) for 3 days. The medium was then changed and the cells were treated with 80 nM TPA (Döşemeci et al. 1988) for another 3 days (6 days of differentiation). For treatments, all differentiated and undifferentiated cells were treated with 25 μM of tBHQ (Alfa Aesar) or equivalent volume of dimethyl sulfoxide DMSO (0.0025%) used as a vehicle for 24 hours before assays were performed.

Neurite length determination

A total of 3×10^4 cells in 12 mL of culture medium were plated in a T-75 flask. After 3 days, images of neurites were taken and counted under the microscope for undifferentiated cells. After differentiation of cells, images were taken at room temperature using Zeiss Axiovert 40 C Microscope with a Zeiss Axiocam as analysis camera and 320x magnification. Determination of soma size and neurite lengths were determined with ImageJ. Extensions with length twice the size of the soma were counted as neurites.

Determination of membrane excitability

12mm round cover slips (Carolina Laboratories) were soaked overnight in nitric acid. Afterwards the cover slips were washed under running MilliQ water for an hour to completely wash out the nitric acid. The slips were subsequently stored in 100% ethanol for at least 30 minutes before using them in cell culture. 4 cover slips were placed in each well of a 6-well plate after the slips were shortly passed through a Bunsen burner to get rid of the ethanol. Cultured cells which adhered to coverslip were transferred to a submerged chamber and perfused by extracellular solution containing:125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 2 Mm $CaCl_2$, 1mM $MgCl_2$, 1.25mM NaH_2PO_4 and 20mM glucose, saturated with 95% O_2, 5% CO_2, at pH 7.4. Cells were visualized using IR-Dodt contrast and fluorescence video-microscopy on either a Slice platform (Scientifica, East Sussex, United Kingdom) or Infrapatch (Luigs and Neumann, Ratingen Germany) on an upright Zeiss microscope (Axio examiner D1, Carl Zeiss Microscopy, LLC, U.S.). Pipettes were fabricated with a 3-4 MΩ tip resistance on a PC-10 Narishige vertical puller. An electrode was filled with an intracellular solution containing: 110 mM potassium gluconate, 40 mM KCl, 10 mM HEPES, 0.1 mM EGTA, 4 mM MgATP, 0.3 mM Na_2GTP, 10 mM phosphocreatine and 0.2%

biocytin at pH 7.2 and osmolarity at 295-305 Osm. Cells were differentiated with either RA or TPA only for 3 or 6 days or a combined treatment of RA/TPA for 6 days. Undifferentiated cells or differentiated cells under different treatments were tested to obtain their electrophysiological parameters including resting membrane potential (RMP) and Resistance of membrane (Rm). A 10 step 1s long protocol with current injection from -20 pA to 70 pA was applied to measure the voltage responses to current injection.

Viable cell counts using trypan blue dye exclusion assay

Cells were seeded in 6-well plates. After 48 hours, undifferentiated cells were treated with 25, 50, 75 and 100 μM of tBHQ for 24 hours. After differentiation, cells were treated with the different concentrations of tBHQ as indicated for 24 hours. The last row of each 6 well plate was used for the control (DMSO treated cells) group. Cell viability was determined by the trypan blue dye exclusion assay. An aliquot of 0.5mL of cell suspension obtained from differentiated and undifferentiated SH-SY5Y cells was mixed with 0.5 mL of 0.4% trypan blue dye and left for 5 minutes at room temperature. The cells were counted using the hemocytometer and the percentage of live cells was determined. The viability of cells after tBHQ treatments were standardized to that of the control group.

GSH Determination using DTNB method

A total of 5 x 10^6 cells were plated in T-75 flask. Cells were grown to confluence (12-15x10^6 cells/flask) before conducting this assay. GSH measurement was assessed for both differentiated and undifferentiated cells using the method as described by Tietze (1969), Hu (1994) and Eyer et al. (2003) with modifications. Cells were washed and scraped out of the flasks in ice-cold phosphate-buffered saline (without calcium and magnesium ions). Cells were then sonicated on

ice in 300 μL EDTA (10 mM) and centrifuged at 14000 rpm at 4˚ for 15 minutes. An aliquot (50 μL) was reserved for measurement of protein content using the Bradford reagent (Bio-Rad; Hercules, USA). Proteins in the supernatant (200 μL) were precipitated with 20% sulfosalicylic acid (65μL) and removed by centrifugation at 10000g for 10 minutes at 4°C. Next, 95μL of supernatant was used for GSH assay, which was conducted in a 96-well plate with 0.5 M Na_2HPO_4, 10mM DTNB and 20% sulfosalicylic acid. The fluorescence intensity was immediately measured in a fluorometer Spectra Max Gemini (Molecular Devices; Sunnyvale, USA) with an excitation wavelength of 412 nm and an emission wavelength of 530 nm. GSH levels were calculated in nmol GSH/mg protein.

GSH measurement, a comparison of DTNB and HPLC

GSH levels in differentiated and undifferentiated SH-SY5Y cells were determined by employing DTNB and HPLC methods to assess the similarities or differences in the results of both methods. Cells were grown to confluence in T-75 flasks. After tBHQ treatments for 24 hours, the cells were washed, scraped and collected with ice-cold PBS. Cells collected from the same flask were divided into two for the determination of GSH using HPLC and DTNB methods. GSH determination using DTNB was performed as stated previously. The HPLC method was done as described by Rebrin et al. (2003, 2007). Cells were immediately placed in ice-cold buffer containing 50 mmol/L potassium phosphate, 2 mmol/L EDTA, and 0.1 mmol/L butylated hydroxytoluene, (pH 7.4) and stored at −80°C. Cells were subsequently homogenized in 10 mM Tris buffer containing 0.32 M sucrose and 1 mM EDTA, (pH 7.1). Aliquots from the homogenized samples were treated with metaphosphoric acid (MPA, Sigma) with a final concentration of 20% (w/v). The mixture of ice-cold 20% (w/v) MPA and the crude homogenates were incubated for 30 minutes and centrifuged for 20 minutes at 14000 × g at 4°C. Supernatants were transferred to autosample vials and were

injected immediately. The mobile phase for the isocratic elution consisted of 25 mM monobasic sodium phosphate, 0.5 mM of 1-octane sulfonic acid, 2.5% acetonitrile, at pH 2.7, adjusted with 85% phosphoric acid. The flow rate was 0.9 ml/min. Calibration standards are prepared, by the dilution of 2 mM stock solutions of GSH and GSSG (oxidized GSH) in 5% (w/v) meta-phosphoric acid. The four-array electrode system used was 1 = +300, 2 = +450, 3 = +600, 4 = +900 mV. Electrode 1 served as a screening electrode to oxidize potentially interfering compounds. Data were collected, analyzed and stored using CoulArray for Windows data analysis software (ESA, Inc.).

GSH was detected in electrodes 2 and 3, while GSSG was monitored in electrodes 3 and 4. GSSG was the last eluting peak after 12 minutes with GSH appearing after 5 minutes.

SOD measurement

The determination of SOD activity was carried out according to manufacturer's instructions (Cayman Chemical Company, MI, USA) for differentiated and undifferentiated cells after treatment with tBHQ or DMSO for 24 hours. This assay utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Cells were harvested and homogenized in cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 20 mM mannitol and 70 mM sucrose. The homogenized samples were then centrifuged at 1500 x g for 5 minutes at 4°C. The supernatant was taken out for assay. To determine the amounts of Cu/Zn-SOD and Mn-SOD in the cells, 3 mM KCN solution was added to the assay mixture to inhibit Cu/Zn-SOD, while Mn-SOD remained unaffected. The reaction mixture was incubated for 20 minutes at room temperature and absorbance was read at 440nm using fluorometer Spectra Max Gemini (Molecular Devices; Sunnyvale, USA). SOD activity was calculated in U/mg protein.

Determination of HO and OGG1 expression using Real-Time PCR

Total RNA was extracted from 5 x 10^6 cells cultured in 6-well plates using Trizol reagent (Invitrogen, USA) after treatment with tBHQ or DMSO for 24 hours. 200 µL of chloroform was added to the sample, vortexed and incubated for 5 minutes, followed by centrifugation at 4°C and 12000 rpm for 15 minutes. The aqueous phase was removed and transferred to a tube containing 500 µL of isopropanol, mixed and incubated overnight at -20°C. Subsequently, samples were spun at 4°C, at 12000 rpm for 15 minutes, the supernatant was discarded and the pellet was resuspended in 100µL Rnase free H_2O followed by incubation at 55°C for 10 minutes. RNA extraction was completed using Total RNA kit I (Omega Bio-Tek, USA). The reverse transcription and real-time PCR were performed using qScript One-Step SYBR Green qRT-PCR Kit, Low ROX (Quanta Biosciences). Human β-actin, OGG1and HMOX reverse and forward primers designed for real-time PCR were synthesized by IDT (Integrated DNA Technologies) according to the following sequences β-actin: forward 5′-CCAACCGCGAGAAGATGA-3′, reverse 5′-TCCATCACGATGCCAGTG-3′, OGG1 forward 5′-CTGCATCCTGCCTGGAGT-3′, reverse 5′-CCTGGGGCTTGTCTAGGG-3′, HO forward 5′-CAGTCAGGCAGAGGGTGATAG-3′, reverse 5′-CCTGCAACTCCTCAAAGAGC-3′. Real time PCR reactions were performed on Mx3005P QPCR system (Agilent Technologies). The expressions of OGG1and HO in tBHQ treated SH-SY5Y cells were determined by the ΔΔCt method as described by Schmittgen and Livak (2008), using DMSO treated SH-SY5Y cells as internal control and β-actin as the reference gene. All target quantities were normalized to the control values and expressed as fold changes relative to the control.

Measurement of OGG1 enzymatic activity

The extraction and activity of OGG1 was performed as described by Bolin et al. (2004). A synthetic oligonucleotide containing oxo8dG 5′-GAACTAGTGOATCCCCCGGGCTGC-3′, where O is oxo8dG (Trevigen Inc., Gaithersburg, MD), was labeled with ^{32}P at the 5′ end using T4 polynucleotide kinase (Roche Diagnostics, Indianapolis, IN). The radiolabeled oligonucleotide was then annealed to its complementary oligonucleotide. An amount of 3, 5 and 10μg of nuclear protein extract and the double-stranded radiolabeled probe were incubated for 2 hours at 37ºC. After the reaction was stopped, the samples were denatured by heating to 100˚C and loaded onto a 20% polyacrylamide gel in 7 M urea and 1x TBE running buffer. Gels were analyzed using FLA-3000 Series Fuji Film Fluorescent Image Analyzer and Image Gauge V3.3 analysis software. The ability of the nuclear extract (3, 5 and 10μg total protein) to remove oxo8dG was expressed as percentage of synthetic probe cleaved (containing oxo8dG) in proportion to total synthetic probe in the reaction (sum of cleaved and uncleaved probe).

Statistical Analysis

Data were expressed as mean ± SEM and analyzed using the student t-test for independent analysis or one way analysis of variance with Turkey's post-hoc analyses as appropriate; $p < 0.05$ was considered statistically significant. All statistical tests were performed using GraphPad ® Prism statistical software (Sorrento, CA).

CHAPTER 3

RESULTS

Introduction

This study employed the use of SH-SY5Y neuroblastoma cells to study the differences in antioxidant response and capacity between undifferentiated (mitotic) and differentiated (post-mitotic) cells. Several evidence have shown that Nrf2 induces the expression of antioxidants after treating cells with tBHQ, which is a major metabolite of 3-tertbutyl-hydroxyanisole (BHA) (Lavoie et al, 2009). In this experiment, 25 µM concentration of tBHQ was used to induce Nrf2 and the levels of antioxidants were determined in undifferentiated and differentiated SH-SY5Y cells.

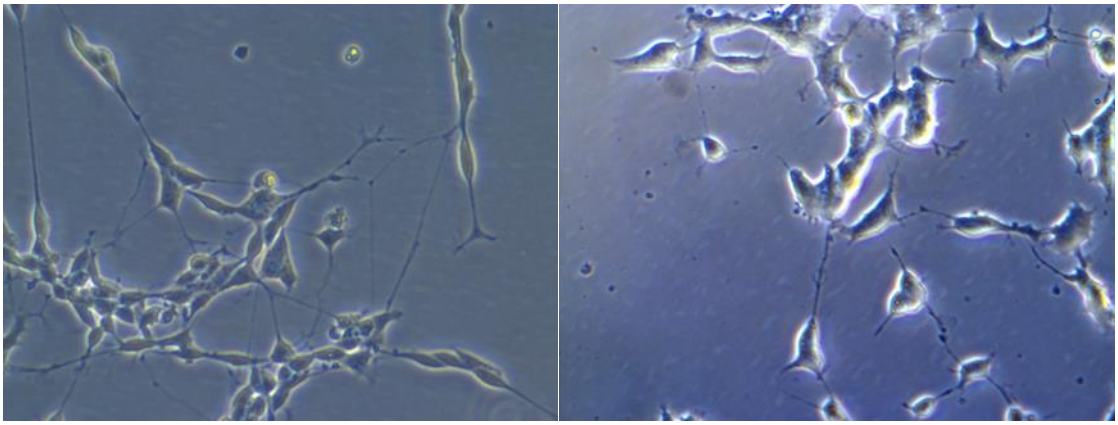
Differentiation and neurite length determination

To ensure that treatment of the cells with RA/TPA induced differentiation, neurite lengths of differentiated and undifferentiated cells were determined. One characteristic of differentiated cells is their ability to grow longer prolongations than undifferentiated cells. After 6 days of differentiation and 3 days of seeding (for undifferentiated cells), images of cells were captured with a Zeiss Axiovert 40 C Microscope and Zeiss Axiocam analysis camera. The acquired images (8A) were then analyzed using ImageJ software to determine lengths of neurites for both undifferentiated and differentiated cells (8B). Differentiated cells had significantly longer neurites than undifferentiated cells.

Figure 8. Neurite length determination.

(A)

Differentiated cells Undifferentiated cells



(B)

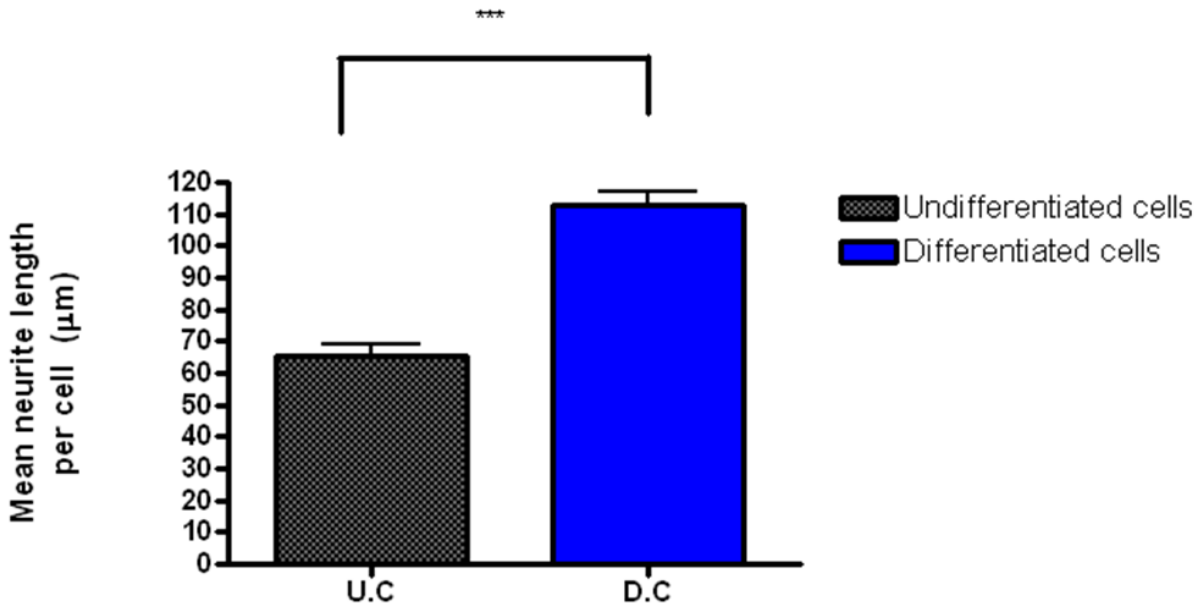


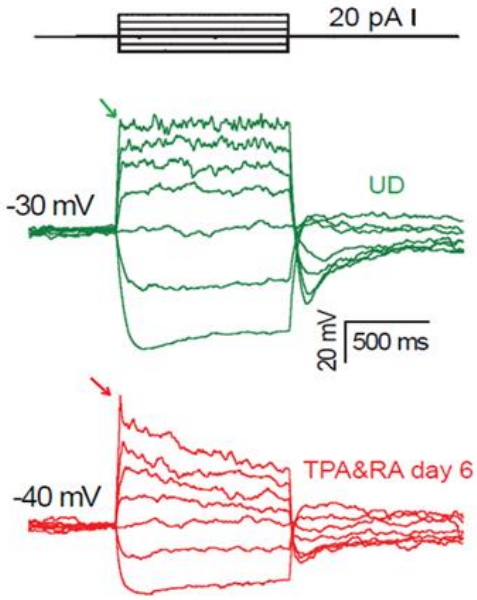
Figure 8. Neurite length determination. The lengths of neurites were determined using ImageJ software and results show mean neurite length pooled from four independent experiments. SH-SY5Y cells differentiated with RA/TPA for 6 days had longer neurite length than undifferentiated cells. Each column represents mean ± S.E.M. (n=57-74 per group), *** indicates significant difference from undifferentiated cells $P<0.0001$; student t-test for independent samples. Images captured at 320x magnification.

Determination of membrane excitability

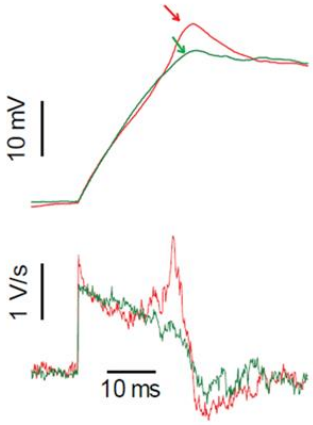
It has been demonstrated that differentiation of SH-SY5Y cells concomitantly induces electrophysiolgical properties which enhance their neuronal characteristics. Differentiated cells develop more excitable membranes than undifferentiated cells with the ability to undergo an action potential. To determine the excitability of differentiated and undifferentiated cells, their resting membrane potential (RMP) and membrane resistance (Rm) were measured. Because both RA and TPA were used in differentiating the cells, for the electrophysiological measurements cells were differentiated with RA or TPA only for either 3 or 6 days and RA/TPA combined treatment for 6 days. This was to ascertain the excitability of the cells when the combined treatment of RA/TPA was used as compared to either treatment alone. In all cases of differentiation, the cells had a more negative resting membrane potentials as compared to undifferentiated cells as seen in Figure 9D. The membrane resistances of all differentiated cells except the cells differentiated with RA for 3 days were lower as compared to undifferentiated cells (Figure 9E).

Figure 9. Electrophysiological properties of differentiated and undifferentiated cells.

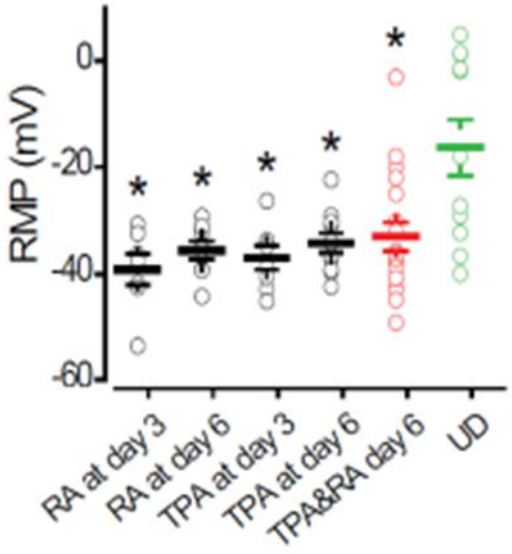
(A)Voltage Responses after Injecting Depolarizing Currents



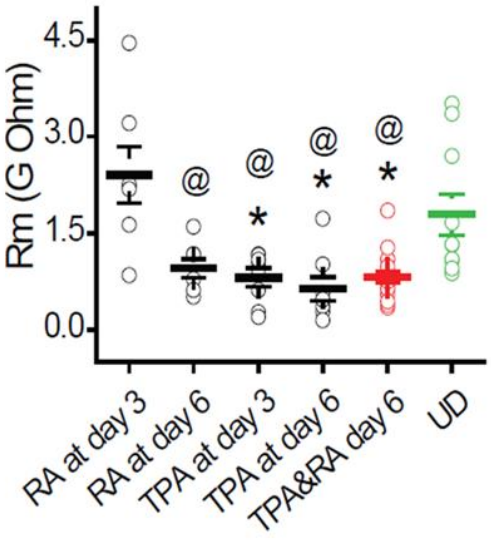
(B) Expanded Voltage Responses



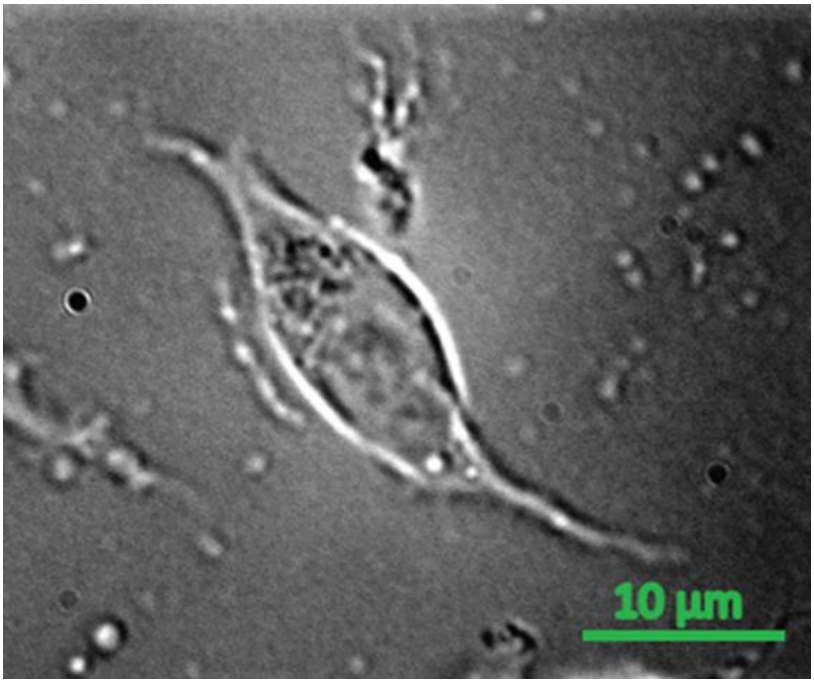
(C) Resting Membrane Potentials



(D) Membrane Resistances



(E) An image of an undifferentiated SH-SY5Y cell used in the electrophysiological analysis



(F) An image of a differentiated SH-SY5Y cell used in the electrophysiological analysis

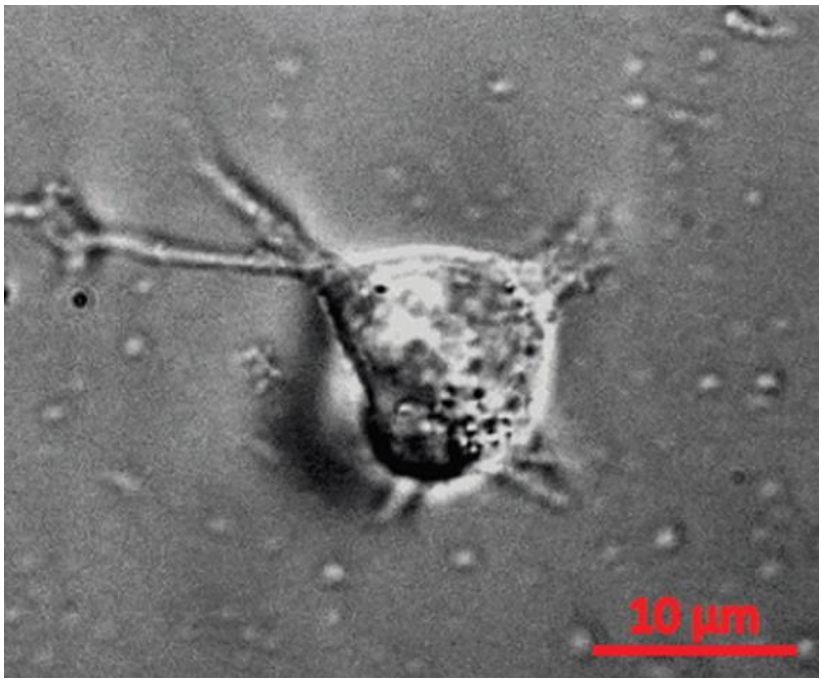


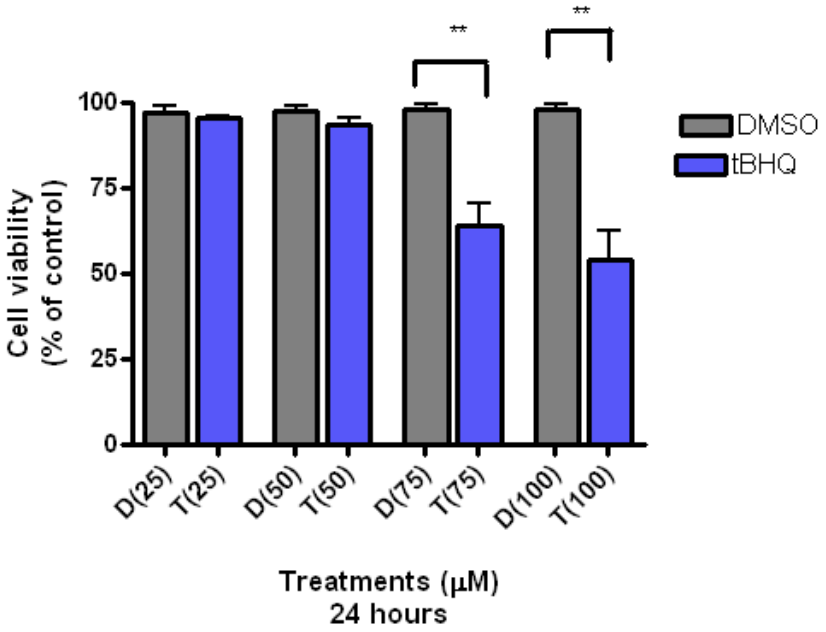
Figure 9. Electrophysiological properties of differentiated and undifferentiated cells. (A) Hyperpolarizing and depolarizing pulses (1 second) delivered at current clamp to test the resting membrane potential and membrane resistance of differentiated and undifferentiated cells. Voltage responses of an undifferentiated cell to pulses shown in A (middle green). Voltage responses of a RA/TPA differentiated cell (day 6) to pulses shown in A (bottom red). (B) Expanded voltage response under 40 pA current step from A as indicated by the green and red arrow (upper); deviation of voltage response showing two rising peak in the RA/TPA induced cell (red trace). (C) Histograms for resting membrane potentials of cultured cells under different treatment conditions. (D) Histograms for membrane resistance of cultured cells under different treatment conditions. (E, F) Representative live IR Dodt contrast images of an (E) undifferentiated or (F) differentiated cell. * Stands for significant difference ($P < 0.05$, n=7-18 per group) compared to undifferentiated cells, @ stands for significant difference ($P < 0.005$) compared to the data for RA at day 3, student t-test for independent samples. The experiments were repeated three times.

Viable cell counts using trypan blue dye exclusion assay

At certain concentrations, tBHQ is known to induce apoptosis (Lavoie et al, 2009, Gharavi and El-Kadi, 2005). To determine that the concentration of tBHQ (25µM) being used in the experiments was not toxic to the cells, cell viability assay was done using different concentrations of tBHQ. SH-SY5Y cells were exposed to 25, 50, 75 and 100 µM concentrations of tBHQ for 24 hours. Afterwards, the trypan blue exclusion assay was done to determine the number of viable cells. Cells which took up the dye were counted as dead. tBHQ did not induce cell death at a concentration of 25 µM in both differentiated and undifferentiated cells as compared to the control (DMSO). At 50 µM concentration, there was no cell death seen in undifferentiated cells (Figure 10A) but cell death in differentiated cells at this concentration was significant (Figure 10B). At concentrations of 75 and 100 µM a significant number of cells died in both undifferentiated and differentiated cells. Thus the 25 µM concentration of tBHQ used for the experiments was not toxic to the cells.

Figure 10. Determination of cell viability for different concentrations of tBHQ

(A)



(B)

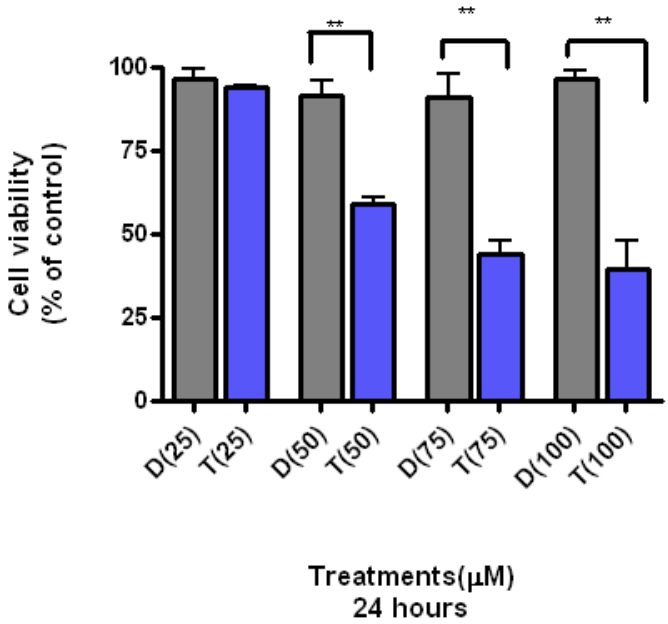


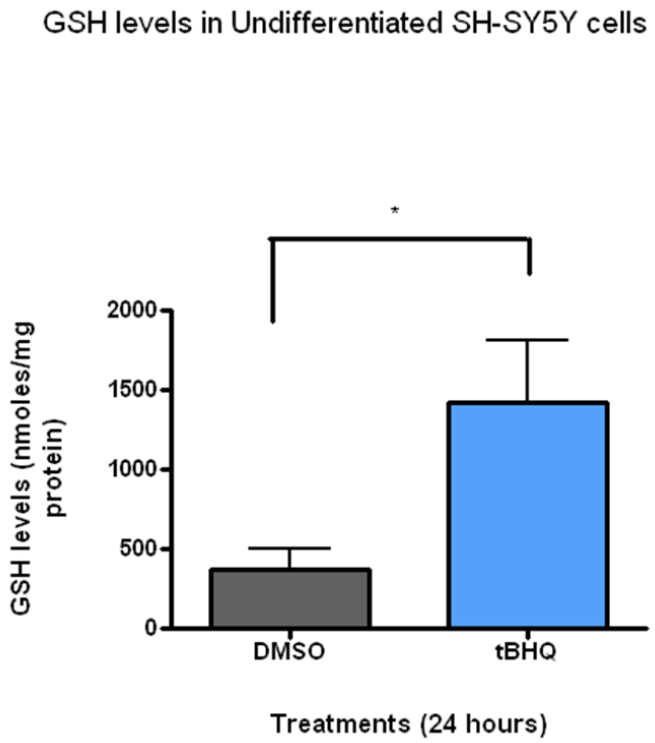
Figure 10. Determination of cell viability for different concentrations of tBHQ. (A) Undifferentiated and (B) differentiated SH-SY5Y cells were treated with 25, 50, 75 and 100 µM of tBHQ for 24 hours and cell viability was determined by trypan blue dye exclusion assay. The experiments were independently repeated three times. Columns represent mean ± S.E.M. (n = 3 per group). ** Significantly different from control, DMSO $P<0.01$; student t-test for independent samples.

GSH Determination using DTNB method

The phenolic antioxidant tBHQ is known to increase GSH levels in many cell types after the activation and subsequent translocation of Nrf2 into the nucleus (Zhang et al., 2006). To determine Nrf2 activity via GSH levels, undifferentiated and differentiated cells were treated with 25 µM of tBHQ for 24 hours and GSH levels were subsequently measured using the DTNB method. Figure 11 (A and B) shows GSH levels in undifferentiated and differentiated cells respectively. In undifferentiated cells, tBHQ caused a significant increase in the levels of GSH. In differentiated cells however, tBHQ treatment did not result in a significant increase in GSH levels.

Figure 11. GSH measurement using DNB method only

(A)



GSH levels in Differentiated SH-SY5Y cells

(B)

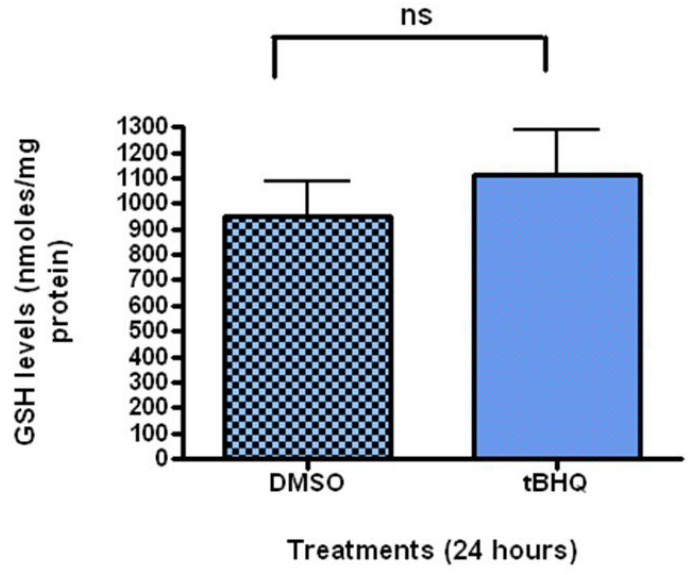


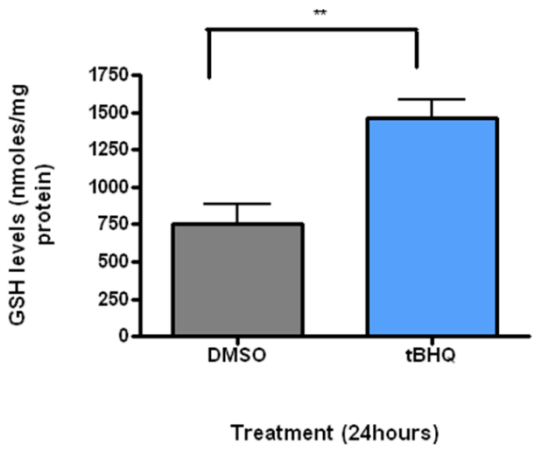
Figure 11. Determination of GSH levels in (A) undifferentiated and (B) differentiated SH-SY5Y cells using the DTNB method after exposing cells to tBHQ for 24 hours. Data is represented as mean ± S.E.M. (n= 5-7 per group) *denote significant difference ($P < 0.05$) as compared to DMSO (control group) treatment, ns not significantly different from control group; one-way Anova with Tukey's all pairs comparison, experiments were independently repeated four times.

GSH measurement, a comparison of DTNB and HPLC

The measurement of GSH levels is mostly done spectrophotometrically using Ellman's reagent (DTNB method). In this method, Ellman's reagent reacts with thiol groups leading to the formation of a thiol-TNB adduct and a concomitant release of an equivalent of 5-thio-2-nitrobenzoic acid (TNB). The quantification of GSH is based on the released TNB which is measured spectrophotometrically at 412 nm (Chen et al., 2008). A major setback in this method of quantification is the interference by compounds which exhibit absorption at the measured wavelength, or that total thiol groups will be measured instead of just GSH levels. One of the solutions to overcoming this problem is using HPLC, which provides a specific and sensitive technique to quantify analytes (Peaston and Weinkove, 2004, Chen et al., 2008). GSH levels were determined in differentiated and undifferentiated cells by HPLC and DTNB methods. Though GSH levels were higher in tBHQ treated undifferentiated and differentiated cells, the levels were not significant as compared to the DMSO control group for the HPLC analysis (Figures 12 and 13). For the DTNB method, GSH was significantly higher in undifferentiated but not in differentiated cells as observed in earlier experiments (Figures 12 and 13).

Figure 12. Determination of GSH levels in undifferentiated SH-SY5Y cells using HPLC and DTNB methods.

(A) GSH measurement in undifferentiated SH-SY5Y cells using DTNB method



(B) GSH measurement in undifferentiated SH-SY5Y cells using HPLC method

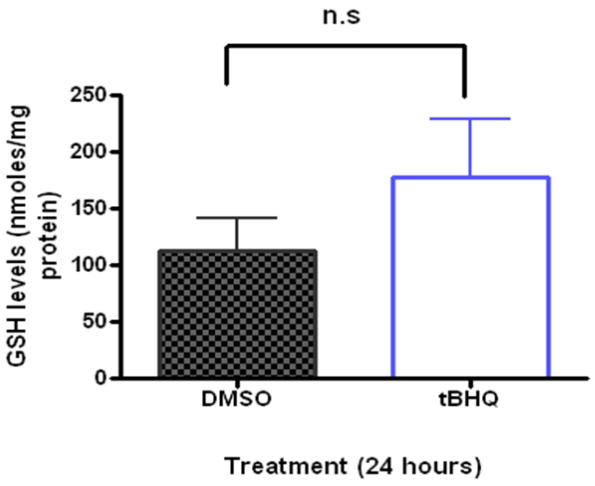
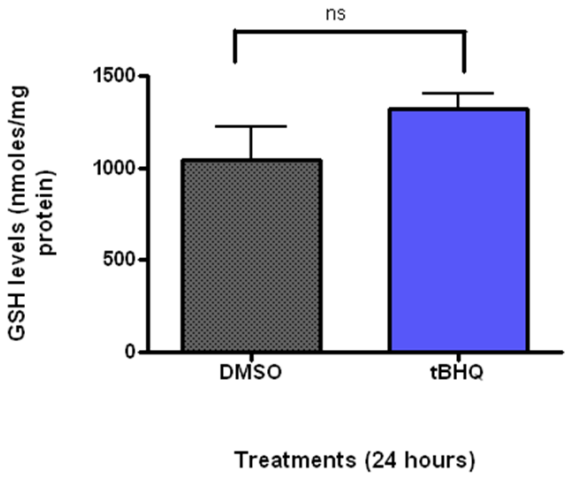


Figure 12. Determination of GSH levels in undifferentiated SH-SY5Y cells using DTNB and HPLC (B) methods for same samples after exposing cells to tBHQ for 24 hours. Data is represented as mean ± S.E.M. (n= 5 per group) **denote significant difference ($P < 0.01$) as compared to DMSO (control group) treatment, ns not significantly different from control group; one-way Anova with Tukey's all pairs comparison, experiments were repeated five times.

Figure 13. Determination of GSH levels in differentiated SH-SY5Y cells using HPLC and DTNB methods.

(A) GSH measurement in differentiated SH-SY5Y cells using DTNB method



(B) GSH measurement in differentiated SH-SY5Y cells using DTNB method

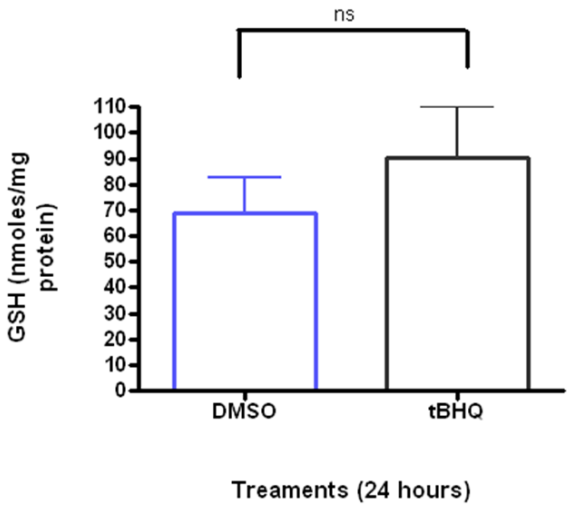


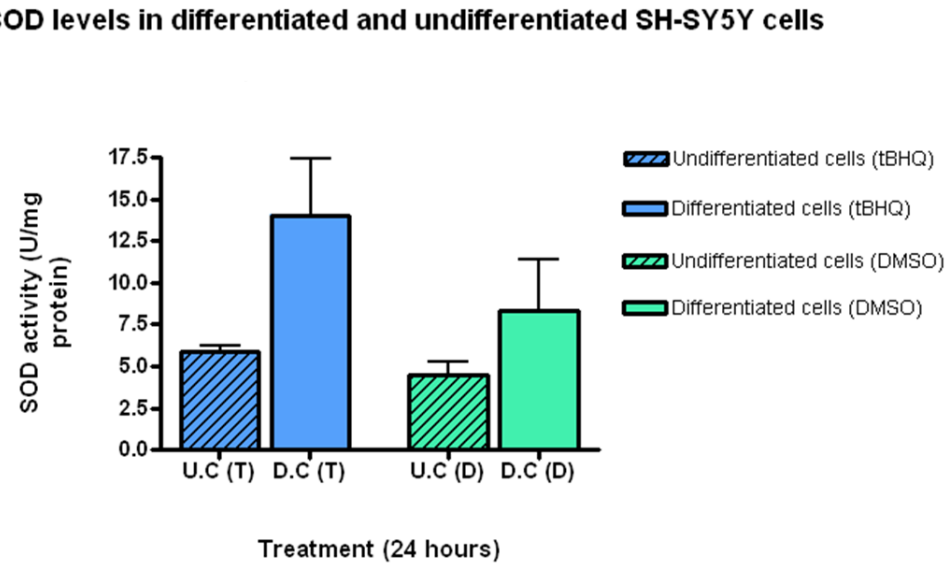
Figure 13. Determination of GSH levels in differentiated SH-SY5Y cells using DTNB (A) and HPLC (B) methods for same samples after exposing cells to tBHQ for 24 hours. Data is represented as mean ± S.E.M. (n=5 per group), ns not significantly different from control group; one-way Anova with Tukey's all pairs comparison experiments were independently repeated five times.

Superoxide dismutases (SOD) measurement

Superoxide dismutases (SOD) are a group of metal-containing enzymes that catalyze the disproportionation of superoxide to hydrogen peroxide and oxygen. SOD are the first and most important line of antioxidant enzyme defense systems against ROS and particularly superoxide anion radicals (Zelko et al., 2002). The promoter regions of Mn-SOD contain consensus binding sites for Nrf2 (Lee et al., 2011), thus, increasing Nrf2 levels will consequently result in an increase in Mn-SOD levels. After undifferentiated and differentiated cells were treated with tBHQ, Cu/Zn- and Mn-SOD levels were determined. The differentiated (both tBHQ treated and control) cells had higher levels of Mn-SOD as compared to undifferentiated (both tBHQ treated and control) cells (Figure 14A). On the other hand, undifferentiated cells had higher levels of Cu/Zn-SOD than differentiated cells (Figure 14B)

Figure 14. Determination of SOD levels in undifferentiated and differentiated SH-SY5Y cells.

(A)



(B)

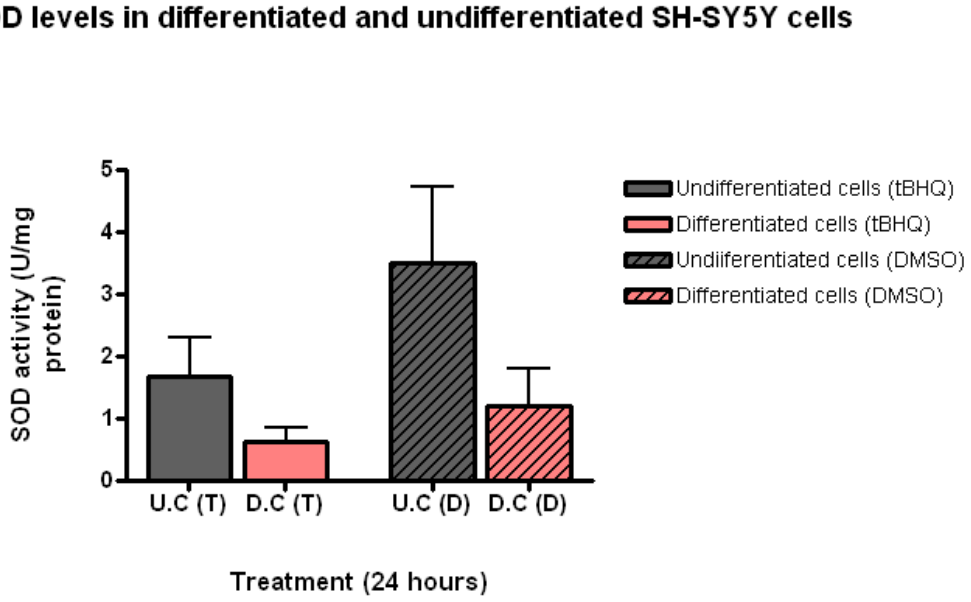


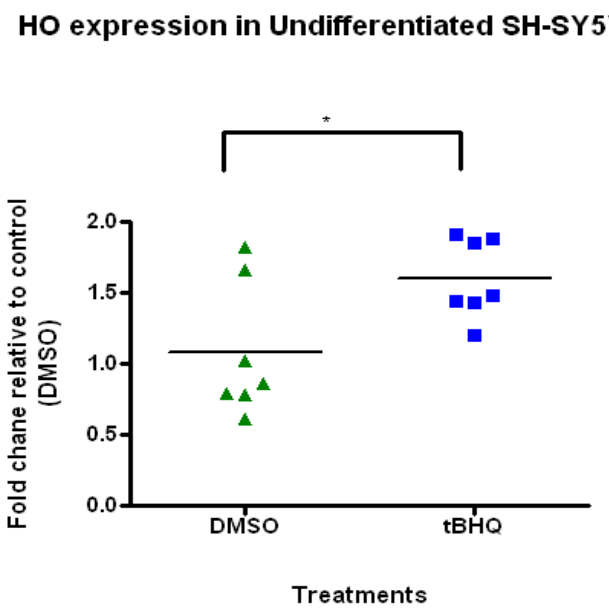
Figure 14. Determination of SOD levels in undifferentiated and differentiated SH-SY5Y cells. Mn-SOD (A) and Cu/Zn-SOD (B) levels were determined in both ndifferentiated and differentiated SH-SY5Y cells. Data is represented as mean ± S.E.M. (n= 4-5 per group); one-way Anova with Tukey's all pairs comparison, experiments were independently repeated two times.

Determination of HO expression using Real-Time PCR

To determine that undifferentiated and differentiated SH-SY5Y cells were responding to Nrf2 induction, HO expression levels were determined using real-time PCR. HO is a prototype enzyme that is induced by Nrf2 (Reichard et al., 2007, Piao et al., 2011). Total RNA was extracted from undifferentiated and differentiated cells cultured in 6-well plates after treatment with tBHQ or DMSO for 24 hours. Determination of HO levels in undifferentiated and differentiated SH-SY5Y cells was determined using the ΔΔCt method. The expression of HO in tBHQ treated undifferentiated and differentiated cells were significantly higher than the DMSO control group (Figure 15 A and B).

Figure 15. Determination of HO expression levels in undifferentiated and differentiated SH-SY5Y cells.

(A)



(B)

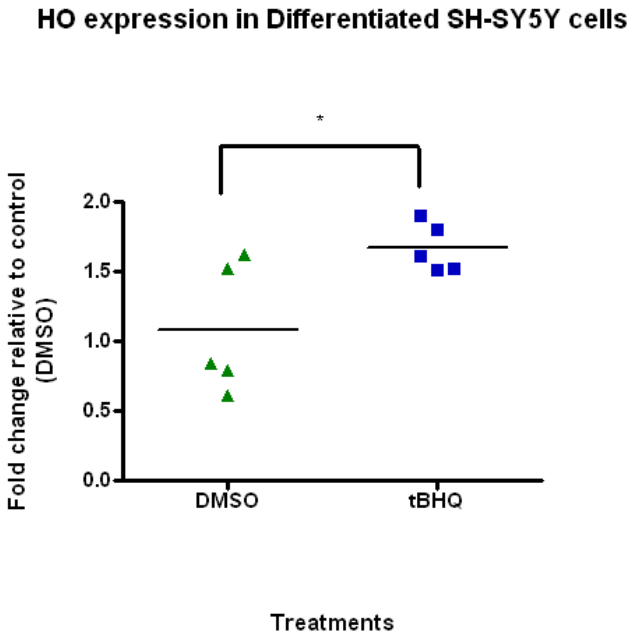


Figure 15. Determination of HO expression levels in undifferentiated (A) and differentiated (B) SH-SY5Y cells. Undifferentiated and differentiated cells were treated with tBHQ for 24 hours and HO expression measured using RT-PCR. Data is represented as mean ± S.E.M. (n=5-7 per group), * indicates significant difference $P<0.05$ compared to control group; student t-test for independent samples, experiments were independently repeated three times.

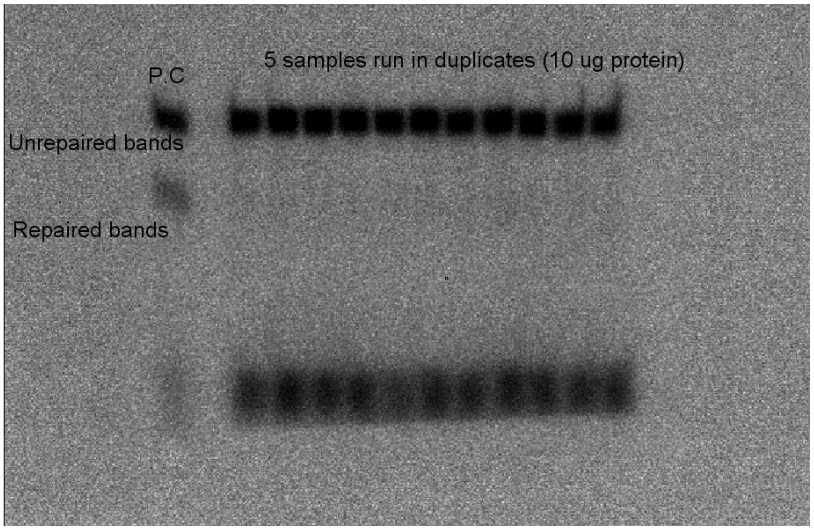
Measurement of OGG1 enzymatic activity

8-Oxoguanine (8-oxoG) is one of the most commonly formed DNA lesions and serves as a cellular marker for both oxidative stress and DNA damage (Piao et al., 2011). 8-Oxoguanine DNA glycosylase 1 (OGG1) is the rate-limiting enzyme involved in the removal of 8-oxoG through the DNA repair mechanism (Piao et al., 2011). Failure to remove 8-oxoG before replication results in G-to-T transversion mutations and studies have revealed direct correlations between defects in the repair of damaged bases and human diseases such as cancer (David et al., 2007). Glycosylase extraction was performed in undifferentiated and differentiated cells and the nuclear extracts were incubated with a radiolabelled oligonucleotide with an oxidized guanine in its sequence. OGG1 activity was expressed as a percentage of synthetic probe cleaved (containing oxo8dG) in proportion to total synthetic probe in the reaction (sum of cleaved and uncleaved probe). Figure 16, shows OGG1 activity in untreated, undifferentiated SH-SY5Y cells. The activity of OGG1 was not enhanced by treatment of cells with tBHQ for 24 hours (Figure 17).

Figure 16. OGG1 enzymatic activity in untreated undifferentiated SH-SY5Y cells

(A)

Gel showing OGG1 enzymatic activity



(B)

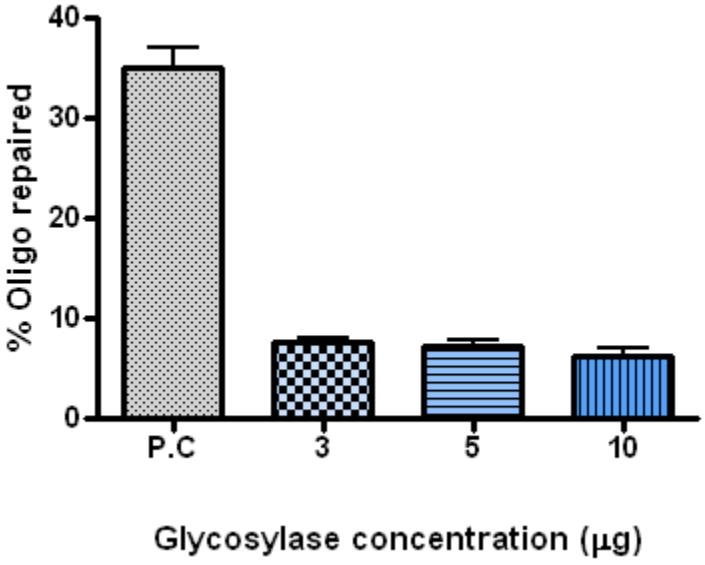
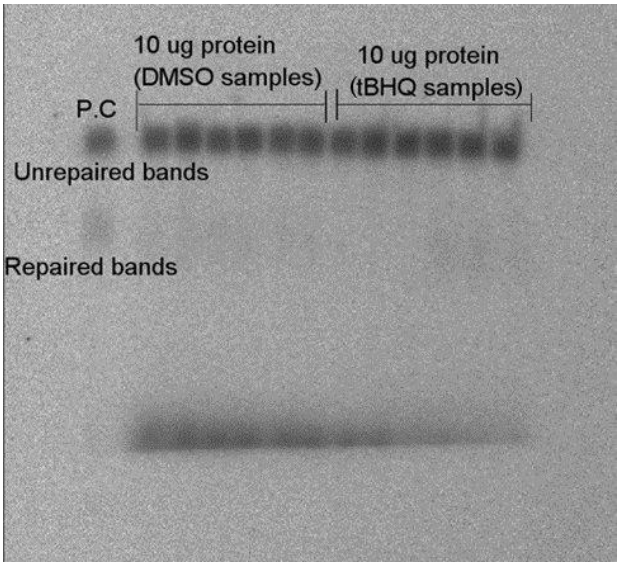


Figure 16. OGG1 enzymatic activity in untreated undifferentiated SH-SY5Y cells. (A) A representation of gels run for OGG1 activity, P.C: Positive control, glycosylase extracted from wild type mouse. (B) Quantitation of gels with different concentrations of glycosylase used (n=10-12 per group), data is represented as mean ± S.E.M.; one-way Anova with Tukey's all pairs comparison, experiments were repeated four times.

Figure 17. OGG1 enzymatic activity in treated undifferentiated SH-SY5Y cells

(A)

Gel showing OGG1 enzymatic activity



(B)

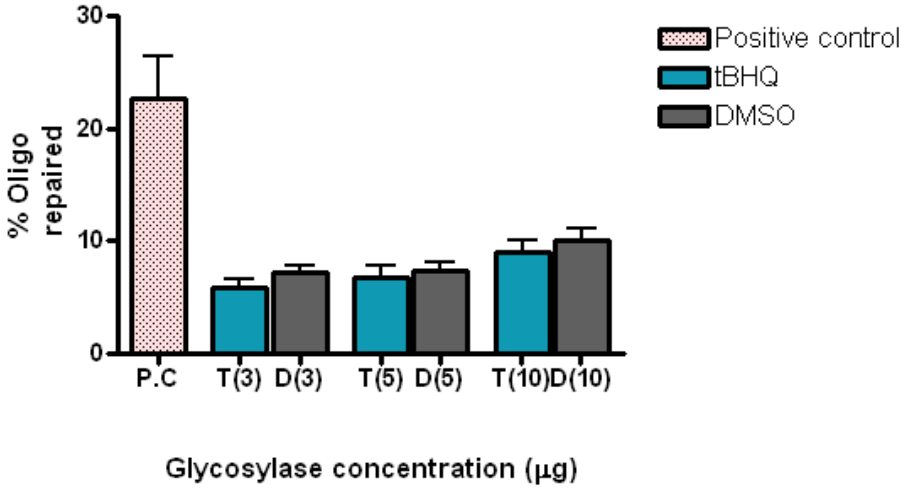


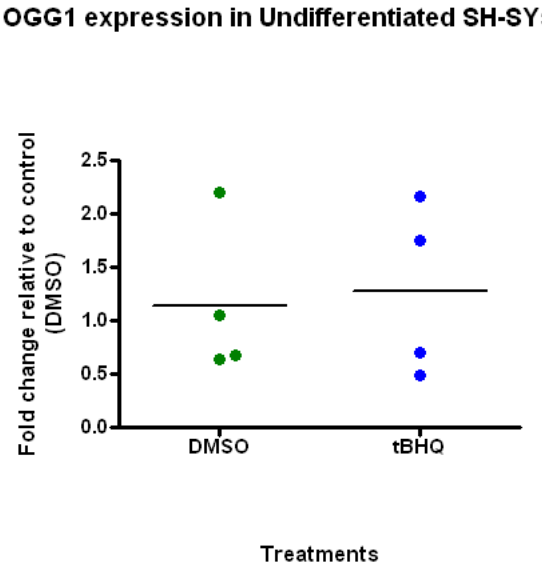
Figure 17. OGG1 enzymatic activity in treated undifferentiated SH-SY5Y cells. (A) A representation of gels run for OGG1 activity, P.C: Positive control, glycosylase extracted from wild type mouse. (B) Quantitation of gels with different concentrations of glycosylase used (n=6-12 per group), data is represented as mean ± S.E.M.; one-way Anova with Tukey's all pairs comparison experiments were repeated four times.

Measurement of OGG1 expression using Real-Time PCR.

Since there was a very low enzymatic activity of OGG1, the expression of OGG1 was subsequently determined in undifferentiated and differentiated SH-SY5Y cells. Cells were cultured and RNA extracted as described in the method section. OGG1 levels were then determined by the ΔΔCt method. There was no significant difference in the expression of OGG1 in tBHQ treated SH-SY5Y cells as compared to the DMSO control group for both undifferentiated and differentiated cells (Figure 18 A and B).

Figure 18. Determination of OGG1 expression levels in undifferentiated and differentiated SH-SY5Y cells

(A)



(B)

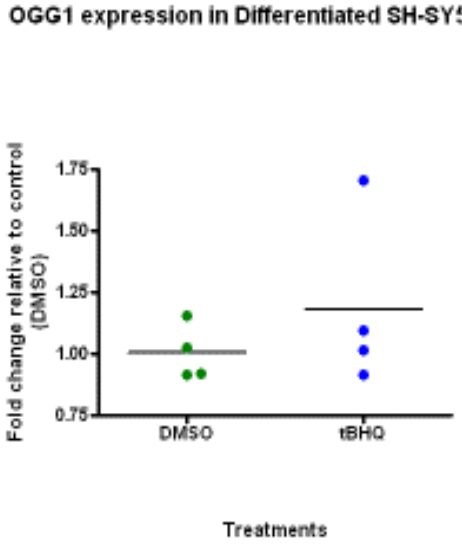


Figure 18. Determination of OGG1 expression levels in (A) undifferentiated and (B) differentiated SH-SY5Y cells. Data is represented as mean ± S.E.M. (n=5-7 per group); one-way Anova with Tukey's all pairs comparison, experiments were repeated two times.

CHAPTER 4

DISCUSSION and CONCLUSION

The objective of this study was to evaluate how differentiation will induce changes in antioxidant response and capacity in a neuronal cell model. The study particularly focused on Nrf2 induction of antioxidants in differentiated and undifferentiated SH-SY5Y neuroblastoma cells. The SH-SY5Y neuroblastoma cell line has been extensively used as cellular models for neurodegeneration, neuroprotection and neurotoxicological studies. Though the cells are oncogenic and mitotic, they can be stimulated to differentiate using different chemical agents as mentioned earlier. When cells differentiate they form a more stable population with an increase in neuronal properties such as release of neurotransmitters and functional changes in Na^+ and K^+ conductance (Tieu et al., 1999). There are a number of studies that have confirmed the neurochemical and morphological differences that exists between differentiated and undifferentiated SH-SY5Y cells. However, a study by Lombet et al. (2001), suggests that RA differentiated SK-N-SH (the parent cell line of SH-SY5Y) showed no significant difference in their expression of ɣ-enolase and differentiated cells do not exhibit significant neuronal properties compared to undifferentiated cells. The question then arises as to whether to use undifferentiated or differentiated cells as models in neuroscience research. Indeed, there are several studies that have either used undifferentiated or differentiated cells and not both in their investigations. However, due to a number of studies that have confirmed the neurochemical and morphological differences between differentiated and undifferentiated cells, we hypothesized that there will be differences in antioxidant response and capacity in differentiated and undifferentiated SH-SY5Y cells.

Previous studies have demonstrated that morphological changes occur after differentiation of SH-SY5Y cells and it is characterized by longer prolongations as compared to undifferentiated cells (Tieu et al., 1999, Xie et al., 2010, Cheung et al., 2009). To establish that our RA/TPA treated cells were morphologically differentiated, the lengths of neurites were determined for differentiated and undifferentiated cells. Consistent with other findings (Presgraves et al., 2004, Encinas et al., 2000, Perez-Juste and Aranda, 1999), the RA/TPA differentiated cells had an increase in process length than undifferentiated cells as shown in Figure 8B.

Furthermore, there is evidence that suggests differentiated SH-SY5Y cells are more excitable and their membrane potential increases relative to undifferentiated cells (Xie et al., 2010). Electrophysiological analysis was done to measure the membrane potentials and resistances of differentiated and undifferentiated cells. The results from the experiment showed that cells differentiated with RA or TPA only for either 3 or 6 days and RA/TPA combined treatment for 6 days had higher resting membrane potentials and lower resistances than undifferentiated cells. Some of the differentiated cells (2/7 in RA day 3; 3/7 in RA day 6; 3/8 in TPA day6; 5/18 in RA&TPA day6) showed a spike-like response (indicative of an action potential), which was not present in any of the undifferentiated cells. A further analysis of the spike response showed two rising peaks upon injection of depolarizing current, which is an indication of voltage dependent channels being expressed in differentiated cells. As shown in Figure 9E, differentiated cells had lower membrane resistances, which shows that differentiated cells had more ion channels than undifferentiated cells. This is in agreement with other studies by Tosetti et al. (1998), Johansson et al. (1996), Toselli et al. (1996) and Brown et al. (1994) that confirmed the presence of ion channels in differentiated cells and resulted in action potentials observed in these cells. The initiation and propagation of action potentials within nerve cells occurs as a result of the activation

of voltage-sensitive sodium channels (Hodgkin and Huxley, 1952). It has been shown that in SH-SY5Y cells, voltage-gated Na+ conductances are present and are involved in the generation of overshooting action potentials in differentiated cells (Påhlmanet et al., 1984, Tosetti et al., 1998). The spikes that were seen in the differentiated cells in this experiment can be attributed to the presence of Na+ channels. After the depolarizing phase of action potential, delayed rectifier K+ channels are responsible for the repolarizing current that brings the membrane potential back to its resting value (Tosetti et al., 1998). Therefore the falling phase of the spikes observed in differentiated cells was due to the presence of K+ conductance in these cells. Though spikes were observed in 5/18 of the RA/TPA differentiated cells, this might be due to the fact differentiation was done for only 6 days as compared to > 15 days as done in a previous experiment by Toselli et al. (1996) and Påhlman et al. (1984).

To evaluate the differences in the antioxidant response and capacity, cells were treated with tBHQ a phenolic antioxidant which is known to increase the levels of antioxidants via the induction of Nrf2. tBHQ, however, can induce cell death at certain concentrations (Lavoie et al, 2009, Gharavi and El-Kadi, 2005). To ensure that the tBHQ concentration being used in our experiments were not toxic to the cells, a cell viability assay was done using different concentrations of tBHQ which were 25, 50, 75 and 100 μM. The different concentrations were chosen based on a previous study by Lavoie et al. (2009), in which cell death was reported at higher concentrations of tBHQ. At concentrations of 25 μM, tBHQ did not induce cell death in both undifferentiated and differentiated SH-SY5Y cells. However at a concentration of 50 μM, tBHQ induced cell death in differentiated cells but not in undifferentiated cells. At concentrations of 75 and 100 μM, a significant cell death was seen in both undifferentiated and undifferentiated cells. tBHQ is known to undergo oxidation-reduction reactions following a dealkylation step, and can act as a pro-oxidant (Nguyen et al.,

2003). When tBHQ is oxidized to its corresponding quinone, tert-butylquinone, it is accompanied by the generation of ROS which induces cell death (Lavoie et al., 2009) and this explains the trend of cell death seen with higher concentrations of tBHQ. Thus, the 25 μM concentration used in our experiments were not toxic to the cells.

GSH is the predominant non-protein thiol in aerobic organisms involved in metabolic and catabolic redox chemistry (Stourman et al., 2008, Townsend et al., 2003 Meister and Anderson, 1983). GSH is regarded as the first line of cellular defense against free radicals and changes in cellular GSH can be utilized as a marker for oxidative stress, (Lu, 2009, Markovic et al., 2007, Han et al., 2006). Nrf2 is known to bind to glutamate-cysteine ligase, the rate limiting enzyme in GSH synthesis and hence increases the levels of GSH (Grant et al., 1997, Piao et al. 2011). In this study, undifferentiated and differentiated SH-SY5Y cells were exposed to 25 μM of tBHQ and GSH levels were determined using DTNB method. The outcome of the experiments showed that in undifferentiated cells, tBHQ induced a significant increase in GSH levels as compared to the DMSO (control) group. In differentiated cells however, the levels of GSH were not significantly increased by tBHQ as compared to the control group. Undifferentiated and differentiated SH-SY5Y cells are mitotic and post-mitotic respectively, therefore they can be likened to the mitotic glial cells and post-mitotic neurons in the brain. Previous studies by Eftekharpour et al. (2000) and Lavoie et al. (2009) revealed that tBHQ could increase glutathione levels in astrocytes but not in neurons. The difference in response between the two cells is due to the fact that expressions of phase II detoxification enzymes are lower in neurons than in astrocytes, with the latter having higher GSH contents (capacity) (Lavoie et al., 2009, Kraft et al., 2004, Murphy et al., 2001, Eftekharpour et al. 2000). This might explain why GSH levels in differentiated cells were not

significantly higher in relation to the control group while levels in undifferentiated cells were significantly higher.

One of the common methods for quantifying GSH is by using the 5, 5'-dithiobis (2-nito-benzoic acid), DTNB method. In this method, GSH is oxidized by DTNB to give GSSG with stoichiometric formation of the yellow di-anion 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB formation is determined at 412 nm (Eyer et al, 2003). This method however is less sensitive, as it fails to distinguish between other thiol groups and interfering compounds which exhibit absorption at the measured wavelength, which is a common phenomenon encountered with biological samples in GSH analysis (Hawkins et al., 2009, Chen et al., 2008). The HPLC-EC method has good specificity for the analysis of glutathione and allows for the detection of GSH and GSSG simultaneously (Yilmaz et al., 2009, Mitton et al., 1994). GSH was quantified in both undifferentiated and differentiated cells using the HPLC- EC method and the results showed lower levels than that observed in the DTNB method. The levels of GSH in both undifferentiated and differentiated cells were not significantly higher than the controls. The higher GSH levels observed in the DTNB method can be attributed to the presence of other thiol groups present in the samples. Nrf2 is known to induce the transcription of thioredoxins by binding to the ARE site in the Trx1 promoter (Im et al., 2012, Kim et al., 2000). Therefore, treatment with tBHQ will also increase the levels of thioredoxins via Nrf2 activation. With the inability of the DTNB method to distinguish between the different thiol groups, it can be concluded that the higher levels of GSH measured using this method was not due to the presence of GSH only, but the presence of other thiol groups.

There are several pathways that mitigate the physiological and pathological effects of ROS in mammalian cells and one of such pathways is the superoxide dismutase (SOD) enzyme, which exists as three genetically distinct isoenzymes (Riley, 1994, Huang et al., 1999). The SOD enzymes

convert superoxide anions to hydrogen peroxide which is then enzymatically removed by catalase and glutathione peroxidase. The extracellular isoform (SOD3) has been shown to be associated with certain cognitive functions and its removal interfered with signaling cascades critical for learning (Thiels et al., 2000, Levin et al., 1998). Mn-SOD (SOD2), which is the primary antioxidant enzyme that scavenges superoxide radicals in the mitochondria, is essential for the survival of aerobic life (Weisiger and Fridovich, 1973 a and b). A study by Hirai et al. (2004) and Keller et al. (1998) showed that Mn-SOD is efficient in protecting against cellular injuries by hypoxia/reoxygenation, prevents neural apoptosis and reduces ischemic brain injury by suppression of peroxynitrite production, lipid peroxidation and mitochondrial dysfunction. Of the SOD family, Mn-SOD is known to be induced by Nrf2. Increase in Nrf2 levels consequently leads to an increase in Mn-SOD by binding to ARE in the promoter region of Mn-SOD (Gong et al., 2012, Zhang et al., 2012, Kwak et al., 2001). In this study, cells were treated with the Nrf2 inducer tBHQ and levels of Cu/Zn-SOD and Mn-SOD were determined. The levels of Mn-SOD were higher in differentiated cells than undifferentiated cells, whereas undifferentiated cells had higher levels of Cu/Zn than differentiated cells. Neurons are particularly known to be susceptible to oxidative stress. On the other hand, they can also be resistant to cytotoxicity induced by oxidative stress via an increase in Mn-SOD expression (Schneider et al., 2011). This could explain the higher contents of Mn-SOD observed in differentiated than in undifferentiated cells.

Nrf2 induction of antioxidants in undifferentiated and differentiated SH-SY5Y cells was further investigated by measuring the expression of HO after cells were treated with tBHQ for 24 hours. The expression of HO was significantly increased in both undifferentiated and differentiated cells. These results demonstrated that undifferentiated and differentiated cells were responding to Nrf2

induction similarly and the different levels of antioxidants observed in undifferentiated and differentiated cells were due to the differences in their antioxidant capacities.

OGG1is a DNA glycosylase involved in the excision of oxidized guanine in the base excision repair (BER). This activity is very important to prevent mutation during DNA replication. Low `activity of OGG1 has been implicated in diseases such as lung cancer, Parkinson's disease, Huntington's disease and Alzheimer's disease (Brower, 2011, Moreira et al., 2008, Yang et al., 2008). In Alzheimer's disease patients in particular, there is a significant BER dysfunction and reduced expression of Uracil-DNA Glycosylase (UDG) and OGG1 activities (Weissman et al., 2007). OGG1 activity in this study was determined by the ability of OGG1 to cleave oxidized guanine in a sequence of a radiolabeled oligonucleotide. Basal OGG1 activity was initially measured without tBHQ treatment in undifferentiated cells and activity was lower as compared to the control. tBHQ treatment in undifferentiated cells did not increase OGG1 activity as compared to the control.

Subsequently, OGG1 expression was determined in undifferentiated and differentiated cells. OGG1 expression was not significantly increased in both undifferentiated and differentiated cells compared to the control group after tBHQ treatment. These results are not very different from a study by Sykora et al. (2013), which showed that undifferentiated and differentiated cells did not show any significant difference in the expression of OGG1.

CONCLUSION

This study has demonstrated that SH-SY5Y cells treated with RA/TPA acquire a more neuronal phenotype as observed in the neurite length measurements and the electrophysiological data.

With respect to tBHQ toxicity, differentiated cells appear to be more susceptible to tBHQ toxicity than undifferentiated cells.

Furthermore, this study confirms that HPLC provides a more sensitive approach in determining GSH levels than the DTNB method.

On the whole, the experiments from this study have showed that undifferentiated and differentiated cells might be similar in their response to Nrf2 induction, in the sense that both phenotypes had higher levels (not always significant) of antioxidants as compared to the control groups. This can be seen in the GSH, Mn-, and Cu/Zn- SOD and OGG1 data. However, their antioxidant capacities are different as observed in GSH and SOD data, with differentiated cells generally having lower antioxidant levels. This confirms several studies that have suggested that neurons tend to have lower antioxidant capacities. Therefore, it can be concluded that differentiation induces changes in the antioxidant capacity of SH-SY5Y cells in a similar manner as observed in neurons. Consequently, differentiated cells will be a better model for studying antioxidant levels in neurons.

Further studies can be conducted with longer periods of differentiation and with different inducers of Nrf2 such as sulphorane and cryptotanshinone to ascertain whether there will be any differences in response as observed in this study.

References

Amouroux R, Campalans A, Epe B, Radicella JP. (2010). "Oxidative stress triggers the preferential assembly of base excision repair complexes on open chromatin regions". Nucleic Acids Res. **38**(9):2878-90.

Aon, Miguel Antonio, Brian Alan Stanley et al. (2012). "Glutathione/thioredoxin systems modulate mitochondrial H_2O_2 emission: An experimental-computational study." J. Gen. Physiol. **139**(6):479-91.

Balmer JE, Blomhoff R. (2002). "Gene expression regulation by retinoic acid". J Lipid Res. **43**(11):1773-808.

Baird L, Dinkova-Kostova AT. (2011). "The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol". **85**(4):241-72.

Bedard K, Krause, K.H (2007). "The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology." Physiol Rev. **87**(1):245-313.

Bolin C, Stedeford T, Cardozo-Pelaez F (2004). "Single extraction protocol for the analysis of 8-hydroxy-2'-deoxyguanosine (oxo8dG) and the associated activity of 8-oxoguanine DNA glycosylase". J Neurosci Methods". **136**(1):69-76.

Brower V. (2011). "Biomarkers: Portents of malignancy". Nature. **471**(7339):S19-21.

Brown NA, Kemp JA, Seabrook GR. (1994). "Block of human voltage-sensitive Na+ currents in differentiated SH-SY5Y cells by lifarizine". Br J Pharmacol. **113**(2):600-6.

Chen Y, Cairns R, Papandreou I, Koong A, Denko NC. (2009). "Oxygen consumption can regulate the growth of tumors, a new perspective on the Warburg effect". PLoS One **4**(9):e7033

Chen XL, Dodd G, Thomas S, Zhang X, Wasserman MA, Rovin BH, Kunsch C. (2006). "Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression". Am J Physiol Heart Circ Physiol. **290**(5):H1862-70

Ciccarone V, Spengler BA, Meyers MB, Biedler JL, Ross RA. (1989). "Phenotypic diversification in human neuroblastoma cells: expression of distinct neural crest lineages". Cancer Res. **49**(1):219-25.

Comhair, Suzy A. A., Erzurum, Serpil C. (2002). "Antioxidant responses to oxidant-mediated lung diseases." Am J Physiol Lung Cell Mol Physiol **283**(2):L246-55

Constantinescu R, Constantinescu AT, Reichmann H, Janetzky B. (2007). "Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y". J Neural Transm Suppl. (72):17-28.

Correa F, Ljunggren E, Mallard C, Nilsson M, Weber SG, Sandberg M. (2011). "The Nrf2-inducible antioxidant defense in astrocytes can be both up- and down-regulated by activated microglia:Involvement of p38 MAPK". Glia. **59**(5):785-99.

Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC. (2009). "Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research". Neurotoxicology. **30**(1):127-35

Clagett-Dame M, McNeill EM, Muley PD. (2006). Role of all-trans retinoic acid in neurite outgrowth and axonal elongation. J Neurobiol. **66**(7):739-56.

Dhar S.K., St Clair D.K. (2012). "Manganese superoxide dismutase regulation and cancer." Free Radic Biol Med **52**(11-12):2209-22.

David SS, O'Shea VL, Kundu S. (2007). "Base-excision repair of oxidative DNA damage". Nature. **447**(7147):941-50.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. (2002). "Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants". Proc Natl Acad Sci U S A. **99**(18):11908-13.

Döşemeci A, Dhallan RS, Cohen NM, Lederer WJ, Rogers TB. (1988). "Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes". Circ Res. **62**(2):347-57.

Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Ceña V, Gallego C, Comella JX. (2000). "Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells". J Neurochem. **75**(3):991-1003.

Eftekharpour E., Holmgren A. and Juurlink B. H. (2000) "Thioredoxin reductase and glutathione synthesis is upregulated by t-butylhydroquinone in cortical astrocytes but not in cortical neurons". Glia **31**(3):241-8.

Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, Reiner E. (2003). "Molar absorption coefficients for the reduced Ellman reagent: reassessment". Anal Biochem. **312**(2):224-7

Fisher JP, Tweddle DA. (2012). "Neonatal neuroblastoma". Semin Fetal Neonatal Med. **17**(4):207-15

Fukai, T., Ushio-Fukai, M. (2011). "Superoxide dismutases: role in redox signaling, vascular function, and diseases." Antioxid Redox Signal **15**(6):1583-606.

Gharavi N, El-Kadi AO. (2005). "tert-Butylhydroquinone is a novel aryl hydrocarbon receptor ligand". Drug Metab Dispos. **33**(3):365-72.

Gong P, Li CS, Hua R, Zhao H, Tang ZR, Mei X, Zhang MY, Cui J. (2012). Mild hypothermia attenuates mitochondrial oxidative stress by protecting respiratory enzymes and upregulating MnSOD in a pig model of cardiac arrest. PLoS One. **7**(4):e35313.

Gómez-Santos C, Ambrosio S, Ventura F, Ferrer I, Reiriz J. (2002). "TGF-beta1 increases tyrosine hydroxylase expression by a mechanism blocked by BMP-2 in human neuroblastoma SH-SY5Y cells". Brain Res. **958**(1):152-60.

Grant, C.M., MacIver, F.H., Dawes, I.W. (1997). "Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast Saccharomyces cerevisiae due to an accumulation of the dipeptide gamma-glutamylcysteine." Mol Biol Cell **8**(9):1699-707.

Habib SL. (2009). "Molecular mechanism of regulation of OGG1: tuberin deficiency results in cytoplasmic redistribution of transcriptional factor NF-YA". J Mol Signal. **4**:8

Han D, Hanawa N, Saberi B, Kaplowitz N. (2006). "Mechanisms of liver injury. III. Role of glutathione redox status in liver injury". Am J Physiol Gastrointest Liver Physiol. **291**(1):G1-7.

Hawkins CL, Morgan PE, Davies MJ. (2009). "Quantification of protein modification by oxidants". Free Radic Biol Med. **46**(8):965-88.

Herman GE. (2000). "Mouse models of human disease: lessons learned and promises to come". ILAR J. **43**(2):55-6.

Hinoi E, Balcar VJ, Kuramoto N, Nakamichi N, Yoneda Y. (2002). "Nuclear transcription factors in the hippocampus". Prog Neurobiol. **68**(2):145-65.

Hirai F, Motoori S, Kakinuma S, Tomita K, Indo HP, Kato H, Yamaguchi T, Yen HC, St Clair DK, Nagano T, Ozawa T, Saisho H, Majima HJ. (2004). "Mitochondrial signal lacking manganese superoxide dismutase failed to prevent cell death by reoxygenation following hypoxia in a human pancreatic cancer cell line, KP4". Antioxid Redox Signal. **6**(3):523-35

Hodgkin AL, Huxley AF. (1952). "Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo". J Physiol. **116**(4):449-72.

Huang, X.J., Song, C.X., Zhong, C.Q., Wang F. S. (2012). "Research progress in the radioprotective effect of superoxide dismutase." Drug Discov Ther **6**(4):169-77.

Huang TT, Carlson EJ, Raineri I, Gillespie AM, Kozy H, Epstein CJ. (1999). "The use of transgenic and mutant mice to study oxygen free radical metabolism". Ann N Y Acad Sci. 893:95-112.

Huang TS, Duyster J, Wang JY. (1995). "Biological response to phorbol ester determined by alternative G1 pathways". Proc Natl Acad Sci U S A. **92**(11):4793-7.

Hudson VM. (2001). "Rethinking cystic fibrosis pathology: the critical role of abnormal reduced glutathione (GSH) transport caused by CFTR mutation". Free Radic Biol Med. **30**(12):1440-61

Hu ML. (1994). "Measurement of protein thiol groups and glutathione in plasma". Methods Enzymol. 233:380-5.

Im JY, Lee KW, Woo JM, Junn E, Mouradian MM. (2012). "DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway". Hum Mol Genet. **21**(13):3013-24

Johansson S, Sundgren AK, Kahl U. (1996). Potential-dependent block of human delayed rectifier K+ channels by internal Na+. Am J Physiol. **270**(4 Pt 1):C1131-44.

Jain, A., Martensson, J., Stole, E., Auld, P.A.M., Meister, A., (1991). "Glutathione deficiency leads to mitochondrial damage in brain". Proc. Natl. Acad. Sci. USA. **88**(5):1913-7.

Jeppesen DK, Bohr VA, Stevnsner T. (2011). "DNA repair deficiency in neurodegeneration". Prog Neurobiol. **94**(2):166-200

Kankofer, M. (2002). "Superoxide dismutase and glutathione peroxidase activities in bovine placenta: spectrophotometric and electrophoretic analysis Revue Méd. Vét **153**(2):121-124.

Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells. **6**(10):857-68.

Keller JN, Kindy MS, Holtsberg FW, St Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, Mattson MP. (1998). "Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction". J Neurosci. **18**(2):687-97.

Kim SN, Kim SG, Park SD, Cho-Chung YS, Hong SH. (2000). "Participation of type II protein kinase A in the retinoic acid-induced growth inhibition of SH-SY5Y human neuroblastoma cells". J Cell Physiol. **182**(3):421-8.

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. (2004). "Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2". Mol Cell Biol. **24**(16):7130-9.

Kolb TM, Davis MA. (2004). "The tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) provokes a prolonged morphologic response and ERK activation in Tsc2-null renal tumor cells". Toxicol Sci. **81**(1):233-42.

Kowaluk EA, Fung HL. (1990). "Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols". J Pharmacol Exp Ther. 255(3):1256-64.

Kraft A. D., Johnson D. A. and Johnson J. A. (2004). "Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occuring preference". J Neurosci. **24**(5):1101-12.

Kwak MK, Itoh K, Yamamoto M, Sutter TR, Kensler TW. (2001). Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1,2-dimethiole-3-thione. Mol Med. **7**(2):135-45.

Lavoie S., Chen Y., Dalton T.P., Gysin R., Cuénod M., Steullet P., Do K.Q. (2009). "Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit." J Neurochem **108**(6):1410-22.

Lee JM, Johnson JA. (2004). "An important role of Nrf2-ARE pathway in the cellular defense mechanism". J Biochem Mol Biol. **37**(2):139-43.

Lee IK, Kang KA, Zhang R, Kim BJ, Kang SS, Hyun JW. (2011). "Mitochondria protection of baicalein against oxidative damage via induction of manganese superoxide dismutase". Environ Toxicol Pharmacol. **31**(1):233-41.

Levin, E. D.; Brady, T. C.; Hochrein, E. C.; Oury, T. D.; Jonsson, L. M.; Marklund, S. L.; Crapo, J. D. (1998). "Molecular manipulations of extracellular superoxide dismutase: functional importance for learning". Behav Genet. **28**(5):381-90.

Li J, Johnson D, Calkins M, Wright L, Svendsen C, Johnson J. (2005) "Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells". Toxicol Sci. **83**(2):313-28.

Li X, Qu ZC, May JM. (2001). "GSH is required to recycle ascorbic acid in cultured liver cell lines". Antioxid Redox Signal. **3**(6):1089-97.

Lindl KA, Jordan-Sciutto KL. (2008). "Examining the endogenous antioxidant response through immunofluorescent analysis of Nrf2 in tissue". Methods Mol Biol. 477:229-43

Lombet A, Zujovic V, Kandouz M, Billardon C, Carvajal-Gonzalez S, Gompel A, Rostène W. (2001). "Resistance to induced apoptosis in the human neuroblastoma cell line SK-N-SH in relation to neuronal differentiation. Role of Bcl-2 protein family". Eur J Biochem. **268**(5):1352-62.

Lu, S.C. (2009). "Regulation of glutathione synthesis." Mol Aspects Med **30**(1-2):42-59.

Lushchak VI. (2012). "Glutathione homeostasis and functions: potential targets for medical interventions". J Amino Acids. 2012:736837

Markovic J, Borrás C, Ortega A, Sastre J, Viña J, Pallardó FV. (2007). "Glutathione is recruited into the nucleus in early phases of cell proliferation'. J Biol Chem. 282(28):20416-24.

Mathews WR, Kerr SW. (1993). "Biological activity of S-nitrosothiols: the role of nitric oxide". J Pharmacol Exp Ther. **267**(3):1529-37.

Mattsson ME, Enberg G, Ruusala AI, Hall K, Påhlman S. (1986). "Mitogenic response of human SH-SY5Y neuroblastoma cells to insulin-like growth factor I and II is dependent on the stage of differentiation". J Cell Biol. **102**(5):1949-54.

McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD (2004). "Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron". J Biol Chem. **279**(30):31556-67

Meister A. and Anderson M. E. (1983). "Glutathione". Annu. Rev. Biochem. **52**, 711–760

Mitton KP, Trevithick JR. (1994). "High-performance liquid chromatography-electrochemical detection of antioxidants in vertebrate lens: glutathione, tocopherol, and ascorbate". Methods Enzymol. 233:523-39.

Morris, C.R., Suh J.H., Hagar W. et al. (2008). "Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease." Blood **111**(1):402-10.

Mohora Maria, Maria Greabu, Corina Muscurel, Carmen Duţă, Alexandra Totan (2007). The sources and the targets of oxidative stress in the etiology of diabetic complications." Romanian J. Biopys **17**(2):63–84.

Moreira PI, Nunomura A, Nakamura M, Takeda A, Shenk JC, Aliev G, Smith MA, Perry G. (2008). "Nucleic acid oxidation in Alzheimer disease". Free Radic Biol Med. **44**(8):1493-505

Motohashi H, Yamamoto M. (2004). "Nrf2-Keap1 defines a physiologically important stress response mechanism". Trends Mol Med. **10**(11):549-57.

Murphy TH, Yu J, Ng R, Johnson DA, Shen H, Honey CR, Johnson JA. (2001). Preferential expression of antioxidant response element mediated gene expression in astrocytes. J Neurochem. **76**(6):1670-8

Nakagawa Y. (2012). "Artificial analogs of naturally occurring tumor promoters as biochemical tools and therapeutic leads". Biosci Biotechnol Biochem. **76**(7):1262-74.

Påhlman S, Ruusala AI, Abrahamsson L, Mattsson ME, Esscher T. (1984). "Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation". Cell Differ. **14**(2):135-44.

Peaston RT, Weinkove C. (2004). "Measurement of catecholamines and their metabolites". Ann Clin Biochem. **41**(Pt 1):17-38.

Perez-Juste G, Aranda A. (1999). "Differentiation of neuroblastoma cells by phorbol esters and insulin-like growth factor 1 is associated with induction of retinoic acid receptor beta gene expression". Oncogene. **18**(39):5393-402.

Piao MJ, Kim KC, Choi JY, Choi J, Hyun JW. (2011). "Silver nanoparticles down-regulate Nrf2-mediated 8-oxoguanine DNA glycosylase 1 through inactivation of extracellular regulated kinase and protein kinase B in human Chang liver cells". Toxicol Lett. **207**(2):143-8.

Presgraves SP, Ahmed T, Borwege S, Joyce JN. (2004). "Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists". Neurotox Res. **5**(8):579-98.

Schmittgen TD, Livak KJ. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. **3**(6):1101-8.

Taguchi K, Motohashi H, Yamamoto M. (2011). "Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution". Genes Cells. **16**(2):123-40.

Toselli M, Tosetti P, Taglietti V. (1996). "Functional changes in sodium conductances in the human neuroblastoma cell line SH-SY5Y during in vitro differentiation". J Neurophysiol. **76**(6):3920-7.

Townsend DM, Tew KD, Tapiero H. (2003). "The importance of glutathione in human disease". Biomed Pharmacother. **57**(3-4):145-55.

Velarde M.C., Flynn J.M., Day N.U., Melov S, Campisi J. (2012) "Mitochondrial oxidative stress caused by Sod2 deficiency promotes cellular senescence and aging phenotypes in the skin." Aging (Albany NY) **4**(1):3-12.

Villacorta L., Azzi A., Zingg J.M. (2007). "Regulatory role of vitamins E and C on extracellular matrix components of the vascular system." Mol Aspects Med **28**(5-6):507-37.

Rebrin I, Kamzalov S, Sohal RS. (2003). "Effects of age and caloric restriction on glutathione redox state in mice". Free Radic Biol Med. **35**(6):626-35.

Rebrin I, Forster MJ, Sohal RS. (2007). "Effects of age and caloric intake on glutathione redox state in different brain regions of C57BL/6 and DBA/2 mice". Brain Res. **1127**(1):10-8.

Reichard JF, Motz GT, Puga A. (2007). "Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1". Nucleic Acids Res. **35**(21):7074-86

Rhinn M, Dollé P. (2012). "Retinoic acid signalling during development". Development. **139**(5):843-58

Sayre L.M., Perry G., Smith M.A., (2008). "Oxidative stress and neurotoxicity." Chem Res Toxicol **21**(1):172-88.

Schneider L, Giordano S, Zelickson BR, S Johnson M, A Benavides G, Ouyang X, Fineberg N, Darley-Usmar VM, Zhang J. (2011). "Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress". Free Radic Biol Med. **51**(11):2007-17

Shi Z.Z., Habib G.M., Rhead W.J., Gahl W.A., He X., Sazer S., Lieberman M.W. (1996) "Mutations in the glutathione synthetase gene cause 5-oxoprolinuria." Nat Genet **14**(3):361-5.

Siow RC, Sato H, Leake DS, Pearson JD, Bannai S, Mann GE (1998). "Vitamin C protects human arterial smooth muscle cells against atherogenic lipoproteins: effects of antioxidant vitamins C and E on oxidized LDL-induced adaptive increases in cystine transport and glutathione". Arterioscler Thromb Vasc Biol. **18**(10):1662-70.

Sofic E, Lange KW, Jellinger K, Riederer P. (1992). "Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease". Neurosci Lett. **142**(2):128-30.

Stourman N.V., Wadington M.C., Schaab M.R, Atkinson H.J., Babbitt P.C., Armstrong R.N., (2008). "Functional Genomics in Escherichia coli: Experimental Approaches for the Assignment of Enzyme Function". ESCEC.

Sykora P, Yang JL, Ferrarelli LK, Tian J, Tadokoro T, Kulkarni A, Weissman L, Keijzers G, Wilson DM 3rd, Mattson MP, Bohr VA. (2013). "Modulation of DNA base excision repair during neuronal differentiation". Neurobiol Aging. **34**(7):1717-27

Thiels, E.; Urban, N. N.; Gonzalez-Burgos, G. R.; Kanterewicz, B. I.; Barrionuevo, G.; Chu, C. T.; Oury, T. D.; Klann, E. (2000). "Impairment of longterm potentiation and associative memory in mice that overexpress extracellular superoxide dismutase". J. Neurosci. 20:7631–7639

Tieu K, Zuo DM, Yu PH. (1999). "Differential effects of staurosporine and retinoic acid on the vulnerability of the SH-SY5Y neuroblastoma cells: involvement of bcl-2 and p53 proteins". J Neurosci Res. **58**(3):426-35.

Tietze F. (1969). "Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues". Anal Biochem. **27**(3):502-22.

Toselli M, Tosetti P, Taglietti V. (1996). "Functional changes in sodium conductances in the human neuroblastoma cell line SH-SY5Y during in vitro differentiation". J Neurophysiol. **76**(6):3920-7.

Tosetti P, Taglietti V, Toselli M. (1998). "Functional changes in potassium conductances of the human neuroblastoma cell line SH-SY5Y during in vitro differentiation". J Neurophysiol. **79**(2):648-58

Vargas MR, Johnson JA. (2009). "The Nrf2-ARE cytoprotective pathway in astrocytes". Expert Expert Rev Mol Med. 11:e17

Weisiger, R.A., Fridovich, I., (1973a). "Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization". J Biol Chem. **248**(13):4793-6.

Weisiger, R.A., Fridovich, I., (1973b). Superoxide dismutase Organelle specificity. J Biol Chem. **248**(10):3582-92.

Weissman L, Jo DG, Sørensen MM, de Souza-Pinto NC, Markesbery WR, Mattson MP, Bohr VA. (2007). "Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnestic mild cognitive impairment". Nucleic Acids Res. **35**(16):5545-55

Wüllner U, Löschmann PA, Schulz JB, Schmid A, Dringen R, Eblen F, Turski L, Klockgether T. (1996). Glutathione depletion potentiates MPTP and MPP+ toxicity in nigral dopaminergic neurones. Neuroreport. **7**(4):921-3.

Xie HR, Hu LS, Li GY. (2010). "SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease". Chin Med J (Engl). **123**(8):1086-92

Yan N., Meister A. (1990). "Amino acid sequence of rat kidney gamma-glutamylcysteine synthetase." J Biol Chem. **265**(3):1588-93.

Yang JL, Weissman L, Bohr VA, Mattson MP. (2008). "Mitochondrial DNA damage and repair in neurodegenerative disorders". DNA Repair (Amst).**7**(7):1110-20

Yilmaz O., Keser S., Tuzcu M., Güvenc M., Çetintas B., Irtegün S., Tastan H., Sahin K., (2009). "A Practical HPLC Method to Measure Reduced (GSH) and Oxidized (GSSG) Glutathione Concentrations in Animal Tissues". J. Anim. Vet. Adv. **8** (2): 343-347

Zelko IN, Mariani TJ, Folz RJ. (2002).Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med. **33**(3):337-49.

Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. (2004). "Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex". Mol Cell Biol. **24**(24):10941-53.

Zhang DD. (2006). "Mechanistic studies of the Nrf2-Keap1 signaling pathway". Drug Metab Rev. **38**(4):769-89.

Zhang R, Chae S, Lee JH, Hyun JW. (2012). "The cytoprotective effect of butin against oxidative stress is mediated by the up-regulation of manganese superoxide dismutase expression through a PI3K/Akt/Nrf2-dependent pathway". J Cell Biochem. **113**(6):1987-97.

.