Novel Neurotrophic Growth Factor Signaling Pathway Mediates Changes in Epigenetics through the Redox and Methylation Influence of EAAT3-Mediated Cysteine Uptake

Thesis Presented

by

Nathaniel Wilkens Hodgson

to

The Bouvé Graduate School of Health Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences with Specialization in Pharmacology

NORTHEASTERN UNIVERSITY
BOSTON, MASSACHUSETTS

August 10th, 2012
Northeastern University
The Graduate School of Bouvé College of Health Sciences

Thesis title: Novel Neurotrophic Growth Factor Signaling Pathway Mediates Changes in Epigenetics through the Redox and Methylation Influence of EAAT3-Mediated Cysteine Uptake

Author: Nathaniel Wilkens Hodgson

Program: Doctor of Philosophy in Pharmaceutical Sciences with a Specialization in Pharmacology

Approval for thesis requirements of the Doctor of Philosophy Degree in Pharmaceutical Sciences

Thesis Committee (Chairman) ____________________________ Date _________
______________________________ Date _________
______________________________ Date _________
______________________________ Date _________
______________________________ Date _________
______________________________ Date _________
______________________________ Date _________

Director of the Bouvé College Graduate School
______________________________ Date _________

Dean ____________________________ Date _________

Copy Deposited in Library ____________________________ Date _________
Table of Contents

List of Figures ................................................................................................................................. vi
List of Figures ................................................................................................................................. vi
List of Tables ...................................................................................................................................... viii
List of Abbreviations ......................................................................................................................... ix
Acknowledgements .......................................................................................................................... xi
Abstract ............................................................................................................................................ xii

I. INTRODUCTION .......................................................................................................................... 1

A. Statement of Problem .................................................................................................................. 1
B. Evolution of an Oxygen Based Redox System .......................................................................... 3
C. Production of Free Radicals ......................................................................................................... 3
D. Oxidative Stress ............................................................................................................................ 4
E. Maintenance of a Reduced GSH ..................................................................................................... 7
F. Glutathione Synthesis .................................................................................................................. 8
G. Cysteine Uptake ........................................................................................................................... 9
H. EAAT3 Function and Regulation ................................................................................................. 10
I. Transsulfuration ............................................................................................................................ 14
J. Methionine Synthase: Structure and Function ........................................................................... 16
K. Neurotrophic Growth Factors ..................................................................................................... 20
L. Methylation, Epigenetics and Differentiation ............................................................................ 21
M. The Redox Environment in the Human Brain ........................................................................... 24
N. Alzheimer’s Disease and Oxidative Stress .................................................................................. 25

SPECIFIC AIMS .............................................................................................................................. 31

II. MATERIALS AND METHODS .................................................................................................. 34
## A. Cell Culture

34

## B. Cell Differentiation

34

## C. Cysteine Uptake

35

## D. Lowry Protein Assay

36

## E. Growth Factor Treatments

36

## F. Aβ Preparations

37

## G. Aβ Treatments

38

## H. Thiol isolation

39

## I. HPLC Thiol and Thioester quantification

40

## J. DNA Isolation

40

## K. DNA Methylation Analysis

40

## L. RNA Isolation

41

## M. Primers

41

## N. cDNA/RT-PCR

41

## O. qRT-PCR Analysis

42

## P. Statistical Methods

43

## III. RESULTS

44

## A. Validation of cysteine uptake in the human neuroblastoma cell line SH-SY5Y

44

## B. Validation of intracellular thiol and thioether quantification

50

## C. Validation of global DNA methylation protocol

51

## D. Specific Aim 1: Investigate neurotrophic growth factor regulation of EAAT3-mediated cysteine uptake and its influence over redox status in cultured neuronal cells

55

## E. Specific Aim 2: Evaluate redox-dependent changes in DNA methylation in cultured SH-SY5Y cells

71

## F. Specific Aim 3: Evaluate changes in EAAT3-mediated cysteine uptake, and intracellular thiol metabolism caused by soluble Aβ oligomers

84
G. Specific Aim 4: Investigate the role of redox-mediated epigenetic changes on differentiation........................................................................................................................................ 101

IV. DISCUSSION .................................................................................................................................................. 108
A. Redox Status in the Human Brain.................................................................................................................. 108
B. EAAT3 is a Controller of Redox in the Human Brain .................................................................................... 112
C. Methylation and Development/Differentiation ............................................................................................. 116
D. Redox, Epigenetics and Neurological Disorders .......................................................................................... 120
E. Aging and Methylation.................................................................................................................................. 123
F. Alzheimer’s disease........................................................................................................................................ 125

V. Future Directions ............................................................................................................................................. 130

VI. REFERENCES .................................................................................................................................................. 132

VII. Appendix ...................................................................................................................................................... 147
List of Figures

Figure 1: A proposed NTGF epigenetic signaling pathway in the brain
Figure 2: Cellular antioxidant reactions
Figure 3: Membrane topology model of the excitatory amino acid transporter
Figure 4: Proposed mechanism for NTGF regulation of EAAT3 and redox in neuronal cells
Figure 5: Sulfur metabolism pathways in neuronal cells
Figure 6: Enzymatic reaction catalyzed by methionine synthase and its subsequent reactivation
Figure 7: Methionine synthase acts as a redox switch
Figure 8: Regulation of gene transcription
Figure 9: Thiols in the brain
Figure 10: Molecular pathology of Alzheimer’s disease
Figure 11: Effects of Aβ oligomers on pathways linked to synaptotoxicity
Figure 12: Treatment course for NTGF experiments
Figure 13: Treatment course for Aβ experiments
Figure 14: Cysteine uptake by SH-SY5Y cells in variable concentrations of cysteine
Figure 15: Time course of cysteine uptake in SH-SY5Y cells
Figure 16: Presence of EAAT1-3 RNA in SH-SY5Y cells
Figure 17: LBTBA decreases cysteine uptake in SH-SY5Y cells
Figure 18: Inhibition of cysteine uptake by LBTBA and Dihydrokainate in SH-SY5Y cells
Figure 19: Typical chromatogram showing the measurement of intracellular thiols
Figure 20: Typical standard curve of measured thiols and thioethers
Figure 21: DNA methylation over 72 hours in SH-SY5Y cells
Figure 22: Time course of the effect of IGF-1 on global DNA methylation in SH-SY5Y cells
Figure 23: Cell survival for 48 hours of IGF-1 treatment
Figure 24: Dose response curve for IGF-1 of EAAT3 mediated cysteine uptake
Figure 25: Dose response curve for BDNF of EAAT3 mediated cysteine uptake
Figure 26: Dose response curve for GDNF of EAAT3 mediated cysteine uptake
Figure 27: Dose response curve for NGF of EAAT3 mediated cysteine uptake
Figure 28: Dose response curve for PDGF of EAAT3 mediated cysteine uptake
Figure 29: Time course of cysteine uptake stimulated by IGF-1 in SH-SY5Y cells
Figure 30: Time course of cysteine uptake stimulated by BDNF in SH-SY5Y cells
Figure 31: Time course of cysteine uptake stimulated by GDNF in SH-SY5Y cells
Figure 32: Time course of cysteine uptake stimulated by NGF in SH-SY5Y cells
Figure 33: Time course of cysteine uptake stimulated by PDGF in SH-SY5Y cells
Figure 34: Cysteine uptake is increased by IGF-1 stimulation and blocked by PI3 kinase inhibition
Figure 35: Time course of the effect of IGF-1 on thiols and thioethers in SH-SY5Y cells
Figure 36: Effect of IGF-1 on thiols and thioethers in SH-SY5Y cells
Figure 37: Effect of PDGF on thiols and thioethers in SH-SY5Y cells
Figure 38: Effect of IGF-1 on global DNA methylation in SH-SY5Y cells
Figure 39: Effect of low cysteine growth conditions on thiols and thioethers in SH-SY5Y cells
Figure 40: Effect of low cysteine growth conditions on global DNA methylation in SH-SY5Y cells
Figure 41: Effect of LBTBA on thiols and thioethers in SH-SY5Y cells
Figure 42: Effect of LBTBA on global DNA methylation in SH-SY5Y cells
Figure 43: Effect of BSO on thiols and thioethers in SH-SY5Y cells
Figure 44: Effect of BSO on global DNA methylation in SH-SY5Y cells
Figure 45: Cysteine uptake is inhibited by conditioned media containing soluble Aβ oligomers
Figure 46: Inhibition of cysteine uptake is prevented by Aβ immunodepletion
Figure 47: Effect of conditioned media containing soluble Aβ oligomers on thiols and thioethers in SH-SY5Y cells
Figure 48: Effect of conditioned media containing soluble Aβ oligomers on the redox state of SH-SY5Y cells
Figure 49: Effect of conditioned media containing soluble Aβ oligomers on the methylation state of SH-SY5Y cells
Figure 50: Effect of conditioned media containing soluble Aβ oligomers on global DNA methylation in SH-SY5Y cells
Figure 51: Cysteine uptake inhibition by Aβ oligomers is ameliorated by IGF-1
Figure 52: Effect of IGF-1 on thiols and thioethers in SH-SY5Y cells exposed to conditioned media containing soluble Aβ oligomers
Figure 53: Effect of conditioned media containing soluble Aβ oligomers +/- IGF-1 on global DNA methylation in SH-SY5Y cells
Figure 54: Phase-contrast micrographs of SH-SY5Y cells grown in media containing Aβ or LBTBA
Figure 55: Effect of conditioned media containing soluble Aβ oligomers on expression of redox and methylation-linked genes in SH-SY5Y cells
Figure 56: Time course of SH-SY5Y cell differentiation
Figure 57: Changes in thiols and thioethers in SH-SY5Y cells during differentiation
Figure 58: Effect of differentiation on the redox state of SH-SY5Y cells
Figure 59: Effect of differentiation on the methylation state of SH-SY5Y cells
Figure 60: Effect of differentiation on the redox potential of SH-SY5Y cells
Figure 61: Effect of differentiation on global DNA methylation in SH-SY5Y cells
Figure 62: Redox based epigenetic signaling
Figure 63: A proposed mechanism for NTGF stimulated cysteine uptake
Figure 64: Changes in DNA methylation during mammalian development
Figure 65: Aβ effects on transsulfuration and methylation
List of Tables

Table 1: Excitatory Amino Acid Transporters
Table 2: Summary of dose response results for NTGF-stimulated cysteine uptake in SH-SY5Y human neuroblastoma cells.
Table 3: Correlation of GSH/GSSG and SAM/SAH ratios with global DNA methylation
Table 4: Correlation of GSH/GSSG and SAM/SAH ratios with global DNA methylation
List of Abbreviations

\(\alpha_7\)-nAcChR: \(\alpha_7\) nicotinic acetylcholine receptor
A\(\beta\): amyloid \(\beta\)
AD: Alzheimer’s disease
Akt: Protein Kinase B
AMPAR: \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor
APP: amyloid precursor protein
BDNF: brain-derived growth factor
BSO: buthionine sulfoximine
CaMKII: calmodulin kinase II
CAT: catalase
Cbl: cobalamin
CBS: cystathionine \(\beta\)-synthase
Cdk5: cyclin-dependent kinase 5
CREB: cAMP-regulatory element binding protein
CSF: cerebrospinal fluid
CYS: cysteine
EAAT: excitatory amino acid transporter
ERK2: extracellular signal-regulated kinase 2
FAD: familial Alzheimer’s disease
G6PD: glucose-6-phosphate dehydrogenase
GCL: glutamate–cysteine ligase
GDNF: glial cell line-derived neurotrophic factor
GCS: \(\gamma\)-glutamylcysteine synthetase
GGT: gamma-glutamyl transferase
Glu: glutamate
Gly: glycine
GPX: glutathione peroxidase
GRX: glutaredoxin
GSH: reduced glutathione
GSK-3\(\beta\): glycogen synthase kinase-3\(\beta\)
GSR: glutathione reductase
GSS: glutathione synthetase
GSSG: oxidized glutathione
GST: glutathione S-transferase
HCY: homocysteine
IGF-1: insulin-like growth factor-1
JNK: c-Jun N-terminal kinase
LNGFR: low-affinity nerve growth factor receptor
MAPK: mitogen-activated protein kinase
MeCbl: methylcob(III)alamin
MET: methionine
MethylTHF: 5-methyltetrahydrofolate
mGluR5: metabotropic glutamate receptor 5
MS: methionine synthase
MTHFR: methylenetetrahydrofolate reductase
mTOR: mammalian target of rapamycin
mTORC: mammalian target of rapamycin complex
MTRR: methionine synthase reductase
NAC: N-acetyl-cysteine
NADP:\(^+\): oxidized nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
NFATc4: nuclear factor of activated T-cells
NGF: nerve growth factor
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NMDAR: N-methyl-D-aspartate receptor
NPC: neuronal precursor cell
NTGF: neurotrophic growth factor
PDGF: platelet-derived neurotrophic factor
PDK: 3-phosphoinositide dependent protein kinase
PI3K: Phosphatidylinositol 3-kinase
PKA: protein kinase A
PLM: phospholipid methylation
PP1: protein phosphatase 1
PP2A: protein phosphatase 2A
PRDX: peroxiredoxin
R-S\(^2\): disulfide
R-SH\(^2\): reduced thiol
ROS: reactive oxygen species
SAM: S-adenosylmethionine
SAH: S-adenosylhomocysteine
SOD: superoxide dismutase
TNFα: tumor necrosis factor-alpha
TXN\(_{ox}\): oxidized thioredoxin
TXN\(_{red}\): reduced thioredoxin
TXNRD: thioredoxin reductase
Acknowledgements

Primarily, I would like to thank my advisor and mentor Dr. Deth for your never-ending support, guidance and encouragement. You are a true role model for a young scientist, and have always encouraged me to look closely at every experiment and to take away every finding it may offer. Your one-on-one tutelage and casual chats about science have meant a lot to me. Your passion for science is inspiring.

I would also like to thank my thesis committee, John Gatley, Robert Schatz, Sunny Zhou and Robert Moir for their time and dedication to making this thesis possible. Your flexibility to fit in meetings, and find time to read my proposal, progress report and thesis is greatly appreciated. Your insightful comments and direction have helped make this thesis what it is.

I am grateful to the students, staff and faculty of the Pharmaceutical Sciences Department. Journal club and the colloquium series have taught me more than any class, thank you for letting me participate.

To my parents: I can’t thank you enough for everything you have done and do for me. You taught me the value of education and have always provided me with everything needed to pursue my goals. I love you.

I would like to thank the following individuals:

My aunt, Heather: for all the support and advice you have shown me over the years. I could not have done this without you. You are the first scientist I ever met and continue to be a role model.

Rachel: for your support and love. Thank you for always being there to make me smile.

My brother, Teddy: you are everything a big brother should be, thank you for always being there for me.

Malav and Christina: you have made my time in lab fun and enjoyable. You are true friends.

Brendan: for the answers to all the questions I’ve asked. I can’t wait to see where your career takes you.

Tom: your drive and focus have pushed me to work harder.

My roommates over the last 5 year, Matt, Dan, Nick, Gage, Garrett and Sean, you have been a huge part of my time here, and always a welcome distraction from science.

Bob Schatz: for always giving me advice and direction. You are a great teacher and mentor; you will be missed by all at Northeastern.
Abstract

The level of oxidative stress increases with age, and abnormal elevations are a hallmark of many neurological and neuropsychiatric disorders, including Alzheimer’s disease. In neurons, the glutamate, aspartate, and cysteine transporter EAAT3 is the predominant transporter of cysteine, the rate-limiting precursor for synthesis of glutathione, the primary intracellular antioxidant. Thus, the regulation of EAAT3 is crucial in maintaining redox balance in neurons, and agents that change its activity can exert important effects on neuronal redox status. Furthermore, since methylation is highly sensitive to redox status, these agents can also modulate methylation status, including DNA methylation, yielding epigenetic consequences.

The redox state of a cell, or the balance of antioxidants and oxidative stress, is an overarching controller of cell fate and function. This balance regulates many cellular pathways. In one such case, the redox sensitive cobalamin-dependent enzyme methionine synthase controls the intersection of the transsulfuration pathway and methylation cycle. Methionine synthase converts homocysteine to methionine, and the redox state of its cobalamin cofactor determines its activity. Oxidation halts activity, leading to increased levels of homocysteine. Under these conditions, the transsulfuration pathway metabolizes homocysteine to cysteine, resulting in glutathione (GSH) synthesis. In such a way, the redox state of a cell determines the fate of homocysteine. A reducing state directs homocysteine through the methylation cycle to produce methionine and then S-adenosylmethionine (SAM), the major methyl donor, thereby increasing methylation capacity. This redox sensitive switch maintains antioxidant levels at the expense of the
methylation cycle. However, in neurons, activity of the transsulfuration pathway is limited, making transport of cysteine by EAAT3 crucial in maintaining redox balance.

Oxidative stress and elevated levels of homocysteine have long been associated with Alzheimer’s disease (AD). The pathogenic protein Aβ, which forms the hallmark plaques in AD brains, causes oxidative stress by reducing copper bound to the copper binding domain of the protein. Recently it has been shown that soluble Aβ oligomers facilitate hippocampal long-term depression by disrupting glutamate uptake by the excitatory amino acid transporter 3 (EAAT3). This link between AD and oxidative stress, elevated homocysteine levels and altered DNA methylation implicates the transsulfuration and methylation pathways as well as EAAT3 and cysteine uptake.

Using SH-SY5Y cells as a model for mature neurons, I show that the intracellular redox state is dependent on EAAT3-mediated cysteine uptake, and sensitive to NTGF regulation. Additionally, the redox state of the cell influences DNA methylation and gene transcription. Furthermore, I show that soluble Aβ oligomers inhibit EAAT3-mediated transport of cysteine, which creates oxidative stress, and disrupts intracellular thiol metabolism creating antioxidant imbalance and changes in DNA methylation and gene transcription. These actions may contribute to the pathology of Alzheimer’s disease.

This work outlines a novel neurotrophic growth factor signaling pathway that uses redox signaling through the mediation of cysteine uptake to influence DNA methylation. This signaling pathway acts in concert with the canonical growth factor activation of transcription factors via PI3K/Akt to change gene transcription. Because the influence of
this novel pathway is global, rather than gene specific, it is able to influence large numbers of genes, and may therefore be an important controller of differentiation.

Overall, this thesis improves our understanding of the mechanism by which neurotrophic growth factors influence redox and the molecular machinery responsible for methylation reactions, including DNA and histone methylation, which regulate gene transcription via their epigenetic effects. This increased understanding provides a novel perspective for the origin of neurological disorders arising over the lifespan and may aid in the development of new treatment approaches for these conditions.
I. INTRODUCTION

A. Statement of Problem

This thesis investigates how growth factors regulate the redox state of a cell, and in turn, how that influences epigenetics. It also examines this relationship in the context of Alzheimer’s disease and investigates a possible link between the growth factor mediated redox signaling and the pathogenic protein in Alzheimer’s disease beta amyloid.

Canonically, growth factors influence cells by binding to a receptor tyrosine kinase, initiating intracellular signaling cascades such as Akt, PI3K, and JAK/STAT or ERK pathways. Many of these signaling pathways achieve their effects through the regulation of transcription factors, resulting in altered transcription of genes. The proposed novel neurotrophic growth factor (NTGF) signaling pathway also affects gene transcription, but uses redox control as a means to influence global DNA methylation and subsequently transcription, instead of transcription factors targeted to individual genes (Fig. 1). Through this proposed novel signaling pathway, NTGFs are able to influence large numbers of genes, and thereby influence cellular differentiation.
**Figure 1: A proposed NTGF epigenetic signaling pathway in the brain.** Growth factors increase EAAT3 mediated cysteine uptake, increasing GSH synthesis. Increased GSH synthesis shifts redox status to a more reducing potential. This shift increases methionine synthase (MS) activity increasing the probability of DNA methylation. DNA methylation leads to changes in gene transcription.

Cellular redox status is primarily regulated by the availability of reduced glutathione (GSH) to donate electrons to oxidized proteins or lipids. Cysteine, the rate-limiting precursor in the synthesis of GSH, is made available by extracellular uptake or by metabolism of homocysteine via the transsulfuration pathway. However, in adult cortical neurons, transsulfuration is limited, and 90% of all available cysteine is transported into the cell by excitatory amino acid transporter 3 (EAAT3) (Watabe et al., 2007). These factors make EAAT3 extremely important in the regulation of redox status in mature cortical neurons. It has previously been shown that oxidative shifts in redox status cause a decrease in the more than 200 methylation reactions, including DNA and histone methylation (Siegmund et al., 2007; Zawia et al., 2009). These changes in methylation broadly affect almost every aspect of cellular metabolism.

Due to the importance of EAAT3 in providing cysteine for the cell’s redox machinery, the regulation of EAAT3 by growth factors will be studied, along with its impact on redox status, DNA methylation and cellular differentiation.
B. Evolution of an Oxygen Based Redox System

With the splitting of water, by photosynthesis, to form atmospheric oxygen (O₂), the environment of the Earth changed for early microbes. Increasing oxygen concentrations, in both the atmosphere and aqueous environments, posed challenges and benefits to microbes living at that time. Oxygen could be used for the first time as the end electron sink in oxidative phosphorylation, greatly increasing the amount of energy derivable from food (Fridovich, 1998). Oxygen also became available for metabolic transformations. It was used to detoxify numerous compounds by mixed function oxidases and became a staple of life for all of Earth’s aerobes. However, utilization of oxygen comes at the cost of the production of free radicals, changes in oxidation states of metals, and the consumption of NAD(P)H, thiols and other compounds essential for metabolic pathways and enzymatic reactions. The need to use oxygen, while at the same time avoiding oxidation, became a driving force for evolution, with many metabolic pathways and cellular mechanisms arising in response to the gradual accumulation of this seemingly simple element. By understanding the evolutionary importance of oxygen, and accompanying oxidative changes, it should not be surprising to think of them as a morphogens able to induce specific cellular responses.

C. Production of Free Radicals

Oxygen is paramagnetic, meaning that it has two unpaired electrons in different pi orbitals that have the same spin (Halliwell and Gutteridge, 1984; Fridovich, 1998). This property of O₂ is the single cause of free radicals and oxidative damage. Paired electrons must have opposite spin quanta, and thus for O₂ to be reduced it cannot accept a pair of electrons with opposite spin, the resulting pairs of electrons would be +/- (normal), and
either +/+ or -/- (highly unfavorable in nature) (Halliwell and Gutteridge, 1984). For this reason, the reduction of oxygen must occur in two additions of single electrons resulting in an intermediate free radical (Halliwell and Gutteridge, 1984). This single electron transfer is slow, and leads to the production of free radicals in biologic systems that involve the reduction of O2. Approximately, 2% – 4% of the oxygen consumed by mitochondria ends up as the free radical superoxide (Watabe et al., 2007).

D. Oxidative Stress

Under normal physiological conditions, reactive oxygen species (ROS) are produced as a byproduct of multiple cellular reactions. ROS include free radical oxygen ions such as superoxide, hydroxyl radicals and organic alkoxy and peroxy radicals and peroxides, such as hydrogen peroxide, peroxynitrite and organic hydroperoxides (Schulz et al., 2000). The balance between oxidized and reduced compounds in a cell at any given moment is called the redox status, or redox state. In humans, ROS are produced from oxidative metabolism, detoxification mechanisms, ionizing radiation and many other sources (Sultana et al., 2009). Damage from ROS includes, but is not limited to, DNA strand breaks and modification, lipid peroxidation, and protein dysfunction and modification (Dringen et al., 2000; Fusco et al., 2007; Sultana et al., 2009). ROS are detoxified by enzymatic reactions such as the conversion of superoxide to hydrogen peroxide by superoxide dismutase and then hydrogen peroxide to water by glutathione peroxidase (Fig. 2). Alternatively, ROS can be reduced by non-enzymatic reactions, using antioxidants such as GSH, and other thiol containing molecules, α-tocopherol (vitamin E), carotenoids, ascorbic acid, bilirubin, uric acid, albumin and nutritional
antioxidants (Fusco et al., 2007; Su et al., 2008). The redox state of a cell is changed when the production of ROS or availability of antioxidants changes.

Figure 2: Cellular antioxidant reactions. Glutathione dependent antioxidant reactions used by the cell to maintain a reduced intracellular potential (Bentley et al., 2008). GSH is a tripeptide containing glutamate, cysteine and glycine.

GSH is the most abundant thiol and most important low molecular weight antioxidant found in cells (Dringen, 2000; Lu, 2009). In most cells the concentration of GSH is maintained at 1–2 mM (Forman et al., 2009). Hepatocytes are responsible for providing much of the GSH found in plasma, and can contain GSH at a concentration up to 10 mM (Aoyama et al., 2008; Forman et al., 2009; Lu, 2009). The sulfhydryl group (–SH) of the middle cysteine residue, of the tripeptide GSH, is involved in reduction and conjugation reactions (Forman et al., 2009). In conjugation reactions, GSH is bound to
xenobiotics, such as quinolones, by various glutathione-S transferases (GSTs) (Fig. 2). These conjugates are then secreted from cells through a membrane transporter such as multidrug resistance proteins (Forman et al., 2009). The major role of GSH, however, is as an antioxidant or reducing agent. GSH is involved in the reduction of both organic and inorganic free radicals and peroxides. In the case of inorganic free radicals, such as superoxide, the free radical is enzymatically converted to a peroxide by superoxide dismutase, and then the peroxide is reduced back to its normal oxidation state by glutathione peroxidase, using GSH as the electron donor (Fig. 2). Glutathione peroxidase is a selenoprotein. This enzyme contains a selenocysteine amino acid at its active site. The selenocysteine is oxidized by peroxide to form SeOH, which is reduced by GSH to form GS-Se (Winterbourn and Metodiewa, 1994; Lu and Holmgren, 2009). Another GSH molecule then reduces the selenocysteine to reactivate the enzyme, releasing GSSG. Glutathione reductase then converts GSSG back to GSH, using NADPH as the reductant (Fridovich, 1998). Selenium is an important redox active element because of its position in the periodic table. It belongs to the chalcogen group along with oxygen and sulfur. Selenium is less electronegative than sulfur which is less electronegative than oxygen, meaning that selenium will reduce sulfur which will reduce oxygen. This transfer of electrons maintains reduced oxygen species in the cell.

Organic peroxides are also reduced by glutathione-dependent mechanisms. Lipid peroxidation, a self propagating chain reaction that causes increased membrane permeability, leading to cellular death, is one such example that is halted by phospholipid hydroperoxide glutathione peroxidase, or GPX4, a GSH-dependent, selenoprotein that can reduce lipid peroxides to lipid alcohols (Forman et al., 2009). GSH also maintains
protein function under oxidative stress. The reaction below illustrates the role GSH plays in reducing oxidized thiol containing proteins (Lu, 2009). These protein-GSH reactions are catalyzed by thiol-transferases.

\[
\text{Protein-SH} + \text{ROS} \rightarrow \text{Protein-SS-R} + 2 \text{GSH} \rightarrow \text{Protein-SH} + \text{GSSG} + 2 \text{H}_2\text{O}
\]

GSH also plays a major role in maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Dringen et al., 2000).

**E. Maintenance of a Reduced GSH**

In healthy cells, roughly 90% of GSH exists in its reduced form. This ratio of reduced and oxidized GSSG is essential in maintaining the redox state of a cell (Schafer and Buettner, 2001). Maintaining reduced GSH is accomplished in part by the enzyme GSH reductase, or GSR. In metabolically active cells, up to 10% of glucose consumed is shuttled through the pentose phosphate pathway to produce NADPH (Schafer and Buettner, 2001). NADPH is the electron donor produced from the catabolism of glucose-6-phosphate in the pentose phosphate pathway. NADPH is used to reduce the FAD moiety present in GSR to make an FADH anion. The FADH anion is used to break disulfide bonds of cysteine residues in GSR which are then used to subsequently reduce the GSSG disulfide bond in a pair of reactions yielding two GSH tripeptides (Asahina et al., 1995). In this way, GSR reduces GSSG to two GSH. However, GSH does not always form disulfide bonds with GSH.

Under certain conditions, proteins containing cysteine residues with accessible sulfhydryl groups can be oxidized. Under such conditions, GSH reacts with the oxidized proteins and forms a protein--GSF disulfide. In a mechanism similar to the reduction of
GSSG to GSH, thioredoxin reduces the protein--GSH disulfide back to the native state protein along with the release of GSH (Arnér and Holmgren, 2000). Again, this mechanism uses NADPH, but not to reduce thioredoxin. Reducing the disulfide bond created to free GSH from the glutathionylated protein is accomplished by Thioredoxin reductase. Thioredoxin reductase is a selenoprotein that uses the electro negativity of a reduced selenocysteine residue to reduce thioredoxin back to an active state (Arnér and Holmgren, 2000; Schafer and Buettner, 2001). Thioredoxin reductase is then reduced back to an active state using NADPH. In addition to the pathways and enzymes dedicated to reducing existing glutathione, another important method of reducing oxidative stress is to increase GSH synthesis.

**F. Glutathione Synthesis**

GSH is a tripeptide comprised of glutamate, cysteine, and glycine (Forman et al., 2009). GSH is synthesized in the cytoplasm in two enzymatic steps. The first reaction forms the dipeptide $\gamma$-glutamylcysteine from glutamate and cysteine, and is carried out by $\gamma$-glutamylcysteine synthetase (GCL). The bond between glutamate and cysteine is through the $\gamma$-carboxyl group of glutamate instead of the normal $\alpha$-carboxyl group (Watabe et al., 2007; Lu, 2009). This reaction is the rate-limiting step in GSH synthesis and is dependent on the availability of cysteine. GCL expression is regulated by Nrf2, a transcription factor that binds to the antioxidant responsive element (ARE), and is up-regulated during times of oxidative stress (Ballatori et al., 2009). In the second reaction, glycine is added to the dipeptide by GSH synthetase to form the complete GSH (Dringen et al., 2000). ATP is required by both enzymes in the synthesis of GSH (Dringen et al., 2000).
G. Cysteine Uptake

In the brain, approximately 90% of the total cysteine uptake is mediated by the excitatory amino acid transporter (EAAT) family of proteins (Shanker et al., 2001). Cysteine is also transported by ASC and System L amino acid transporter systems, however, studies have shown that these transport systems are not active in cysteine uptake in neurons in the brain (Himi et al., 2003; Aoyama et al., 2008; Watabe et al., 2008). Cystine, the oxidized dithiol form of cysteine and an important contributor in maintaining GSH levels in astrocytes, is transported by the Xc- transporter, but this potential source of cysteine uptake does not play a role in neuronal cysteine availability (Himi et al., 2003; Aoyama et al., 2008).

<table>
<thead>
<tr>
<th>Major Cell Type</th>
<th>EAAT1</th>
<th>EAAT2</th>
<th>EAAT3</th>
<th>EAAT4</th>
<th>EAAT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS Distribution</td>
<td>Glia</td>
<td>Glia</td>
<td>Neuron</td>
<td>Neuron</td>
<td>Neuron</td>
</tr>
<tr>
<td>Alternative Name</td>
<td>GLAST</td>
<td>GLT-1</td>
<td>EAAC1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Excitatory Amino Acid Transporters. The CNS distribution and alternative names for each of the five EAATs.

Five members of the EAAT family have been identified, glutamate-aspartate transporter (GLAST or EAAT1), glutamate transporter 1 (GLT-1 or EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 (Danbolt, 2001). GLAST and GLT-1 are found in astrocytes, oligodendrocytes and other glial cells (Holmseth et al., 2012). EAAT4 and EAAT5 are restricted to cerebellar Purkinje cells and the retina (Danbolt, 2001; Watabe et al., 2007; Aoyama et al., 2008). EAAC1 is uniquely and exclusively localized to neurons (Holmseth et al., 2012). Therefore, EAAC1 is the only transporter capable of
importing cysteine into mature human neurons (Danbolt, 2001; Himi et al., 2003; Aoyama et al., 2008; Watabe et al., 2008; Li et al., 2010).

H. EAAT3 Function and Regulation

EAAT3 is a glutamate, aspartate and cysteine transporter. Glutamate transport is coupled to cotransport of three Na⁺ ions and 1 H⁺, and counter transport of 1 K⁺ ion (Zerangue and Kavanaugh, 1996). EAAT3 is found diffusely localized across the entire cell membrane, unlike other glutamate transporters that localize at the synapse (Nieoullon et al., 2006; Watabe et al., 2007, 2008; Holmseth et al., 2012). Knockdown of EAAT1 or 2 result in an increase in extracellular glutamate, whereas a knockdown of EAAT3 has no effect on glutamate concentrations (Watabe et al., 2008). Additionally, the preferential transport of cysteine over glutamate by EAAT3 has identified the primary role of EAAT3 to be cysteine uptake (Bendahan et al., 2000; Danbolt, 2001; Watabe et al., 2007, 2008). The importance of EAAT3 as a cysteine transporter is confirmed by the low levels of GSH and vulnerability to oxidative stress observed in EAAT3 gene-deficient mice (Watabe et al., 2007).
Figure 3: Membrane topology model of the excitatory amino acid transporter. This model of an EAAT transporter shows the 8 transmembrane domains as well as the active loop structure between transmembrane domains 7 and 8 that carries glutamate, aspartate or cysteine into the cell. This model also shows the GTRAP regulatory site on the c-terminal tail (Kanai and Hediger, 2003).

EAAT3 is primarily sequestered in intracellular vesicles, with roughly 20% of the transporter localized at the cell surface during normal conditions (Nieoullon et al., 2006; Sheldon et al., 2006; Aoyama et al., 2008). Increased translocation of EAAT3 to the cell surface is known to occur in response to three signaling mechanisms: Akt/PI3K activation, PKC activation, and MAP kinase activation. through inhibition of the glutamate transport associated protein for EAAC1 (GTRAP3-18) (Himi et al., 2003; Sheldon et al., 2006; Watabe et al., 2008).

Binding of NTGFs to receptor tyrosine kinases triggers the phosphatidylinositol 3-kinase (PI3K) /Akt signal transduction system (Fig. 3) (Góra-Kupilas and Joško, 2005;
Frost and Lang, 2007). A $^{502}\text{YVN}^{504}$ motif in the carboxyl-terminal of the EAAT3 protein is necessary for NTGF dependent increased cell surface expression in response to Akt/PI3K activation (Sheldon et al., 2006). The tyrosine residue in this motif has not been found to be phosphorylated during activation by PI3K/Akt. During NTGF/PI3K/Akt activation, increased cell surface expression results from increased insertion and not decreased endocytosis of the transporter, and is dependent on the mTOR complex mTORC2 (Lin et al., 2001a; Watabe et al., 2008; Almilaji et al., 2012).

Figure 4: Proposed mechanism for NTGF regulation of EAAT3 and redox in neuronal cells. Binding of neurotrophic growth factors to receptor tyrosine kinases causes activation of PI3 kinase. PI3 kinase phosphorylates PIP2 to PIP3, an activator of the PDK1. PDK1 phosphorylates Akt, also Akt can be phosphorylated my mTORC. Akt activation leads to increased surface expression of EAAT3, and increased cysteine uptake. Increased intracellular cysteine leads to increased GSH synthesis and a shift in the redox state to a more reducing potential.

Activation of PKCα causes phosphorylation of serine 465 that precedes the increase in cell surface expression, and this activation both increases insertion and decreases endocytosis (Sheldon et al., 2006; Aoyama et al., 2008; Watabe et al., 2008).
Involvement of the $^{502}$YVN$^{504}$ motif during PKC activation is unclear, with studies claiming both its necessity and its uninvolvement (Huang and Zuo, 2003; Huang et al., 2006; Sheldon et al., 2006; Watabe et al., 2007; Aoyama et al., 2008). PKC$\varepsilon$ activation doesn’t change cell surface expression but may increase EAAT3 activity (Aoyama et al., 2008).

GTRAP3-18 is an ER membrane bound protein with a molecular weight of 22.5 kD (Lin et al., 2001a; Butchbach et al., 2003; Watabe et al., 2008). GTRAP3-18 interacts with EAAT3 at its carboxyl-terminal intracellular domain (Lin et al., 2001a; Butchbach et al., 2003). GTRAP3-18 acts as a negative regulator of EAAT3 by sequestering it within intracellular membrane compartments and inhibiting its trafficking to the cell surface (Lin et al., 2001a; Watabe et al., 2008). GTRAP3-18 inhibits the trafficking of EAAT3 to the cell surface by inhibiting Rab1, a member of the Ras small GTPase superfamily involved in intracellular trafficking (Maier et al., 2009; Li et al., 2010). Chronic morphine, methyl-$\beta$-cyclodextrin and retinoic acid have all been shown to decrease EAAT3 glutamate uptake by up to 50% by up-regulating GTRAP3-18 (Lin et al., 2001a; Butchbach et al., 2003; Watabe et al., 2007, 2008). In addition, knockdown of GTRAP3-18 with antisense RNA leads to an increase in EAAT3 cell surface expression, and an increase in intracellular GSH (Watabe et al., 2008). It has also been shown that GTRAP3-18 regulates the glycosylation of EAAT3, changing its affinity for glutamate (Nieoullon et al., 2006). The role of this glycosylation in cysteine transport is unknown.
Isoflurane and other volatile anesthetics have been shown to up-regulate EAAT3 transcription and increase glutamate uptake (Huang and Zuo, 2003). This up-regulation is independent of both PKC and PI3K, however, and phosphorylation of serine 465 is critical for isoflurane-induced EAAT3 cell surface expression (Huang and Zuo, 2003; Huang et al., 2006). Intravenous anesthetics show no effect on EAAT3 expression, and while the δ-opioid receptor decreases cell surface expression of EAAT3 (Yun et al., 2006; Aoyama et al., 2008; Watabe et al., 2008). In other studies, the transcription factor regulatory factor X1 (RFX1) was shown to increase both EAAT3 expression and activity (Ma et al., 2006).

Arginine 447 is an important amino acid involved in the selectivity of substrate for the transporter (Bendahan et al., 2000). Mutation of the arginine residue at position 447 to cysteine made the transporter specific for cysteine, and electroneutral with no cotransport of sodium (Bendahan et al., 2000). Paradoxically, oxygen radicals and hydrogen peroxide inhibit EAAT3, possibly by direct interaction with the transport process (Watabe et al., 2008).

I. Transsulfuration

In times of oxidative stress, one way to elevate GSH is to augment cysteine levels, which can be accomplished by two mechanisms: transport of cysteine from outside the cell or by intracellular conversion of homocysteine (HCY) to cysteine. The thiol amino acid HCY is formed by the methionine cycle, and is converted to cysteine through a process termed transsulfuration.
In the liver 50% of the cysteine needed for GSH synthesis originates from methionine by the transsulfuration pathway (Fig. 5) (Aoyama et al., 2008; Lu, 2009). In this pathway, homocysteine from the methylation cycle is converted to cysteine via the intermediate cystathionine (Dringen et al., 2000; Forman et al., 2009). The enzyme cystathionine beta-synthase (CBS) initiates transsulfuration with the condensation of serine and homocysteine to form cystathionine (Banerjee et al., 2003). SAM is a positive modulator of CBS, while oxidative stress decreases the activity of the enzyme (Banerjee et al., 2003). Cystathionine is then cleaved to form cysteine and alpha-ketobutyrate by cystathionine gamma-lyase, completing transsulfuration.

In mature neurons, transsulfuration is not a significant source of cysteine. In primary neuron culture experiments, incubation with methionine does not increase the cellular GSH level (Aoyama et al., 2008). In the brain, extracellular cysteine levels are maintained by astroglial cells, which can release up to 10% of their intracellular GSH per hour (Dringen et al., 2000). The ectoenzyme γ-glutamyl transpeptidase cleaves GSH to a γ-glutamyl residue and the dipeptide cysteinylglycine (Dringen et al., 2000). Cysteinylglycine is then broken down to cysteine and glycine, amino acids. Glial cells contain an active transsulfuration pathway and are consistently synthesizing and exporting GSH (Dringen et al., 2000). Glial cells also can take up cystine for synthesis of GSH (Aoyama et al., 2008).
Figure 5: Sulfur metabolism pathways in neuronal cells. Red text highlights the transsulfuration pathway, ending with the formation of GSH. Blue text features dopamine-stimulated phospholipid methylation, utilizing MS. The orange text shows cysteine uptake into neurons via the EAAT3 transporter. Regeneration of methylcobalamin through the formation of glutathionylcobalamin and the addition of SAM is shown in green.

J. Methionine Synthase: Structure and Function

Aside from being metabolized to cysteine, homocysteine is also an important regulatory metabolite in the methylation cycle. The methylation cycle produces SAM, the methyl donor to the 209 methyltransferases in the human body. Homocysteine is methylated to methionine by the enzyme methionine synthase. Methionine synthase is a folate and cobalamin-dependent enzyme that catalyzes the methylation of homocysteine to methionine. The enzyme is comprised of five domains, four of which bind homocysteine, methylfolate, cobalamin, and SAM (Leclerc et al., 1996; Chen et al.,
1997; Bandarian et al., 2003). The fifth domain, known as the cap domain, links the folate- and cobalamin-binding domains. It also functions to cover and protect the cobalamin co-factor from oxidation by limiting access of ROS and electrophiles from the surrounding redox environment (Zhao et al., 2001).

Methionine is subsequently adenosylated to SAM via the ATP-dependent enzyme methionine adenosyltransferase (MAT). SAM is utilized in the methylation of membrane phospholipids, nucleic acids, proteins and biogenic amines and a wide variety of other substrates. SAH is formed when the methyl group from SAM is enzymatically added to another molecule by a methyltransferase (Zhao et al., 2001). SAH is hydrolyzed to homocysteine and adenosine by the enzyme SAH hydrolase. The hydrolysis of SAH is reversible with thermodynamics favoring SAH formation (Williams and Schalinske, 2010), and SAH is a strong inhibitor of methylation reactions. Thus, homocysteine can be remethylated to methionine to support methylation, converted to cystathionine via the transsulfuration to support GSH synthesis, or be converted to SAH to inhibit methylation.

Cobalamin is an essential cofactor for methionine synthase activity and its oxidation state regulates the activity of the enzyme. Figure 6 illustrates the oxidation state of the cobalamin cofactor during the reaction and regeneration of the cofactor in the event it is oxidized. 5-methyltetrahydrofolate (MTHF) transfers a methyl group to the cob(I)alamin forming methylcob(III)alamin or simply methylcobalamin (Dixon et al., 1996) (Fig. 6). Methylcobalamin donates the methyl group to the thiol group of homocysteine forming methionine (Banerjee et al., 1990) (Fig. 6). Cob(I)alamin is then ready to accept another methyl group from MTHF and continue methylating homocysteine (Evans et al., 2004).
As cob(I)alamin waits to be remethylated by MTHF it is easily oxidized to cob(II)alamin (Kräutler, 2012). Cobalamin oxidation inactivates methionine synthase. The reactivation requires methionine synthase reductase to reduce the cobalamin in the presence of SAM which donates a methyl to reform the methylcobalamin methionine synthase active enzyme (Kräutler, 2005, 2012) (Fig. 7). Additionally, cob(II)alamin is converted to hydroxocobalamin in the presence of superoxide anion by SOD. Hydroxocobalamin reacts spontaneously with GSH to form glutathionylcobalamin, the glutathionylcobalamin can then be converted to methylcobalamin, using SAM by methionine synthase (Fig 7).

Any event that increases the time that the cobalamin cofactor spends as cob(I)alamin increases the chances of its oxidation, and the inactivation of methionine synthase. Decreasing the availability of MTHF just does that. Limiting dietary folate, or

Figure 6: Enzymatic reaction catalyzed by methionine synthase and its subsequent reactivation. Figure from Wilson et al. (Wilson et al., 1999).
problems reducing methylenetetrahydrofolate to MTHF by methylenetetrahydrofolate reductase have been shown to decrease the activity of methionine synthase (Chan et al., 2008; Banerjee et al., 2009).

Figure 7: Methionine synthase acts as a redox switch. Methionine synthase contains a redox active methylcobalamin cofactor. Under oxidative stress, this cofactor becomes oxidized and halts the activity of methionine synthase. Under these conditions, homocystine can be condensed with serine to form cystathionine and then cystine to support GSH synthesis. Only when the redox state is restored does the favorable GSH/GSSG ratio allow for the glutathionylation of oxidized cobalamin, and then methylation of the glutathionylcobalamin to reactivate the enzyme.
K. Neurotrophic Growth Factors

NTGFs consist of a family of endogenous proteins that are able to affect the growth and survival of developing neurons and the maintenance of mature neurons (Deister and Schmidt, 2006). These factors are, in part, responsible for differentiation of neural precursor cells (NPCs) to neurons or glial cells, neurite growth, pruning of dendritic spines, cell survival and many other processes (Deister and Schmidt, 2006; Madduri et al., 2009). NTGFs consist of neurotrophins such as NGF and BDNF, GDNF family of ligands such as GDNF, and neuropoietic cytokines (Deister and Schmidt, 2006). IGF-1 and PDGF are not always classified as NTGFs because of their systemic effects; however, these two growth factors have been shown to be crucial for normal brain development and function, and in this proposal will be included in the classification of NTGF (Ye and D’Ercole, 2006; Frost and Lang, 2007; Nguyen et al., 2010).

BDNF, NGF, PDGF, GDNF and IGF-1 all bind to a receptor tyrosine kinase (Cross and Dexter, 1991). BDNF and NGF bind to Trk receptors, BDNF to TrkB and the low-affinity nerve growth factor receptor (LNGFR), also known as p75, and NGF to TrkA (Patapoutian and Reichardt, 2001). GDNF binds to the RET receptor (Madduri et al., 2009). IGF-1 binds to the IGF-1 receptor, and PDGF depending on the combination of the two subunits, A and B, which make up the complete PDGF will bind to the alpha or beta PDGF receptor (Yu et al., 1995; Ye and D’Ercole, 2006; Frost and Lang, 2007). All of these NTGFs are known activators of the PI3K/Akt pathway, with IGF-1 being the most potent (Yu et al., 1995; Mograbi et al., 2001; Patapoutian and Reichardt, 2001; Deister and Schmidt, 2006; Ye and D’Ercole, 2006; Frost and Lang, 2007; Madduri et al., 2009; Nguyen et al., 2010)
In the case of NTGFs, the PI-3 kinase pathway is activated by the binding of the NTGF to its receptor tyrosine kinase. The binding of the NTGF causes the receptor to dimerize, if it is not already, and auto-phosphorylate tyrosine residues located in the intracellular domain of the receptor. PI3K then complexes with these phosphorylated tyrosines. PI3K becomes active in this complex, and phosphorylates PIP2 to PIP3 (Góra-Kupilas and Joško, 2005; Rexhepaj et al., 2007; Nguyen et al., 2010). PIP3 is deactivated back to PIP2 by the phosphatase PTEN. PIP3 binds to phosphoinositide dependent protein kinase (PDK1) which then activates Akt (PKB) (Góra-Kupilas and Joško, 2005; Rexhepaj et al., 2007; Koeberle and Bähr, 2008). Activation of Akt is known to promote neuronal survival, proliferation and cell growth (Frost and Lang, 2007).

L. Methylation, Epigenetics and Differentiation

While all cells contain the same DNA, differential gene expression is accomplished by an ensemble of regulatory events, collectively described as epigenetic mechanisms, in concert with the promoter or repressor effects of transcription factors. Methylation of DNA at CpG sites and modifications of histone tail regions are primary epigenetic mechanisms, reversibly producing graded suppression of gene expression (Martin and Zhang, 2007). DNA methylation is the addition of a single carbon methyl group from the methyl donor, SAM, to the cytosine pyridine ring. This methylation reaction forms 5-methylcytosine (5meC), catalyzed by DNA methyltransferases (DNMTs).

It is well-recognized that epigenetic regulation is essential for X-chromosome inactivation, genetic imprinting, cellular homeostasis, silencing of DNA elements,
chromatin remodeling, and is a primary driving force for development (Pogribny and Beland, 2009; Heyn et al., 2012). These modifications are both stable and dynamic. Epigenetic patterns can be inherited across generations, but de novo CpG methylation and demethylation is also constantly occurring, providing genomic plasticity (Santos and Dean, 2004; Hitchler and Domann, 2007; Martin and Zhang, 2007).

In simplistic terms, development can be viewed as a series of highly orchestrated decisions which guide cells to change their functional activity, divide into similar cells or differentiate into a novel cell type. Changes in epigenetic status underlie many of these dynamic decisions, and the ordered sequence of normal development is guided by DNA-encoded information. However, external factors, occurring during the early in utero environment or during postnatal development, can adversely impact the course of epigenetic regulation, with adverse consequences (Hitchler and Domann, 2007). In the brain, NPCs differentiate to become the three major cell types comprising the CNS, neurons, astrocytes and oligodendrocytes (Juliandi et al., 2010). Recent research has shown that epigenetics plays a central role, along with growth factors, in the differentiation of NPCs (Hirabayashi and Gotoh, 2010; Juliandi et al., 2010). Furthermore, given the centrality of epigenetics, agents or conditions affecting DNA or histone methylation are highly likely to cause changes in cellular differentiation.
DNA and histone methylation utilizes SAM produced by the methylation cycle (Fig. 8) (Santos and Dean, 2004; Martin and Zhang, 2007). Together these reactions exert broad control over cellular function. SAH, a product of methylation reactions, retains high affinity for methyltransferase enzymes and serves as a methylation inhibitor (Weir and Scott, 1999; Clarke, 2002). Increasing the availability of SAM and/or decreasing SAH increases the probability of methylation reactions occurring. Since SAH is reversibly converted to homocysteine by S-adenosylhomocysteine hydrolase, increased levels of homocysteine also inhibit methylation, and activities of the two enzymes acting on homocysteine (methionine synthase and cystathionine-beta-synthase) therefore exert an important influence over methylation (Weir and Scott, 1999). Thus, an increase in methionine synthase activity will potentially increase DNA methylation, while a decrease
has the opposite effect. Since MS activity is highly sensitive to cellular redox status, DNA methylation status is likewise highly sensitive to redox, with MS (along with cystathionine-beta-synthase) providing the crucial link.

The high sensitivity of MS activity to inactivation by ROS or other electrophiles provides a mechanism for cells to regulate their redox status. Inactivation of MS by oxidative stress increases the conversion of homocysteine to cysteine, augmenting synthesis of GSH. Increased GSH levels help restore redox balance, allowing methylation to return to normal. Changes in gene expression during periods of MS inactivation can also contribute to restoration of redox balance, illustrating the close metabolic relationship between these factors.

This suggests that factors regulating sulfur metabolism and cysteine uptake can influence methylation and epigenetics.

**M. The Redox Environment in the Human Brain**

The brain exists within a closed compartment, shielded by the blood-brain barrier, and surrounded by cerebrospinal fluid (CSF). However, the level of the sulfur containing amino acid cysteine, which is rate-limiting for GSH synthesis, is remarkably lower (>10-fold) than plasma (Fig. 9), implying a scarcity of antioxidant resources (Castagna et al.; Ballatori et al., 2009; Forman et al., 2009). This low antioxidant capacity is especially important since the brain utilizes 20% of the oxygen consumed by the body, although it represents only 2% by weight (Dringen et al., 2000). The scarcity of cysteine and GSH is the basis of a metabolic interdependence between astrocytes and neurons with consequences for regulation of neuronal redox status. Astrocyte-released GSH is
hydrolyzed to cysteine and taken up by neurons via the EAAT3 allowing synthesis of neuronal GSH (Dringen, 2000; Watabe et al., 2008). This metabolic interdependence allows antioxidant resources to be stored and released in a local manner, while maintaining the generally low levels in CSF. As a result, neurons are highly dependent upon astrocyte-derived cysteine for maintenance of their redox status, and the gating of cysteine uptake by EAAT3 becomes an exceptionally powerful mechanism for regulating redox status in neurons.

![Image](image.png)

**Figure 9: Thiols in the brain.** The human brain contains low levels of cysteine and GSH, as compared to blood. Neurons rely on cysteine from astrocyte-derived GSH to make their own GSH and maintain redox balance. GSH levels in astrocytes are 4-fold higher than in neurons (Castagna et al.; Sun et al., 2006).

**N. Alzheimer’s Disease and Oxidative Stress**

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder causing cognitive decline, impaired attention and short-term memory. As AD progresses, further cognitive degeneration such as impaired language, facial recognition, and hearing is
manifested. Pathologically, this results from the accumulation of beta amyloid $\text{A}\beta$, and hyperphosphorylated tau. Tau hyperphosphorylation causes dysfunction of the neuronal cytoskeleton, leading to formation of neurofibrillary tangles and the loss of intracellular transport capacity. Tau is dephosphorylated by PP2A, a protein phosphatase, and it’s inhibition leads to increase Tau phosphorylation (Battaglia-Hsu et al., 2009).

Accumulation of neurofibrillary tangles causes neuroinflammation (Trojanowski and Lee, 1995). Neurodegeneration starts in the entorhinal cortex and the basal ganglia, and spreads to the hippocampus, and eventually to other parts of the cortex (Mutter et al., 2010). Due to the early cholinergic neuron loss in the cortex, cognitive functions such as short term memory are the first to be affected (Swerdlow, 2007).

$\text{A}\beta$ is formed by the sequential cleavage of the amyloid precursor protein (APP) by either $\alpha$-, or $\beta$-secretase followed by $\gamma$-secretase. When $\alpha$-secretase first cleaves APP, the cleavage releases a non-amyloidogenic soluble fragment. When $\beta$-secretase cleavage of APP is followed by $\gamma$-secretase cleavage, $\text{A}\beta$ is released. Cleavage via $\gamma$-secretases generates peptides between 39-42 amino acids long, with $\text{A}\beta_{40}$ being the most common and $\text{A}\beta_{42}$ being the most amyloidogenic (Swerdlow, 2007) (Fig. 10). $\gamma$-secretase is comprised of nicastrin, APH-1, presenilin-1 and PEN-2, these proteins together form the holoenzyme.
Figure 10: Molecular pathology of Alzheimer’s disease. APP is cleaved to form Aβ42 by γ-secretase. Aβ42 aggregates to form low order oligomers which ultimately develop into visible amyloid plaques. The accumulation of these aggregates causes the formation of paired helical formations (PHFs) comprised mostly of Tau. PHFs go on to disrupt cellular trafficking and the neuronal cytoskeleton and cause neuronal dysfunction and death. This loss of neuronal function and cell death causes dementia (Karran et al., 2011).

The etiology of Alzheimer’s disease (AD) is complex and poorly understood. The first clues to the cause of AD came from early genetic studies in families with high rates of AD. These families carrying mutations causing familial AD (FAD) were all shown to have mutations in either APP or one of the presenilin genes (Obulesu et al., 2011). Most commonly however, AD is not caused by these FAD mutations. Many genetic risk factors for AD have been identified. For example, mutations in apolipoprotein E (ApoE) have been reported to be a risk factor in 50% of late onset cases, and effect the clearance
and oligomerization of Aβ (Beyer et al., 2005; Castellano et al., 2011; Cerf et al., 2011). The genetic evidence for Aβ's role in Alzheimer's disease is very strong, but the pathological role of Aβ, and mechanistic role in neuronal death is still largely debated and questioned.

The toxic Aβ oligomer hypothesis for Alzheimer’s disease has recently been proposed and has attracted considerable research. The Aβ oligomer hypothesis helps resolve the lack of correlation between amyloid plaques and cognitive impairment or neurodegeneration (Benilova et al., 2012). Early research on the proteins isolated from the brains of AD patients showed protofibrils with a smaller diameter than the amyloid fibrils that made up the amyloid plaques. These protofibrils were shown to be oligomeric species of Aβ, and linked the monomeric Aβ to the observed plaque deposits (Walsh et al., 1997). Currently many oligomeric species from soluble monomers to soluble oligomers to insoluble oligomers to plaques have been isolated from the brains of Alzheimer’s patients and the soluble species more strongly correlate with disease symptoms than plaques (Li et al., 2009; Mc Donald et al., 2010). Importantly, these oligomeric species have been shown to cause cell death and disrupt various cellular processes in vitro and in vivo (Fig. 11) (Walsh et al., 2002; Li et al., 2009).
Figure 11: Effects of Aβ oligomers on pathways linked to synaptotoxicity. Aβ has been shown to alter glutamate receptor-dependent cascades, activate caspases, and upregulate α7-nAcCh receptors leading to disruption of LTP and LTD. Aβ induced calcium release activates calcineurin and NFATc4 and causes dystrophic changes in neuritis and spine loss. Additionally, Aβ stabilizes NMDA receptors causing excitotoxicity (Benilova et al., 2012).

In brain tissues from AD patients, presence of the ε4 allele of ApoE correlates with decreased GSH levels, as compared with those of age-matched controls (Aoyama et al., 2008). The methionine synthase polymorphism A2756G is a risk factor for AD (Beyer et al., 2003). Other studies have found elevated levels of HCY in the plasma, and decreased levels of SAM in the CSF of AD patients, which further implicates an alteration of the transsulfuration pathway in AD patients (Swerdlow, 2007; Mosconi et al., 2008; Sultana et al., 2009). Also a hallmark of Aβ, is its ability to induce inflammation and free oxygen radical production (Su et al., 2008). 4-Hydroxynonenal (4-HNE), a lipid peroxidation metabolite and indicator of oxidative stress, is elevated in the
serum of Alzheimer’s disease patients (Swerdlow, 2007; Sultana et al., 2009). In a mouse model of AD, over expression of amyloid precursor protein (APP) caused a decrease in the protein level of EAAT3 (Nieoullon et al., 2006). Also, the knock down of EAAT3 in mice reveals a brain phenotype mimicking that of AD patients, including neuronal cell death and decreased working memory (Watabe et al., 2007; Sultana et al., 2009). Recently, soluble oligomers of amyloid beta have been shown to inhibit glutamate uptake by EAAT3 (Li et al., 2009). Taken together, this evidence connects dysfunctional EAAT3 and elevated levels of oxidative stress with Alzheimer’s disease.
SPECIFIC AIMS

Specific Aim 1: Investigate neurotrophic growth factor regulation of EAAT3-mediated cysteine uptake and its influence over redox status in cultured neuronal cells.

Under normal conditions, only 20% of EAAT3 is at the plasma membrane, with the remainder being located in endoplasmic vesicles. Similar to the insulin receptor, activation of tyrosine kinase-linked receptors and the PI3 kinase-signaling pathway recruits additional transporters to the cell surface and augments cysteine uptake. Importantly, this implies that neurotrophic growth factors, which activate PI3 kinase, may play an important role in EAAT3 regulation. To investigate this role, cysteine uptake will be measured in SH-SY5Y human neuroblastoma cells using $[^{35}\text{S}]$-labeled cysteine. Dose-response and time-response relationships will be determined for brain-derived growth factor (BDNF), glial cell-derived growth factor (GDNF), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF). Inhibitors of the tyrosine kinase/PI3 kinase and MAP kinase pathways will be used to probe the importance of these signaling pathways in growth factor response. In parallel studies, the intracellular thiols and thioethers involved in GSH synthesis, and the methylation cycle (cysteine, cysteine, GSH, GSSG, homocysteine, homocystine, cystathionine, methionine, S-adenosylmethionine and S-adenosylhomocysteine) will be measured in cell lysates by HPLC coupled with an electrochemical detector. These
measurements will allow us to evaluate the redox status of the cell as regulated by growth factor effects on EAAT3.

**Specific Aim 2: Evaluate redox-dependent changes in DNA methylation in cultured SH-SY5Y cells.**

Measurement of changes in intracellular thiol levels and global CpG DNA methylation will allow us to characterize the effects of NTGF induced changes in redox status on DNA methylation. Cellular redox will be altered by 1. Culturing cells in media containing low cysteine, similar to CSF levels. 2. Addition of NTGFs found to be effective in Aim 1. 3. Treatment with buthionine sulfoximine (BSO), a specific γ-glutamylcysteine synthetase inhibitor, which blocks GSH synthesis. 4. Treatment with LBTBA, an inhibitor of EAAT3 mediated cysteine uptake.

**Specific Aim 3: Evaluate changes in EAAT3-mediated cysteine uptake, and intracellular thiol metabolism caused by soluble Aβ oligomers, the pathogenic protein found in the brains of Alzheimer’s Disease (AD) patients.**

The effects of soluble Aβ oligomers on EAAT3-mediated transport of cysteine, oxidative stress, and intracellular thiol metabolism will be studied. Soluble Aβ oligomers concentrated from culture media containing Aβ secreted by the 7PA2-CHO, APP-expressing cell line will be used. NGTFs, found to be effective in Aim 1, will be added to SH-SY5Y neuroblastoma cells cultured in media with or without soluble Aβ oligomers to evaluate the effectiveness of NGTFs to normalize cysteine transport, oxidative stress and intracellular thiol metabolism.
Specific Aim 4: Investigate the role of redox-mediated epigenetic changes on differentiation.

Measurement of intracellular thiols and global CpG DNA methylation during the differentiation of SH-SY5Y cells will allow us to view the changes redox and methylation which accompany cellular differentiation. SH-SY5Y cells will be differentiated using the commonly used protocol of treatment with retinoic acid followed by BDNF treatment (Encinas et al., 2000). Intracellular thiols, global CpG DNA methylation and neurite number, a marker of neuronal differentiation, will be measured prior to differentiation and at three differentiation time points.
II. MATERIALS AND METHODS

A. Cell Culture

SH-SY5Y human neuroblastoma cells were purchased commercially from ATCC®. Cells were grown as monolayers in 10 cm standard tissue culture dishes, containing 10 mL of alpha-modified Minimum Essential Medium (α-MEM) supplemented with 1% penicillin-streptomycin-fungizone (antibiotics) and 10% fetal bovine serum (FBS). Cultured cells were maintained in an incubator chamber with 5% CO₂ at 37°C. For cell passage, confluent cells were detached with 1.5 mL trypsin-EDTA solution. Cells were resuspended in 10 mL fresh medium and seeded at low density in 10 cm standard tissue culture dishes. For most experiments, cells were plated and incubated for 24 hours then media was switched to low serum media (1%) FBS for an additional 24 hours prior to use. For cell lines, experiments utilized cells between 5-30 passages, with no significant differences in results.

B. Cell Differentiation

SH-SY5Y neuroblastoma cell line cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM l-glutamine, penicillin (20 units/ml), streptomycin (20 mg/ml), and 10% (vol/vol) heat-inactivated fetal calf serum (Hyclone, Logan Utah). Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂. Cells were seeded at an initial density of 10⁴ cells/cm² in culture dishes (Corning) previously coated with 5 mL of 0.05 mg/ml collagen (Sigma). All-trans retinoic acid (RA) (Sigma) was added the day after plating at a final concentration of 10 μM in DMEM with 1% fetal calf serum. After 5 days in the presence
of RA, cells were washed three times with DMEM and incubated with 50 ng/ml BDNF (Sigma) in DMEM (without serum) for different intervals.

C. Cysteine Uptake

SH-SY5Y human neuroblastoma cells were plated in six-well standard tissue culture plates containing 2 mL of media for 48 hours before the assay. Confluent cells were treated and incubated for various time-points. Media was aspirated after pretreatment and then cells were washed with 600 μL of 37°C Hanks Buffered Salt Solution (HBSS). Non-radioactive HBSS was aspirated, replaced with 600 μL of 37°C HBSS containing radiolabeled cysteine ([35S]cysteine, (1 μCi/1 mL)), 10 μM unlabeled cysteine (saturates the system) and 100 μM DTT (reducing agent), and incubated for 5 minutes. The [35S]cysteine/HBSS mixture was aspirated and treatment was terminated with 2x washes of ice-cold HBSS. Cells were then lysed with 600 μL of dH2O, scraped, collected in 1.5 mL microcentrifuge tubes, and sonicated for 10 seconds. 100 μL of each sample was aliquoted for a Lowry protein assay. 200 μL of each sample (in triplicate) was aliquoted into scintillation vials with 4 mL of scintillation fluid, vortexed, and counted for radioactivity with a Perkin Elmer scintillation counter. Samples were normalized against protein content. This protocol is based on the method developed by Chen and Swanson.(Chen and Swanson, 2003)
D. Lowry Protein Assay

Protein concentrations were determined by the modified Lowry method for protein quantification using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

E. Growth Factor Treatments

SH-SY5Y human neuroblastoma cells were plated in six-well standard tissue culture plates for cysteine uptake experiments of 10 cm$^2$ Petri dishes for thiol experiments. Cells were grown in normal media (10% FBS) for 24 hours after which the media was replaced by low-serum media (1% FBS) for another 24 hours totaling 48 hours before the assay. Confluent cells were pretreated with wortmannin 100 mM or PD98059 10 μM for one hour followed by treatment with NTGF 10 nM for 60 minutes for cysteine uptake, 2 hours for thiol measurements, or 24 hours for DNA methylation measurements.
Figure 12: Treatment course for NTGF experiments. Diagram showing cell culture conditions and treatment times and during NTGF experiments.

F. Aβ Preparations

Aβ preparations were obtained from the Selkoe lab at the Center for Neurologic Disease, Harvard Medical School. Soluble Aβ oligomers were prepared using the following protocol. Secreted human Aβ peptides were collected and prepared from the conditioned medium (CM) of a CHO cell line (7PA2) that stably expresses human APP751 containing the V717F AD mutation. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, and 200 mg/ml G418 for selection. Upon reaching ~95% confluency, the cells were washed and cultured overnight (~15 h) in serum-free medium. CM was collected, spun at 1500 × g to remove dead cells and debris, and stored at 4°C. The CM was concentrated 10-fold with a YM-3 Centricon filter.
Aliquots of concentrated CHO and 7PA2 CM were stored at −80°C. Immunodepleted conditioned media was produced by mixing polyclonal Aβ antiserum AW7 (1:100) with conditioned media and incubated at RT for 2 hours on an orbital shaker. The mixture was then mixed with Protein A Sepharose and again incubated at RT this time for 4 hours on an orbital shaker. The Sepharose Aβ immunocomplexes were then removed by centrifugation. (Li et al., 2009).

G. Aβ Treatments

SH-SY5Y human neuroblastoma cells were plated in six-well standard tissue culture plates or 10 cm² petri dishes 48 hours before the assay. Cells were grown in normal media (10% FBS) for 24 hours after which the media was replaced by low-serum media (1% FBS) for another 24 hours totaling 48 hours before the assay. Confluent cells were treated with 10% of the culture volume of 10x concentrated CHO conditioned media, or with 10x concentrated 7PA2 conditioned media for 2 hours for cysteine uptake or thiol measurements, and 24 hours for DNA methylation studies. In experiments examining the effects of IGF-1 supplementation, IGF-1 was applied 1 hour prior to the addition of Aβ oligomers.
**Figure 13: Treatment course for Aβ experiments.** Diagram showing cell culture conditions and treatment times and during Aβ experiments.

**H. Thiol isolation**

Treatments were carried out using either 6-well cell culture plates or 10 cm standard round dishes. Confluent cells were pretreated in culture media as indicated for individual experiments. After treatments, media was aspirated and the cells were washed 2x with 1 mL of ice-cold HBSS. HBSS was aspirated from the cells and 0.5 mL ice-cold dH2O was added to each well. Cells were scraped from the dish and suspended in dH2O. 100 μL of homogenate was used to determine protein content using a modified Lowry Protein Assay. The cell suspension was sonicated for 15 seconds on ice and an equal volume of 0.4 N perchloric acid solution was added. Sonicates were then spun at 13,000 RPM on a tabletop microcentrifuge for 60 min. 100 μL of sample was added to a conical micro autosampler vial, blown with nitrogen, capped and kept at 4°C in the autosampler cooling tray. Samples were normalized against protein content.
I. HPLC Thiol and Thioester quantification

100 μL of thiol sample was added to a conical micro autosampler vial, blown with nitrogen, capped and kept at 4°C in the autosampler cooling tray. 10 μL of sample was injected onto an Agilent Eclipse XDB-C8 (3 x 150mm; 3.5 μm) and Agilent Eclipse XDB-C8 (4.6 x 12.5mm; 5 μm) guard column by the autosampler. Samples were eluted using the following step gradient: 0-9 min 0% B, 9-19 min 50% B, 19-30 min 50% B, then equilibrate column with 5% B for 12 min at a flow rate of 0.6 mL/min. Mobile phase A consists of 25 mM Sodium phosphate, 1.4 mM 1-Octanesulfonic acid in dH₂O, adjusted to pH 2.65 with phosphoric acid. Mobile phase B consists of 50% Acetonitrile, 50% dH₂O, 25 mM Sodium phosphate, 1.4 mM 1-Octanesulfonic acid in dH₂O, adjusted to pH 2.65 with phosphoric acid. Thiols were measured using the ESA CoulArray with BDD analytical cell model 5040 electrochemical detector at an operating potential of 1500 mV. Samples were normalized against protein content.

J. DNA Isolation

DNA from cell culture for the analysis of DNA methylation was harvested and isolated using the DNeasy® Blood & Tissue Kit from QIAGEN® according to the provided protocol. Isolated DNA was quantified using a ND-1000 NanoDrop spectrophotometer. Only 1μL of DNA is needed for quantification on the NanoDrop.

K. DNA Methylation Analysis

Isolated DNA from SH-SY5Y cell culture was adjusted to a concentration of 50 ng/mL, 100 ng of DNA was used for each assay. Assessment of the global DNA
methylation status was accomplished using the MethylFlash™ Methylated DNA Quantification Kit from Epigentek (Farmingdale, NY). The methylated fraction of DNA was identified using 5-methylcytosine monoclonal antibodies and quantified by an ELISA-like reaction. The levels of methylated DNA were calculated using the OD intensity on a microplate reader at 450 nm.

L. RNA Isolation

Cells were maintained and plated in six-well culture dishes as described above. RNA was isolated using the RNAqueous®-4PCR kit from Ambion®. Isolated RNA was treated with DNase, as stated in the extended RNA isolation protocol, followed by sample quantification using a ND-1000 NanoDrop spectrophotometer.

M. Primers

All custom primers were designed using the Invitrogen OligoPerfect™ Designer to have between 50-60% GC content, an annealing temperature of 60°C and a length of 20 bases. Primer sets were checked for primer-dimer formation and each primer was specific for the desired template. Full primer sequences can be found in the Appendix (Table 1). GAPDH primers were uniform in expression and validated in qRT-PCR studies; therefore, the housekeeping gene GAPDH is an appropriate internal and loading control.

N. cDNA/RT-PCR

cDNA synthesis and subsequent PCR amplification was performed using the Cloned AMV First-Strand cDNA Synthesis Kit and Platinum® Taq DNA Polymerase High Fidelity from Invitrogen™. cDNA synthesis used 1 μg RNA, 10 mM dNTP mix, 50 μM oligo primers and q.s. with dH2O to a final volume of 12 μL. Samples were denatured
at 65°C for 5 minutes and then placed on ice. Cloned AMV RT (15 units/μL), RNaseOUT™ (40 U/μL), 0.1 M DTT, 5x cDNA synthesis buffer and dH₂O in a final volume of 8 μL were used in the second part of the reaction. RT-PCR was performed using 2 μL of cDNA with 50 mM MgSO₄, 10 mM dNTP mix, 5 U/μL Taq DNA polymerase, dH₂O and 10 μM sense and antisense primers in a final volume of 50 μL. 40 cycles were performed in the PTC-100 thermocycler from MJ Research, using 1 min/kb extension time at 68°C. The primer annealing temperature was 60°C.

Equal amounts of PCR products were run on a 10% pre-cast TBE gel and electrophoresed for 90 minutes at 200 V and 10-18 mA. After electrophoresis, gels were agitated on a shaker with SYBR Safe™ DNA Gel Stain for 30 minutes and then visualized using a UV transilluminator.

O. qRT-PCR Analysis

qRT-PCR was performed on duplicate samples using the ABI Prism 7000 Sequence Detection System (Applied Biosystems™). The assay was run in 96-well optical reaction plates. qRT-PCR used 3 μL of diluted cDNA template, 10 μM sense and antisense primers, 12.5 μL SYBR® Green PCR Master Mix from Applied Biosystems™, and dH₂O in a final volume of 25 μL. The following thermal parameters were used: incubate 2 min at 50°C, followed by 10 min at 95°C, and then 40 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 45 sec, followed by a final extension of 72°C for 5 min. No template controls were run on each plate and dissociation curves were generated to determine any non-specific products. All data was analyzed using the ∆∆Ct method and normalized to GAPDH.
P. Statistical Methods

Statistical analyses were carried out using Graph Pad Prism® version 5.01. The Student’s t-test for independent means was used to test for significant differences between control and experimental groups. Data was expressed as mean ± standard error of the mean (SEM). Best-fit values, including correlation coefficients, were calculated using non-linear and linear regression models.
III. RESULTS

A. Validation of cysteine uptake in the human neuroblastoma cell line SH-SY5Y.

To determine the conditions used for the measurement of cysteine uptake, uptake media containing concentrations of cysteine ranging from 10 nM-1 mM was investigated (Fig. 14). At each concentration of cysteine, non-specific uptake was subtracted from total uptake. Non-specific uptake was assayed using a parallel uptake experiment kept on ice during the measurement time period. Uptake media containing a concentration of 10 μM cysteine was chosen for use in all subsequent uptake experiments. This value agrees with similar cysteine uptake protocols (Shanker et al., 2001; Chen and Swanson, 2003; Himi et al., 2003).

The time allowed for cysteine uptake was also investigated. Cysteine uptake was allowed to proceed for 0-10 min (Fig. 15). Uptake reached a plateau after 5 minutes. Thus, all subsequent cysteine uptake experiments were preformed in uptake media containing cysteine at a concentration of 10 μM, for 5 minutes.
Figure 14: Cysteine uptake by SH-SY5Y cells in variable concentrations of cysteine. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake with increased concentrations of cysteine in the uptake media n=6. Nonspecific binding was subtracted from total cysteine uptake.

Figure 15: Time course of cysteine uptake in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 5 min n=6. Nonspecific binding was subtracted from total cysteine uptake.
To identify the transporters which could participate in cysteine uptake, primers were designed to all of the excitatory amino acid transporters, EAAT1-5. PCR studies were performed with RNA from cultured SH-SY5Y human neuroblastoma cells. Custom primers were designed using the Invitrogen OligoPerfect™ Designer to have between 50-60% GC content, an annealing temperature roughly 60°C and a length of 20 bases. Primer sets were checked for primer-dimer formation, and target specificity in NCBI’s Primer Blast. Full primer sequences can be found in Appendix I. RNA was isolated from cellular extracts using the RNAqueous® - 4PCR kit from Ambion and treated with DNase to remove trace amounts of DNA. See Appendix II for protocol. PCR results in SH-SY5Y cultured cells show RNA encoding EAAT1-3 (Fig. 16). mRNA coding for EAAT3 was found to be transcribed at the highest levels followed by EAAT1 at a level of less than half. EAAT2, 4 and 5 were observed a near baseline levels.
Figure 16: Presence of EAAT1-3 RNA in SH-SY5Y cells. EAAT mRNA levels from SH-SY5Y neuroblastoma cells were probed using qRT-PCR with primers designed for each EAA transporter. n=3.

To pharmacologically characterize the participation of cysteine transport of EAAT1 and EAAT3 in SH-SY5Y cells, the well-characterized EAAT3-specific inhibitor L-beta-threo-benzyl-aspartate (LBTBA) was utilized (Esslinger et al., 2005). A concentration-dependent inhibition of cysteine uptake by LBTBA was observed (Fig. 17). All concentrations of LBTBA tested yielded significant inhibition of cysteine uptake, with $10^{-5}$ M inhibiting over 99% of cysteine uptake, and an IC$_{50}$ of $1.86 \times 10^{-7}$ M. These data suggested that the EAAT transporter system was the major route of cysteine uptake in SH-SY5Y neuroblastoma cells. Additionally cysteine uptake was measured in the presence of LBTBA or dihydrokainate, a non-specific EAAT inhibitor (Fig. 18). LBTBA
inhibited cysteine uptake with an IC_{50} of $3.13 \times 10^{-8}$ M while dihydrokainate inhibited with an IC_{50} of $1.56 \times 10^{-5}$ M. Because LBTBA inhibited cysteine uptake with equal or greater efficacy of dihydrokainate, the participation of other EAAT transporters involved in cysteine uptake can be ruled out. These data suggested that the EAAT3 transporter is the major route of cysteine uptake in SH-SY5Y neuroblastoma cells.

![Graph showing LBTBA decreases cysteine uptake in SH-SY5Y cells.](image)

**Figure 17: LBTBA decreases cysteine uptake in SH-SY5Y cells.** Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a dose dependent inhibition of cysteine uptake with uptake media containing increasing concentrations of LBTBA \( n=6 \). Asterisks (*) show a significant difference \( p<0.05 \) from control. Nonspecific binding was subtracted from total cysteine uptake.
Figure 18: Inhibition of cysteine uptake by LBTBA and Dihydrokainate in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed Cysteine uptake was decreased in the presence of uptake media containing LBTBA or Dihydrokainate. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.
B. Validation of intracellular thiol and thioether quantification

Running and isolation conditions were optimized for electrochemical HPLC detection of cellular thiols and thioethers from SH-SY5Y cell lysates. Column loads of 1-200 ng cysteine, cystine, cystathionine, GSH, HCY, methionine, GSSG, homocystine, SAH and SAM were used to optimize the running conditions for resolution and quantification. Figure 19 shows a prepared cell lysate spiked with 10 ug/mL of each thiol or thioether measured.

Figure 19: Typical chromatogram showing the measurement of intracellular thiols. Cysteine, cystine, cystathionine, glutathione, homocysteine, methionine, oxidized glutathione homocystine S-adenosylhomocysteine and S-adenosylmethionine are resolved in a single HPLC separation and measured by electrochemical detection.

Figure 20 shows a sample standard curve for each thiol or thioether ranging from 25-200 ng. The $R^2$ value for each trend line is above 0.99, showing the accuracy of the detection method.
**Figure 20: Typical standard curve of measured thiols and thioethers.** Standard curves for cysteine, cystine, cystathionine, glutathione, homocysteine, methionine, oxidized glutathione, homocystine, S-adenosylhomocysteine and S-adenosylmethionine.

C. Validation of global DNA methylation protocol.

As cells divide, their DNA is demethylated, and then remethylated. To remove these confounding factors from our DNA methylation studies, we grew cells to confluence for 24 hours in media supplemented with 10% FBS. After 24 hours the cells were switched to 1% FBS. DNA methylation was then measured after 24, 48 and 72 hours after media was switched to low serum media (Fig. 21).
Figure 21: DNA methylation over 72 hours in SH-SY5Y cells. Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. DNA was isolated after 24, 48 and 72 hours in 1% FBS growth media. n=3. Asterisks (*) show a significant difference (p<0.05) from control.

No changes in DNA methylation were observed 24-72 hours after growth in low serum media. To confirm we could observe a change in DNA methylation under the conditions being tested, we measured DNA methylation after 2, 6, 12, 24 and 48 hours of IGF-1 (10 nM) treatment (Fig. 22).
Figure 22: Time course of the effect of IGF-1 on global DNA methylation in SH-SY5Y cells. Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. Cells were treated for 2, 6, 12, 24, and 48 hours with IGF-1 (10nM). n=3. Asterisks (*) show a significant difference (p<0.05) from control.

IGF-1 significantly increased DNA methylation after 24 hours and was persistent after 48 hours. Measurement after 48 hours was used because it allowed a 24 hour time period for cells to adjust to the low serum media, and then an additional 24-hour time period for treatments.

To confirm that cell number did not change over the treatment time, cells were counted 2, 12, 24, 48 and 72 hours after cell media was switched over to low serum media with or without IGF-1 (10 nM) (Fig. 23).
Figure 23: Cell survival for 48 hours of IGF-1 treatment. SH-SY5Y cells were counted using a Bio Rad TC10 automated cell counter. Cells were initially grown for 24 hours in media supplemented with 10% FBS then switched over to media containing 1% FBS prior to treatment. Cells were counted prior to, and after 2, 12, 24, 48 and 72 hours with IGF-1 (10nM). Media was replaced every 24 hours. n=3.

Cell number did not change over the time tested. These results show that global DNA methylation is stable over the time and conditions tested, and changes in DNA methylation are unlikely to be caused by cell division.
D. Specific Aim 1: Investigate neurotrophic growth factor regulation of EAAT3-mediated cysteine uptake and its influence over redox status in cultured neuronal cells.

Studies have shown that EAAT3 is regulated by pathways similar to those of the glucose transporter Glut4. Activation of the PI3 kinase signaling pathway by insulin leads to increased surface expression of Glut4, and increased glucose uptake (Cong et al., 1997). It has been reported that under normal conditions, only 20% of EAAT3 is active at the plasma membrane, with the remainder being located in the endoplasmic reticulum (Lin et al., 2001a; Watabe et al., 2008). Previous studies have shown that growth factor activation of tyrosine kinase-linked receptors recruits additional EAAT3 transporters to the cell surface and augments glutamate uptake (Lin et al., 2001a; Watabe et al., 2007). To test whether or not cysteine uptake was regulated by similar pathways, growth factor-stimulated cysteine uptake was examined. Five different NTGFs: IGF-1, BDNF, GDNF, NGF, and PDGF, were tested for their ability to augment cysteine uptake.

Dose response curves for NTGF stimulation of cysteine uptake were initially determined (Fig. 23-28). SH-SY5Y cells were treated for 1 hour with NTGFs, with concentrations ranging from 100 pM to 1 μM under low fetal bovine serum conditions (1% FBS). Low serum conditions were used in place of normal 10% FBS medium supplementation to decrease background growth factor stimulation from growth factors contained in FBS.
Figure 24: Dose response curve for IGF-1 of EAAT3 mediated cysteine uptake. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a stimulatory dose response curve for IGF-1. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.

Figure 25: Dose response curve for BDNF of EAAT3 mediated cysteine uptake. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a stimulatory dose response curve for BDNF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.
Figure 26: Dose response curve for GDNF of EAAT3 mediated cysteine uptake. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a stimulatory dose response curve for GDNF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.

Figure 27: Dose response curve for NGF of EAAT3 mediated cysteine uptake. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a stimulatory dose response curve for NGF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.
Figure 28: Dose response curve for PDGF of EAAT3 mediated cysteine uptake. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed a stimulatory dose response curve for PDGF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from control.

NTGFs were able to robustly and potently stimulate cysteine uptake. IGF-1 more than doubled cysteine uptake, and stimulated uptake with an EC\textsubscript{50} of 0.73 nM. Near-maximum stimulation of cysteine uptake was observed with 10 nM IGF-1, and this concentration was used in subsequent IGF-1 experiments. The summary of results for the other NTGFs is included in Table 2.

<table>
<thead>
<tr>
<th>NTGF</th>
<th>EC\textsubscript{50} (nM)</th>
<th>Efficacy (% increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>0.73</td>
<td>107</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.32</td>
<td>44</td>
</tr>
<tr>
<td>GDNF</td>
<td>0.92</td>
<td>53</td>
</tr>
<tr>
<td>NGF</td>
<td>152</td>
<td>70</td>
</tr>
<tr>
<td>PDGF</td>
<td>0.34</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 2: Summary of dose response results for NTGF-stimulated cysteine uptake in SH-SY5Y human neuroblastoma cells.
Time course experiments for NTGF stimulated cysteine uptake were carried out for radiolabeled cysteine uptake measurements after 0, 10, 30, 60, 120 and 240 minutes of incubation with NTGFs under low serum conditions (Figs 29-33).

Figure 29: Time course of cysteine uptake stimulated by IGF-1 in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 60 min when stimulated by 10nm IGF-1. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from time 0.
Figure 30: Time course of cysteine uptake stimulated by BDNF in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 30 min when stimulated by 10nM BDNF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from time 0.

Figure 31: Time course of cysteine uptake stimulated by GDNF in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 60 min when stimulated by 10nM GDNF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from time 0.
Figure 32: Time course of cysteine uptake stimulated by NGF in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 30 min when stimulated by 1μM NGF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from time 0.

Figure 33: Time course of cysteine uptake stimulated by PDGF in SH-SY5Y cells. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line showed an increase in uptake over time reaching a plateau after 30 min when stimulated by 1nM PDGF. n=6. Nonspecific binding was subtracted from total cysteine uptake. Asterisks (*) show a significant difference (p<0.05) from time 0.
For each NTGF, near maximal uptake was achieved after 60 minutes of incubation. IGF-1, BDNF, GDNF and NGF all significantly increased uptake after 30 minutes of treatment. PDGF increased uptake after 60 minutes of treatment.

IGF-1 most effectively stimulated cysteine uptake and had potency similar to the GDNF, BDNF and PDGF. It also stimulated cysteine uptake by 30 minutes and near maximally by 60 minutes. For these reasons, IGF-1 was used in subsequent experiments looking at the effects of NTGF mediated cysteine uptake.

To investigate the signaling pathways activated by NTGFs which lead to stimulation of cysteine uptake, SH-SY5Y neuroblastoma cells were pretreated with the PI3 kinase inhibitor wortmannin or the MEK1 inhibitor PD98059 for 60 minutes prior to IGF-1 stimulation. Radiolabeled cysteine uptake was measured 60 minutes after IGF-1 treatment (Fig. 34). Pretreatment was carried out under low serum conditions, 1% FBS.
Figure 34: Cysteine uptake is increased by IGF-1 stimulation and blocked by PI3 kinase inhibition. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma cell line. Cells were pretreated with wortmannin (100 nM), a PI3K inhibitor or PD98059 (10 μM), a MEK1 inhibitor. Cysteine uptake was stimulated with IGF-1 (10nM), n=6. Asterisks (*) show a significant difference (p<0.05) from control, (#) shows a significant difference (p<0.05) from IGF-1 treated group.

IGF-1 significantly increased cysteine uptake, which was blocked by wortmannin, but not blocked by PD98059. The inhibition of IGF-1 stimulated cysteine uptake by wortmannin indicates the involvement of the PI3 kinase pathway. The absence of effect by PD98059 indicates that the MAP kinase pathway is not involved in NTGF stimulated cysteine uptake at these time points.
After observing the effect of IGF-1 on cysteine uptake in SH-SY5Y cells, the intracellular effects on thiols and thioethers involved in GSH synthesis were investigated. Intracellular thiols were measured using HPLC linked to an electrochemical detector. A time course for IGF-1-mediated changes in intracellular thiols was produced using data from five different time points 30 minutes, 1, 2, 4 and 48 hours and control values (Fig. 35). Cell growth media was changed to low-serum media (1% FBS) 24 hours prior to measurement, at the initiation of the experiment and again after 24 hours of treatment.

**Figure 35: Time course of the effect of IGF-1 on thiols and thioethers in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 30, 60, 120 and 240 minutes with IGF-1 (10 nM) prior to measurement, n=6. Control values for intracellular thiols (nM/mg protein) were CYS 108.2, cystine 19.9, cystathionine 14.1, GSH 18.5, HCY 1.8, homocystine 2.9, MET 4.1, GSSG 0.4, SAM 6.6 and SAH 0.9. Asterisks (*) show a significant difference (p<0.05) from the control group.
Maximal changes for cysteine, cystathionine, GSH, homocystine, methionine, GSSG, SAM and SAH were observed after 120 minutes of treatment with IGF-1 (Fig. 35). Maximal changes in the levels of homocysteine and SAH were observed at 240 minutes of IGF-1 treatment. As there was the greatest change in intracellular thiol metabolism after 2 hours of IGF-1 treatment, subsequent experiments measuring intracellular thiols after NTGF treatment used a 2-hour treatment. Additionally, changes in thiols persisted after 48 hours of treatment.

To examine the effect of IGF-1-stimulated cysteine uptake on intracellular thiol metabolism, SH-SY5Y neuroblastoma cells were treated with IGF-1 for 2 hours prior to measurement. To determine the important signaling pathways activated by IGF-1 that are involved in these changes, intracellular thiols were measured after 1 hour pretreatments with wortmannin (100 nM) or PD98059 (10 μM) followed by a 2 hour treatment with IGF-1 (10 nM) (Fig. 36). Cell growth media was changed to low-serum media (1% FBS) 24 hours prior to measurement.
Figure 36: Effect of IGF-1 on thiols and thioethers in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were pretreated for 1 hour with wortmannin (100 nM), or PD98059 (10 μM), and then treated for 2 hours with IGF-1 (10 nM) prior to measurement, n=6. Control values for intracellular thiols (nM/mg protein) were CYS 104.4, cystine 22.1, cystathionine 15.2, GSH 19.1, HYC 1.6, homocystine 3.4, MET 3.5, GSSG 0.4, SAM 6.2 and SAH 1.0. Asterisks (*) show a significant difference (p<0.05) from control, (#) shows a significant difference (p<0.05) from IGF-1 treated group.

IGF-1 stimulation increased intracellular levels of cysteine, GSH, methionine and SAM, and decreased cystathionine, homocystine, and GSSG. These data, along with the data showing that IGF-1 stimulates cysteine uptake (Fig. 24) suggest that IGF-1 decreases the oxidation state, as shown by the increase in GSH and decrease in GSSG, decreases transsulfuration, as shown by the decrease in cystathionine, and increases the methylation capacity, as shown by the increase in SAM, of the cell (Fig. 36).
PI3 kinase inhibition with 100 nM wortmannin prior to IGF-1 stimulation decreased cysteine, and GSH, and increased cystathionine, homocysteine, SAM and SAH levels when compared to the untreated control. Additionally, PI3 kinase inhibition decreased cysteine, GSH, methionine and SAM, and increased cystathionine, homocysteine, homocystine, and SAH levels when compared with the IGF-1 treated group. PI3 kinase inhibition had an opposing effect to IGF-1 stimulation in intracellular thiol levels of cysteine, cystathionine and homocystine, and had a normalizing effect in methionine and SAM. These data, along with the data that show that IGF-1 stimulated cysteine uptake is blocked by wortmannin (Fig.34) show that PI3 kinase inhibition increases the oxidation state, as shown by the decrease in GSH, increases transsulfuration, as shown by the increase in cystathionine, and decreases the methylation capacity, as shown by the increase in SAH a potent inhibitor of methylation (Fig. 36).

MAP kinase inhibition with 10 μM PD98059 prior to IGF-1 stimulation increased cysteine, methionine, and SAM, and decreased homocystine levels when compared to the untreated control. MAP kinase inhibition prior to IGF-1 stimulation also normalized cystathionine and GSH levels, and decreased homocystine methionine and SAM when compared to the IGF-1 stimulated group (Fig. 36). These data, along with the data that showing that IGF-1 stimulated cysteine uptake is not blocked by PD98059 (Fig. 34), suggest that MAP kinase inhibition does not block the changes in cysteine uptake, or intracellular thiols mediated by IGF-1 stimulation. The pattern of change in the levels intracellular thiols was similar between the IGF-1 treated group and the PD98059 and IGF-1 treated group revealing that MAP kinase inhibition does not block the effects of IGF-1 mediated changes in intracellular thiol metabolism.
To examine the role of NTGFs in intracellular thiol metabolism further, the previous experiment was replicated using PDGF as the cysteine-stimulating NTGF instead of IGF-1. Again, SH-SY5Y neuroblastoma cells were treated with PDGF for 2 hours prior to measurement. Again to investigate the pathways involved, intracellular thiols were also measured after 1 hour pretreatments with wortmannin (100 nM) or PD98059 (10 μM) followed by a 2 hour treatment with PDGF (1 nM) (Fig. 28). Cell growth media was changed to low-serum media (1% FBS) 24 hours prior to measurement.

![Graph showing the effect of PDGF on thiols and thioethers in SH-SY5Y cells.](image)

**Figure 37: Effect of PDGF on thiols and thioethers in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were pretreated for 1 hour with wortmannin (100 nM), or PD98059 (10 μM), and then treated for 2 hours with PDGF (10 nM) prior to measurement, n=6. Control values for intracellular thiols (nM/mg protein) were CYS 104.4, cystine 22.1, cystathionine 15.2, GSH 19.1, HCY 1.6, homocystine 3.4, MET 3.5, GSSG 0.4, SAM 6.2 and SAH 1.0. Asterisks (*) show a significant difference (p<0.05) from control, (#) shows a significant difference (p<0.05) from PDGF treated group.

68
The pattern of intracellular thiols changed by PDGF stimulation was the same as IGF-1 stimulation. PDGF stimulation increased intracellular levels of cysteine, GSH, methionine and SAM, and decreased cystathionine, homocystine, and GSSG. However, the magnitude of change was smaller when compared with IGF-1 stimulation. These data, along with the data that shows that PDGF stimulates cysteine uptake (Fig. 28), suggest that PDGF decreases the oxidation state, decreases transsulfuration, and increases the methylation capacity of the cell similarly to IGF-1 stimulation (Fig. 37).

PI3 kinase inhibition with 100 nM wortmannin prior to PDGF stimulation decreased cysteine, and GSH similarly to the IGF-1 experiment, but failed to increase cystathionine, homocysteine, SAM and SAH levels. However, PI3 kinase inhibition decreased cysteine, GSH, methionine and SAM, and increased cystathionine, and SAH levels when compared with the PDGF treated group, similarly to the IGF-1 experiment, but did not increase homocysteine and homocysteine. These discrepancies seem to be caused by the lower stimulation efficacy over control by PDGF, and higher variability in the thiol levels. The thiol pattern and cysteine uptake data (Fig. 28) suggest that PI3 kinase inhibition prior to PDGF stimulation increases the oxidation state, as shown by the decrease in GSH, but normalizes transsulfuration and the methylation capacity of the cell (Fig. 37).

Similar to PDGF stimulation alone, MAP kinase inhibition with 10 μM PD98059 prior to PDGF stimulation increased cysteine, GSH, methionine, and SAM, and decreased homocysteine, homocystine and GSSG levels when compared to the untreated
control. MAP kinase inhibition prior to IGF-1 stimulation decreased GSH levels when compared to the IGF-1 stimulated group (Fig. 37). These data, along with the data that show that PDGF stimulated cysteine uptake is not blocked by PD98059 (Fig. 34), suggest that MAP kinase inhibition does not block the change in cysteine uptake, or intracellular thiols mediated by PDGF stimulation.

These data taken together show that NTGFs stimulate cysteine uptake, and that stimulation is dependent on PI3 kinase activity, but not MAP kinase activity. Furthermore, the intracellular thiol data show that NTGFs decrease the oxidation state, decrease transsulfuration and increase the methylation capacity of SH-SY5Y human neuroblastoma cells.
E. Specific Aim 2: Evaluate redox-dependent changes in DNA methylation in cultured SH-SY5Y cells.

The link between oxidative stress and impaired DNA methylation has been implicated in many diseases including AD, atherosclerosis, many cancers and psychiatric disorders (Pogribny and Beland, 2009). It has been hypothesized that oxidative shifts in redox status cause a decrease in the roughly 1000 methylation reactions, including DNA methylation, carried out by the 209 methyltransferases in humans, by decreasing the availability of the methyl donor SAM (Siegmund et al., 2007; Deth et al., 2008; Hinterberger and Fischer, 2012).

To investigate the link between redox status, methylation capacity and DNA methylation, intracellular thiol levels and DNA methylation were measured under various conditions of cysteine availability, NTGF stimulation, and oxidative stress.

Above the link between IGF-1 stimulation, cysteine uptake and intracellular thiol metabolism has been illustrated. IGF-1 increases cysteine uptake resulting in a more reduced oxidation state and increased SAM availability (Fig. 36). To examine the effect of these changes on epigenetics, global DNA methylation was measured in SH-SY5Y cells treated with IGF-1 for 24 hours. IGF-1 treatment for 24 hours was used because 5-meC accumulated over 24 hours (Fig 22), and was not dependent on cell division as cell numbers were not changed over the 24-hour treatment time (Fig. 23).
To examine the effects of IGF-1, and the signaling pathways involved, on global DNA methylation SH-SY5Y cells were seeded at high concentration and grown for 24 hours in media containing 10% FBS to reach confluence. Complete confluence was needed to arrest cell division which could affect DNA methylation results. After the 24 hour growth period, cell media was switched to low serum (1% FBS) media for 24 hours prior to the experiment. Cells were untreated, treated with IGF-1 for 24 hours or pretreated with wortmannin (100 nM) or PD98059 (10 μM) for 1 hour followed by IGF-1 for 24 hours to determine the important signaling pathways activated by IGF-1 involved (Fig. 38). DNA was isolated immediately after treatments and global DNA methylation was measured using a high affinity ELISA assay.

**Figure 38: Effect of IGF-1 on global DNA methylation in SH-SY5Y cells.**
Quantification of 5-methylcytosine using ELISA in a human neuroblastoma cell line. Cells were treated for 24 hours with IGF-1 (10nM), Wortmannin 100nM, or PD98059 10μM. n=6. Asterisks (*) show a significant difference (p<0.05) from control.
IGF-1 (10 nM) stimulation increased global DNA methylation by 24%, which was blocked by PI3 kinase inhibition by wortmannin (100 nM), but not MAP kinase inhibition by PD98059 (10 μM). These data suggest that activation of the PI3 kinase, but not the MAP kinase pathway by IGF-1 is necessary for NTGF-mediated DNA methylation. These changes support the hypothesis that DNA methylation is increased by the increase in the SAM/SAH ratio caused by the increase in IGF-1 mediated cysteine uptake. These data suggest that as the redox potential is shifted to a more reducing condition, as shown previously by the increase in GSH/GSSG ratio (Fig. 35) methionine synthase activity is increased, as shown by the increase in the SAM/SAH ratio (Fig. 35), and this increase in methylation capacity causes an increase in DNA methylation.

The availability of cysteine in the brain is much more limited than in the rest of the body. In the blood circulating cysteine is about 100-fold higher than in the CSF. To investigate the consequences of limited cysteine availability on intracellular thiol levels, SH-SY5Y cells were seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was switched to either normal media containing 570 μM cysteine and 10% FBS or media containing no cysteine and 10% FBS for 24 hours (Fig. 39). Intracellular thiols and DNA were isolated 24 hours after the growth media was switched. Intracellular thiols were measured by HPLC-EC and global DNA methylation was measured using a high affinity ELISA assay. This treatment length difference reflects the time needed for methylation capacity to be manifested as a change in DNA methylation, as shown in the DNA methylation time course study with IGF-1.
Serum supplementation was critical at maintaining cell viability, attempts to grow cells in media containing no cysteine and 1% FBS resulted in cell viabilities of less than 50% (data not shown).

Figure 39: Effect of low cysteine growth conditions on thiols and thioethers in SH-SY5Y cells. Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown for 24 hours in alpha MEM (570 μM cysteine) with 10% FBS or alpha MEM (no cysteine) with 10% FBS. n=6. Control values for intracellular thiols (nM/mg protein) were CYS 108.4, cystine 24.2, cystathionine 14.1, GSH 20.0, HYC 1.5, homocysteine 3.7, MET 3.5, GSSG 0.44, SAM 6.4 and SAH 1.1. Asterisks (*) show a significant difference (p<0.05) from control.

Low cystine growth media reduced intracellular levels of cysteine, cystine, GSH, homocysteine, homocystine, methionine, SAM and SAH, and increased cystathionine, while GSSG was unchanged. Despite the large changes in many of the thiols measured, GSH only decreased by roughly 10%. The 130% increase in cystathionine, and decreases
in all of the metabolites of the methylation cycle suggest that these cells are metabolizing homocysteine to cysteine and further to GSH to maintain the redox balance of the cell.

**Figure 40: Effect of low cysteine growth conditions on global DNA methylation in SH-SY5Y cells.** Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. Cells were grown for 24 hours under control, alpha MEM (570 μM cysteine + 10% FBS) or low cysteine media, alpha MEM (0 cysteine + 10% FBS). n=6. Asterisks (*) show a significant difference (p<0.05) from control.

One consequence of the shift on intracellular thiol metabolism caused by limiting the availability of extracellular cysteine was its effect on DNA methylation status (Fig. 40). Low cysteine growth media reduced global 5-MeC DNA methylation by 46%. This shift in DNA methylation is not dependent on a shift in SAM/SAH, as that ratio actually increased under low cysteine growth conditions. However, this decrease in DNA methylation is probably caused by the decrease in availability of SAM rather than the shift in SAM/SAH ratio.

To further investigate how cysteine availability affects intracellular thiol metabolism and DNA methylation, SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach
confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS for the 24 hours prior to measurement (Fig. 41). Cells were then treated for either 2 hours with LBTBA (10 μM) for intracellular thiol measurement or for 24 hours for DNA methylation measurement. Intracellular thiols were measured by HPLC-EC and global DNA methylation was measured using a high affinity ELISA assay.

![Figure 41: Effect of LBTBA on thiols and thioethers in SH-SY5Y cells.](image)

Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with LBTBA (10μM). n=6. Control values for intracellular thiols (nM/mg protein) were CYS 128.3, cystine 20.1, cystathionine 18.2, GSH 15.8, HYC 1.1, homocystine 3.2, MET 3.7, GSSG 0.32, SAM 8.4 and SAH 0.94. Asterisks (*) show a significant difference (p<0.05) from control.

Inhibition of cysteine uptake by LBTBA reduced intracellular levels of cysteine, and methionine and increased cystathionine, SAM and SAH. Inhibition of cysteine
uptake showed a different thiol pattern than limiting extracellular cysteine. Limiting extracellular cysteine decreased the metabolites of the methylation pathway, while inhibiting cysteine uptake increased homocysteine by almost 500% and SAH by 350%. Deadenosylation of SAH to homocysteine is reversible, and accumulation of homocysteine has been shown to increase SAH (Fuso et al., 2005). SAH is a potent inhibitor of methylation reactions, and its accumulation is probably responsible for the observed decrease in DNA methylation status (Fig. 42).

![Graph showing effect of LBTBA on global DNA methylation in SH-SY5Y cells.](image)

**Figure 42: Effect of LBTBA on global DNA methylation in SH-SY5Y cells.** Quantification of 5-methylcytosine using ELISA in a human neuroblastoma cell line. Cells were treated for 24 hours with LBTBA (10μM). n=6. Asterisks (*) show a significant difference (p<0.05) from control.

LBTBA reduced 5-MeC DNA methylation by 37%. This shift in DNA methylation is likely caused by the accumulation of SAH. These data show that DNA methylation can be decreased by limiting the availability of SAM, or by increasing SAH.
The discrepancies in the intracellular thiol data between cells grown in low cysteine media, and those treated with the EAAT3 inhibitor LBTBA are possibly a result of the different treatment times and growth conditions. In experiments where cells were treated with growth factors or small molecules, cells were grown to confluence for 24 hours in media containing 10% FBS, then the media was replaced with media containing 1% FBS for 24 hours before the start of the experiment. In those experiments, treatments were added either 2 hours prior to measurement of intracellular thiols, or 24 hours prior to DNA isolation for methylation experiments. The low cysteine media growth conditions presented a problem. The cells were again grown to confluence for 24 hours in media containing 10% FBS, however the media could not be replaced with media containing 1% FBS as this killed many of the cells. Therefore the low cysteine containing media had to be added 24 hours after seeding the cells, and 24 hours prior to both measurements. Thus, the intracellular thiol measurements for the low cysteine growth condition were measured after 24 hours in limited cysteine, while the LBTBA treatment only lasted for 2 hours. It is possible the cells grown under low cysteine growth conditions depleted the available methionine in the growth media resulting in the observed decreases in the methylation pathway metabolites. It is unlikely the cells treated with for 2 hours with LBTBA would deplete the available methionine in the growth media in that time period, and thus could supplement the methylation cycle with methionine from the media. Additionally, the cell growth media for the limited cysteine experiments contained 10% FBS over the entire growth and experiment time, where the cells used in the LBTBA experiments were only initially grown in 10% FBS containing media for the first 24 hours of growth. Therefore, each of these experiments must be looked at independently.
Increasing cysteine uptake with IGF-1 caused DNA methylation to increase, and decreasing cysteine uptake by limiting its availability or inhibiting EAAT3 caused DNA methylation to decrease. Because, cysteine is the limiting precursor in GSH synthesis, the role of GSH synthesis in DNA methylation was investigated, using BSO, an inhibitor of γ-glutathionylcysteine ligase. Again, SH-SY5Y cells were seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with normal media containing 1% FBS for the 24 hours prior to the experiment (Fig. 43). Cells were treated for either 2 hours with BSO (100 μM) for intracellular thiol measurement or for 24 hours for DNA methylation measurement. Intracellular thiols were measured by HPLC-EC and global DNA methylation was measured using a high affinity ELISA assay.
**Figure 43: Effect of BSO on thiols and thioethers in SH-SY5Y cells.** Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with BSO (100 μM). n=6. Control values for intracellular thiols (nM/mg protein) were CYS 128.3, cystine 20.1, cystathionine 18.2, GSH 15.8, HCY 1.1, homocystine 3.2, MET 3.7, GSSG 0.32, SAM 8.4 and SAH 0.94. Asterisks (*) show a significant difference (p<0.05) from control.

Inhibition of glutathione synthase by BSO (100 μM) reduced intracellular levels of GSH, homocysteine, methionine and SAM and increased cysteine, cystine, cystathionine, GSSG and SAH (Fig. 43). The reduction of GSH by roughly 50% by BSO dramatically decreased the GSH/GSSG ratio and the SAM/SAH ratio. A GSH/GSSG ratio favoring a reducing potential inside the cell is necessary for methionine synthase function. Increasing the oxidation state of the cell decreases methionine synthase activity and increases the activity of cystathionine-β-synthase to condense serine and homocysteine to cystathionine. These two redox switches decrease methylation and
increase transsulfuration, respectively, to alleviate oxidative stress by directing the methylation cycle metabolite HCY to cysteine and then GSH.

![Graph showing 5-Methylcytosine levels](image)

**Figure 44: Effect of BSO on global DNA methylation in SH-SY5Y cells.** Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. Cells were treated for 24 hours with BSO (100 μM). n=6. Asterisks (*) show a significant difference (p<0.05) from control.

BSO reduced 5-MeC DNA methylation by 24% (Fig. 44). This shift in DNA methylation is likely caused by decrease in the SAM/SAH ratio caused by the increase in oxidation state. These data show that DNA methylation can be decreased by lowering the available GSH in the cell.

GSH/GSSG and SAM/SAH ratios were calculated for each of the treatment groups and correlated to DNA methylation (Table 3).
Table 3: Correlation of GSH/GSSG and SAM/SAH ratios with global DNA methylation. Asterisks (*) show a significant correlation (p<0.05). n=24.

<table>
<thead>
<tr>
<th></th>
<th>GSH/GSSG</th>
<th>SAM/SAH</th>
<th>DNA Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.484</td>
<td>5.995</td>
<td>0.303</td>
</tr>
<tr>
<td>IGF-1</td>
<td>64.153</td>
<td>13.647</td>
<td>0.376</td>
</tr>
<tr>
<td>BSO</td>
<td>10.568</td>
<td>0.837</td>
<td>0.252</td>
</tr>
<tr>
<td>LBTBA</td>
<td>31.190</td>
<td>2.555</td>
<td>0.209</td>
</tr>
<tr>
<td>R</td>
<td>0.805</td>
<td>0.921</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

IGF-1 treatment increased both the GSH/GSSG and SAM/SAH ratios, and BSO and LBTBA both decreased the GSH/GSSG and SAM/SAH ratios compared to the control group. Additionally, both GSH/GSSG and SAM/SAH ratios correlated to DNA methylation with R-values of 0.805 and 0.921 respectively. These data show that global DNA methylation is regulated by the redox state of the cell. Furthermore, NTGFs are able to utilize this redox regulated methylation pathway by altering cysteine uptake in a PI3 kinase dependent manner. Data from the limited cysteine growth condition was not used in these correlations because of the different growth conditions and treatment times.

The preceding data suggests that impaired cysteine uptake decreases GSH synthesis, which triggers an increase in transulfuration, shown by increased levels in cystathionine. The negative shift in the GSH/GSSG ratio leads to the inhibition of methionine synthase, shown by elevated levels of homocysteine and decreased levels of methionine. This inhibition leads to an increase in SAH, a potent inhibitor of SAM-dependent methylation reactions, resulting in accumulation of SAM. The decrease in methionine synthase activity, reflected by the increase in homocysteine, may reflect the GSH requirement for synthesis of methylcobalamin. Cobalamin is the cofactor of...
methionine synthase which accepts the methyl-group from 5-methyltetrahydrofolate (5-MTHF), and adds it to homocysteine to form methionine. During oxidative stress, it is more likely that Cbl(I), the oxidation state of cobalamin that readily forms methylcobalamin, is oxidized to Cbl(II), which cannot be methylated, thus inhibiting MS activity. The oxidation of cobalamin denotes a redox sensitive switch for controlling the methylation cycle.
F. Specific Aim 3: Evaluate changes in EAAT3-mediated cysteine uptake, and intracellular thiol metabolism caused by soluble Aβ oligomers.

Studies have implicated dysfunction in the transsulfuration pathway, and thiol metabolism associated with AD (Beyer et al., 2003; Hamel et al., 2008). Other studies have shown that soluble oligomers of Aβ inhibit glutamate uptake by EAAT3 in neurons (Li et al., 2009). If soluble oligomers of Aβ inhibit cysteine uptake, as they do glutamate, these observations taken together make a possible link between EAAT3-mediated cysteine uptake, decreased intracellular cysteine, dysfunctional sulfur metabolism and AD. To determine the effect of AD-associated Aβ oligomers on cysteine uptake, soluble Aβ oligomers from a CHO cell line that stably expresses hAPP with the V717F amyloidogenic AD mutation (7PA2 cells) were used. These cells release monomeric and oligomeric Aβ species into the culture medium in the absence of insoluble aggregates (Podlisny et al., 1995; Walsh et al., 2002; Li et al., 2009).

To test whether or not cysteine uptake is inhibited by soluble oligomers of Aβ, SH-SY5Y cells were seeded at high density and grown for 24 hours in media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, media was replaced with media containing 0.1%, 1% or 10% of a 10x concentrate of either CHO or 7PA2-conditioned media, and 1% FBS for the 2 hours prior to radiolabeled cysteine uptake measurement (Fig. 45).
Figure 45: Cysteine uptake is inhibited by conditioned media containing soluble Aβ oligomers. Radiolabeled cysteine uptake in SH-SY5Y human neuroblastoma cells was decreased in a dose-dependent manner when incubated with Aβ oligomer-containing 7PA2 conditioned media. Cells were treated with conditioned media for 2 hours n=6. Asterisks (*) show a significant difference (p<0.05) from matched CHO cell conditioned media group.

7PA2 conditioned media reduced cysteine uptake in a dose-dependent fashion (Fig. 45). To confirm that the Aβ oligomers were responsible for cysteine uptake inhibition, conditioned media from 7PA2 cells that was immunodepleted of Aβ was also tested. SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either CHO or 7PA2-conditioned media, or CHO or 7PA2-conditioned media that had been immunodepleted of Aβ oligomers, and 1% FBS for the 2 hours prior to radiolabeled cysteine uptake.
measurement. Immunodepletion of soluble Aβ oligomers using the anti-Aβ antibody AW7 restored cysteine uptake (Fig. 46).

![Graph showing L-[35S]-Cysteine Uptake](image)

**Figure 46: Inhibition of cysteine uptake is prevented by Aβ immunodepletion.** Radiolabeled cysteine uptake experiments in SH-SY5Y human neuroblastoma cells. Cells were treated for 2 hours with Aβ oligomer containing 7PA2 conditioned media, control CHO conditioned media, or immunodepleted conditioned media n=6. Asterisks (*) show a significant difference (p<0.05) from matched CHO conditioned media group.

After confirming that Aβ inhibits EAAT3 mediated cysteine uptake in SH-SY5Y, the intracellular thiol metabolism was examined. SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either CHO or 7PA2-conditioned media, or CHO or 7PA2-conditioned media that had been immunodepleted of Aβ oligomers, and 1% FBS 2 hours
prior to intracellular thiol measurement Intracellular thiols were measured by HPLC-EC (Fig. 47).

**Figure 47: Effect of conditioned media containing soluble Aβ oligomers on thiols and thioethers in SH-SY5Y cells.** HPLC isolation and electrochemical detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with 7PA2 or CHO conditioned media or conditioned media immunodepleted of Aβ using the AW7 anti-Aβ polyclonal antiserum n=6. Control values for intracellular thiols (nM/mg protein) were CYS 107.2, cystine 18.6, cystathionine 19.2, GSH 16.4, HCY 1.2, homocystine 3.0, MET 4.0, GSSG 0.30, SAM 7.2 and SAH 0.89. Asterisks (*) show a significant difference (p<0.05) from matched CHO conditioned media group.

Exposure to soluble Aβ decreased intracellular cysteine, GSH and methionine, and increased cystathionine, homocysteine, SAM and SAH. To assess the change in redox potential and methylation capacity associated with the shifts in intracellular thiol levels, the ratios of GSH/GSSG and SAM/SAH were calculated.
Figure 48: Effect of conditioned media containing soluble Aβ oligomers on the redox state of SH-SY5Y cells. The GSH/GSSG ratio was calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with 7PA2, CHO conditioned media or conditioned media immunodepleted of Aβ using the AW7 anti-Aβ antibody. n=6. Asterisks (*) show a significant difference (p<0.05) from matched CHO conditioned media group.

Figure 49: Effect of conditioned media containing soluble Aβ oligomers on the methylation state of SH-SY5Y cells. The SAM/SAH ratio was calculated from the HPLC/electrochemical measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with 7PA2, CHO conditioned media or conditioned media immunodepleted of Aβ using the AW7 anti-Aβ antibody. n=6. Asterisks (*) show a significant difference (p<0.05) from matched CHO conditioned media group.
The decrease in the GSH/GSSG ratio indicates a shift to a more oxidative state (Fig. 48). The decrease in the SAM/SAH ratio indicates a decrease in the methylation capacity (Fig 49). This shift is caused by the accumulation of SAH, a potent inhibitor of SAM-dependent methylation reactions. This shift in SAM/SAH ratio reduces the probability for methylation reactions such as DNA methylation. To confirm the change in methylation capacity, global DNA methylation was measured in the presence of soluble Aβ oligomers. SH-SY5Y cells were seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either CHO or 7PA2-conditioned media, or CHO or 7PA2-conditioned media that had been immunodepleted of Aβ oligomers, and 1% FBS for the 24 hours prior to DNA methylation measurement (Fig. 50). Global DNA methylation was measured using a high affinity ELISA assay.
Figure 50: Effect of conditioned media containing soluble Aβ oligomers on global DNA methylation in SH-SY5Y cells. Quantification of 5-methylcytosine using ELISA in a human neuroblastoma cell line. Cells were treated for 24 hours with 7PA2, or CHO conditioned media or conditioned media immunodepleted of Aβ using the AW7 anti-Aβ antibody. n=6. Asterisks (*) show a significant difference (p<0.05) from matched CHO conditioned media group.

5-methylcytosine decreased after treatments of soluble Aβ oligomer containing 7PA2 conditioned media. Immunodepletion of soluble Aβ oligomers using the anti-Aβ antibody AW7 restored global DNA methylation to control levels.

After showing that soluble oligomers of Aβ inhibited cysteine uptake, and demonstrating that IGF-1 could stimulate cysteine uptake, the ability of IGF-1 to stimulate cysteine uptake in the presence of soluble oligomers of Aβ was investigated. If the soluble oligomers of Aβ directly blocked cysteine uptake by directly inhibiting EAAT3, then IGF-1 should not be able to stimulate cysteine uptake in their presence. If soluble oligomers of Aβ interfere with the regulation of EAAT3, then IGF-1 stimulation
might be able to increase cysteine uptake in their presence. To investigate this, SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (100 nM), and 1% FBS for the 2 hours prior to radiolabeled cysteine uptake measurement (Fig. 51).
Figure 51: Cysteine uptake inhibition by Aβ oligomers is ameliorated by IGF-1. Radiolabeled cysteine uptake experiments in the SH-SY5Y human neuroblastoma. Cells were treated for 2 hours with control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (100 nM) n=6. Asterisks (*) show a significant difference (p<0.05) from the CHO-CM group, (#) shows a significant difference (p<0.05) between the 7PA2-CM and 7PA2-CM + IGF-1 treatments.

IGF-1 was able to stimulate cysteine uptake in both the control and soluble Aβ oligomer-containing conditioned media treatment groups. Importantly, IGF-1 increased cysteine uptake in the cells treated with soluble oligomers of Aβ to levels just shy of control. This shows that IGF-1 is able to ameliorate EAAT3 inhibition by soluble oligomers of Aβ. The effect of IGF-1 was dependent on PI3 kinase signaling, as its effect was not seen in the presence of the PI3 kinase inhibitor wortmannin.
After showing that IGF-1 could stimulate cysteine uptake in SH-SY5Y cells in the presence of soluble oligomers of Aβ, the intracellular thiol metabolism was examined. SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (100 nM), and 1% FBS for the 2 hours prior to intracellular thiol measurement. Intracellular thiols were measured by HPLC-EC (Fig. 52).
Figure 52: Effect of IGF-1 on thiols and thioethers in SH-SY5Y cells exposed to conditioned media containing soluble Aβ oligomers. Electrochemical/HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were treated for 2 hours with control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (100 nM) n=6. Control values for intracellular thiols (nM/mg protein) were CYS 107.2, cystine 18.6, cystathionine 19.2, GSH 16.4, HCY 1.2, homocysteine 3.0, MET 4.0, GSSG 0.30, SAM 7.2 and SAH 0.89. Asterisks (*) show a significant difference (p<0.05) from the CHO-CM group, (#) shows a significant difference (p<0.05) between the 7PA2-CM and 7PA2-CM + IGF-1 treatments.

Exposure to soluble Aβ oligomers decreased intracellular cysteine, GSH and methionine, and increased cystathionine, homocysteine, SAM and SAH compared to the cells treated with CHO conditioned media (Fig. 52). IGF-1 when added to the cells treated with CHO conditioned media increased cysteine, methionine and SAM and decreased cystathionine when compared to the cells treated with CHO conditioned media alone. IGF-1 when added to the cells treated with soluble Aβ oligomer containing conditioned media increased methionine and SAM and decreased homocysteine and
GSSG when compared to the cells treated with CHO conditioned media alone. When compared to cells treated with soluble Aβ oligomer containing conditioned media alone, IGF-1 increased cysteine and GSH to control values, and increased methionine to above control values, and decreased cystathionine, homocysteine and SAH to control levels and homocystine and GSSG to below control values. This effect by IGF-1 was either decreased or eliminated by the PI3 kinase inhibitor wortmannin, showing that this effect is dependent on the PI3 kinase pathway.

These data, along with the cysteine uptake data show that IGF-1 is able to decrease the oxidative shift caused by the soluble oligomers Aβ, and prevent the decrease in methylation capacity. Next, the effect of IGF-1 on global DNA methylation in cells treated with soluble Aβ oligomer containing conditioned media was investigated.

Again, SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and grown for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (WMN; 100 nM), and 1% FBS for the 24 hours prior to DNA methylation measurement (Fig. 53). Global DNA methylation was measured using a high affinity ELISA assay.
Figure 53: Effect of conditioned media containing soluble Aβ oligomers +/- IGF-1 on global DNA methylation in SH-SY5Y cells. Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. Cells were treated for 2 hours with control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media, CHO conditioned media and IGF-1 (10 nM), 7PA2 conditioned media and IGF-1 (10 nM), or 7PA2 conditioned media, IGF-1 (10 nM) and wortmannin (WMN; 100 nM) n=6. Asterisks (*) show a significant difference (p<0.05) from the CHO-CM group, (#) shows a significant difference (p<0.05) between the 7PA2-CM and 7PA2-CM + IGF-1 treatments.

Global DNA methylation was decreased after treatment with soluble Aβ oligomer-containing 7PA2 conditioned media. However, IGF-1 stimulation restored global DNA methylation to control levels in the presence of soluble Aβ oligomers. GSH/GSSG and SAM/SAH ratios were calculated for each of the treatment groups and correlated to DNA methylation (Table 4).
Table 4: Correlation of GSH/GSSG and SAM/SAH ratios with global DNA methylation. Asterisks (*) show a significant correlation (p<0.05). n= 18.

<table>
<thead>
<tr>
<th></th>
<th>GSH/GSSG</th>
<th>SAM/SAH</th>
<th>DNA Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.720</td>
<td>6.045</td>
<td>0.279</td>
</tr>
<tr>
<td>Aβ</td>
<td>31.035</td>
<td>3.299</td>
<td>0.199</td>
</tr>
<tr>
<td>Aβ + IGF-1</td>
<td>43.258</td>
<td>8.015</td>
<td>0.280</td>
</tr>
<tr>
<td>R</td>
<td>0.961</td>
<td>0.918</td>
<td>*</td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

Oligomers of Aβ decreased both the GSH/GSSG and SAM/SAH ratios, but IGF-1 was able to rescue the GSH/GSSG and SAM/SAH ratios back to levels similar to the control group. Additionally, both GSH/GSSG and SAM/SAH ratios correlated to DNA methylation with R-values of 0.961 and 0.918 respectively.

These data show that global DNA methylation is correlated to the redox state and methylation capacity of the cell. Furthermore, Aβ alters this redox regulated methylation pathway by altering cysteine uptake. Together, these data show that soluble oligomers of Aβ inhibit cysteine uptake, cause an oxidizing shift in the redox potential of the cell, and decrease the methylation capacity. Furthermore, these data show that treatment of cells with IGF-1 concurrently with soluble oligomers of Aβ ameliorate these effects and restore normal DNA methylation. It was noticed that the cells changed phenotype during the 24-hour treatment with conditioned media containing soluble oligomers of Aβ (Fig. 54).
Figure 54: Phase-contrast micrographs of SH-SY5Y cells grown in media containing Aβ or LBTBA. Cells were treated for 24 hours with control CHO conditioned media, Aβ oligomer containing 7PA2 conditioned media or LBTBA (10 μM). Bar = 50 um.

Phase-contrast images were taken using the same treatment schedule as the DNA methylation study, with the exception that cells were plated at an initial density of density of 10^4 cells/cm^2 in culture dishes coated with 5 mL 0.05 mg/ml collagen. A clear change in morphology was observed between cells grown in conditioned media and conditioned media containing oligomers of Aβ. The 7PA2 morphology was mimicked by treatment with LBTBA (10 μM). The above images suggest that the changes in cysteine uptake, redox, and DNA methylation caused by Aβ containing media cause cellular differentiation, or at least a change in morphology.

To confirm that the changes in DNA methylation caused actual changes in gene transcription, mRNA was isolated from cells treated with soluble oligomers of Aβ. SH-SY5Y cells were again seeded at high concentration and grown for 24 hours in normal media containing 10% FBS to reach confluence. After the 24 hour growth period, cell media was replaced with media containing 1% FBS and cells were allowed to grow for an additional 24 hours prior to the experiment. After 24 hours in 1% FBS containing media, cell media was replaced with media containing 10% of a 10x concentrate of either control CHO conditioned media or Aβ oligomer containing 7PA2 conditioned media, and
1% FBS for the 24 hours prior to mRNA isolation. mRNA was probed using qRT-PCR with primers designed for a panel of redox and methylation-linked genes including BDNF, Nrf-2, Nlgn-3, DRD4, MAT2a, CBS, CGL, MS, the catalytic and modulatory subunits of GCL, EAAT3, Line-1, COMT, GSS, GSR and DNMT3a.

**Figure 55: Effect of conditioned media containing soluble Aβ oligomers on expression of redox and methylation-linked genes in SH-SY5Y cells.** Expression of mRNA levels from SH-SY5Y neuroblastoma cells were probed using qRT-PCR with primers designed for a panel of redox and methylation-linked genes. Genes were normalized to β-actin and then to control gene expression n=4. Asterisks (*) show a significant difference (p<0.05) from the CHO-CM treated cells.

Soluble oligomers of Aβ increased BDNF, Nrf-2, CBS, MS, the catalytic and modulatory domains of GCL, and EAAT3, and decreased Nlgn-3, DRD4, MAT2a,
LINE-1, COMT, GSR and DNMT3a. These data show that changes in global DNA methylation are associated with changes in DNA transcription.
G. Specific Aim 4: Investigate the role of redox-mediated epigenetic changes on differentiation.

Studies have shown the importance of epigenetic changes in the differentiation of cells that accompanies development and the differentiation of stem cells and neuroblastoma cells in culture (Hirabayashi and Gotoh, 2010; Stallings et al., 2011; Armstrong, 2012). However, the link between redox, differentiation, and DNA methylation has never been studied. To see if changes in redox status changes along with DNA methylation and differentiation, SH-SY5Y cells were differentiated in culture using retinoic acid.

SH-SY5Y neuroblastoma cells were grown in DMEM supplemented with 10% FBS. Cells were seeded at an initial density of $10^4$ cells/cm$^2$ in culture dishes coated with 5 mL 0.05 mg/ml collagen. After 24 hours of growth, media was changed to media containing all-trans retinoic acid (10 μM) in DMEM with 1% FBS. Media was changed every 24 hours. After 5 days in the presence of RA, cells were washed three times with DMEM and incubated with 50 ng/ml BDNF in DMEM (without serum) for 1 day. Cells were photographed over the 7-day differentiation time period (Fig. 56).
Figure 56: Time course of SH-SY5Y cell differentiation. Phase-contrast micrographs of SH-SY5Y cells growing in complete medium (DMEM with 10% fetal calf serum) without additives (A) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1(B) 3 (C) or 5 days (D). Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7 (E). Bar = 50 um.

Images of the cells from days 0-7 show a clear change in morphology. On day 0, the SH-SY5Y cells grow in clusters, rapidly dividing, forming balls of cells. After ATRA is added and the serum supplementation is dropped to 1%, the cells stop rapidly dividing and start to spread into monolayers and develop dendritic branches. The length and number of these dendritic processes increase through day 7. By day, 7 cells have small round soma and long dendrites, and have completed differentiating into neuronal-like cells. Intracellular thiol metabolism was studied during the differentiation time period and measured at days 0, 1, 3, 5 and 7 (Fig. 57).
Figure 57: Changes in thiols and thioethers in SH-SY5Y cells during differentiation. Electrochemical HPLC isolation and detection of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown in complete medium (DMEM with 10% fetal calf serum) without additives (Day 0) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1, 3 or 5 days. Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7. n=3. Asterisks (*) show a significant difference (p<0.05) from day 0.
Intracellular levels of cysteine and cystine were decreased only on day 1 of measurement; levels of cystathionine were increased on days 1 and 3, GSH was decreased on days 3 and 5, homocysteine, SAH were increased on days 1, 3 and 5, homocysteine was increased on day 5, GSSG was increased on days 1, 3, 5 and 7 and methionine and SAM were decreased on days 1, 3 and 5. In an overall trend, intracellular thiols suggested a pattern of oxidative stress and reduced methylation capacity from day 1 to day 5 that was restored to normal by day 7. The GSH/GSSG and SAM/SAH ratios and the redox potential of the cells at the different differentiation time points were calculated. The redox potential was calculated using a method derived from (Schafer and Buettner, 2001).

Figure 58: Effect of differentiation on the redox state of SH-SY5Y cells. The GSH/GSSG ratio was calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown in complete medium (DMEM with 10% fetal calf serum) without additives (Day 0) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1, 3 or 5 days. Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7 n=3. Asterisks (*) show a significant difference (p<0.05) from day 0.
Figure 59: Effect of differentiation on the methylation state of SH-SY5Y cells. The SAM/SAH ratio was calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown in complete medium (DMEM with 10% fetal calf serum) without additives (Day 0) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1, 3 or 5 days. Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7 n=3. Asterisks (*) show a significant difference (p<0.05) from day 0.

Figure 60: Effect of differentiation on the redox potential of SH-SY5Y cells. The redox potential was calculated using from electrochemical HPLC measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were grown in complete medium (DMEM with 10% fetal calf serum) without additives (Day 0) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1, 3 or 5 days. Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7 n=3. Asterisks (*) show a significant difference (p<0.05) from day 0.
The GSH/GSSG ratio decreased from day 0 to day 1 by over 50% and stayed decreased by 50% during the differentiation time period. The redox potential rapidly changed from -224 mV to -198 mV during the first day in differentiation media, and slowly decreased to -204 mV through the remainder of the differentiation protocol. Interestingly the SAM/SAH ratio initially dropped from 10 to 3, and stayed below 4 until day 7 when the ratio returned to day 0 levels. The complete recovery of the SAM/SAH ratio is interesting, as it is observed concurrently with only a partial recovery of the GSH/GSSG ratio and redox potential. To compare redox potential and SAM/SAH ratio with DNA methylation, global DNA methylation was measured in DNA isolated at each time point during differentiation.
Figure 61: Effect of differentiation on global DNA methylation in SH-SY5Y cells. Quantification of 5-methylcytosine using ELISA in a human neuroblastoma cell line. DNA was isolated from cells grown in complete medium (DMEM with 10% fetal calf serum) without additives (Day 0) or in differentiation medium (DMEM with 1% FCS and 10 μM RA) for 1, 3 or 5 days. Cells were then grown in serum free media (DMEM with no FCS and 50 ng/mL BDNF) on day 7. n=6. Asterisks (*) show a significant difference (p<0.05) from day 0, # show a significant difference (p<0.05) from day 5.

DNA methylation was decreased at day 1 through day 7; however, there was a partial recovery on day 7. The SAM/SAH ratio correlates with the global DNA methylation better than the GSH/GSSG ratio or calculated redox potential. These data show that major redox and methylation changes accompany all-trans retinoic acid induced differentiation of SH-SY5Y cells.
IV. DISCUSSION

A. Redox Status in the Human Brain.

The human brain is a unique organ which sets humans apart from every other organism on earth. The brain’s ability to record, store and recall memories is a feat accomplished by nearly 4 billion years of evolution. This nearly incomprehensible amount of time spent tinkering with the genes that now make humans human has given rise to an organ with roughly 100 billion neurons making an estimated 100-500 trillion synapses (Drachman, 2005). This incredible organ organizes itself over time constantly forming and pruning synapses to perform its functions. Neurotrophic factors play an important role in this development and organization. The secretion of NTGFs from one part of the brain causes axons to grow towards that area and form synapses. PDGF is involved in cell proliferation and cell migration in the embryonic brain and IGF-1 regulates plasticity in the hippocampus (Cross and Dexter, 1991; Baker et al., 1993; Llorens-Martín et al., 2009). These factors are, in part, responsible for differentiation of NPCs to neurons or glial cells, neurite growth, pruning of dendritic spines, cell survival and many other processes (Deister and Schmidt, 2006; Madduri et al., 2009).

Neurons in the adult human brain are non-dividing and are generally non-replaceable. Keeping neurons from dividing is critical for retaining synapses and axonal networks. Neuronal division would erase all of this stored information and memory. Therefore, it is necessary to keep neurons in a stable, non-mitotic state. The human brain has evolved many mechanisms to maximize the longevity of this neuronal network.
Oxidative stress is one way the brain has evolved to keep cells out of the cell cycle (Kruman, 2004). The human brain is a unique redox environment, maintaining a lower redox capacity than the rest of the body. The CSF contains much lower levels of cysteine and GSH than the blood (Castagna et al.). Adult human neurons partially block the transsulfuration pathway; this block is observable by the high levels of cystathionine in the brain compared to other tissues, and the brains of other organisms (Tallan et al., 1958). SH-SY5Y cells behave similarly, in experiments that induced oxidative stress and increased transsulfuration, cystathionine accumulated (Figs. 41, 43 and 47). However, this accumulation of cystathionine was unexpected as SH-SY5Y cells supposedly have an active transsulfuration pathway (Jurkowska et al., 2011). This thesis appears to be the first to report intracellular levels of cystathionine in SH-SY5Y cells, so the activity of the level of transsulfuration activity is largely unexplored.

A partial or full block of transsulfuration in neurons makes EAAT3 mediated cysteine uptake the primary or even the only source of cysteine in mature human neurons. As cysteine is the rate-limiting precursor to GSH synthesis, this makes EAAT3 a profound and highly dynamic controller of antioxidant capacity and redox status, and the limitation in antioxidant capacity makes the brain much more sensitive to oxidative changes. Others have shown that the unique redox environment of the brain is important for keeping neurons from dividing (Currais et al., 2009). My studies show that the modulation of cysteine uptake by NTGF stimulation is able to change intracellular thiol levels and shift the redox potential of the cell (Figs. 36 and 37). Furthermore, I show that there are epigenetic consequences to this shift in redox and those changes result in altered gene transcription and morphology (Fig. 62). Others have shown that NTGFs can
stimulate EAAT3 mediated glutamate uptake, and have shown it to be PI3 kinase
dependent (Sims et al., 2000; Xia et al., 2006). The role of EAAT3 dependent cysteine
uptake is also supported by studies that show decreased glutathione levels and increased
neurodegeneration in the brains of EAAC1 knockout mice (Aoyama et al., 2006).

**Figure 62: Redox based epigenetic signaling.** Growth factors increase EAAT3
mediated cysteine uptake, increasing GSH synthesis. Increased GSH synthesis shifts
redox status to a more reducing potential. This shift increases methionine synthase
activity increasing the probability of DNA methylation. DNA methylation leads to
changes in gene transcription. Protein methylation and changed gene transcription cause
changes in protein function and phenotype.
The neuroblastoma cell culture system used in these studies is a conservative system with a much greater redox capacity than neurons in the human brain. SH-SY5Y cells are able to produce cysteine from homocysteine, at least on a limited scale, unlike neurons in the human brain, and cell culture media has more than 250 times the cysteine, compared to CSF (Castagna et al.; Sun et al., 2006; Jurkowska et al., 2011). These differences make neurons more sensitive to changes in oxidative pressures. I would therefore expect changes in redox-sensitive methylation in response to NTGFs to be more exaggerated in vivo, as compared to the cell culture conditions I employed.
B. EAAT3 is a Controller of Redox in the Human Brain

The importance of EAAT3 as a cysteine transporter has been overlooked for decades. Its discovery by homology mapping with other glutamate transporters initiated a long sequence of events that obscured the true importance of this transporter. Recently, studies in Japan, led by Drs. Koji Aoyama and Masahiko Watabe, and in the United States by Raymond Swanson, have shed light on the ability of EAAT3 to transport cysteine. Studies mapping the localization of EAAT3 in the brains of rats show its ubiquitous expression in all areas of the brain, even in areas where there is no glutamatergic signaling (Holmseth et al., 2012). In glutamatergic neurons, EAAT3 is not confined to the synapse, but expressed over the entire soma (Nieoullon et al., 2006). These findings strongly suggest a non-glutamate function of EAAT3. Additionally, studies, in which EAAT3 has been knocked-down in the brains of rats show oxidative stress, but not increased extracellular levels of glutamate (Watabe et al., 2007). Finally, studies have shown that EAAT3 preferentially transports cysteine over glutamate (Bendahan et al., 2000; Danbolt, 2001; Chen and Swanson, 2003). Despite these findings EAAT3 is still classified as a glutamate transporter, and observations of aberrant EAAT3 activity or regulation and oxidative stress in disease go largely unnoticed and unstudied (Li et al., 2010).

This thesis shows how EAAT3-mediated cysteine uptake is regulated by the PI3 kinase pathway. However, most of the research done on EAAT3 regulation investigates glutamate transport and signaling. NTGFs have been shown to stimulate EAAT3-mediated glutamate uptake in a variety of conditions and models (Alesutan et al., 2010; Sopjani et al., 2010; Almilaji et al., 2012). These experiments have shown that under
different conditions signaling pathways such as the Akt/PI3 kinase, MAP kinase and PKC pathways can increase surface expression of EAAT3 at the cell surface (Himi et al., 2003; Sheldon et al., 2006; Watabe et al., 2008). In acute studies, the Akt/PI3 kinase and MAP kinase pathways are shown to be most active, with PKC activity playing a role in long term regulation (Watabe et al., 2007). My studies show that NTGF stimulation of cysteine uptake is PI3 kinase-dependent, and not dependent on MAP kinase activity (Fig. 34). This differs from the reported literature, and is unexplained by this work. The test conditions could differ, or the MAP kinase pathway could only regulate glutamate uptake. The localization of cell signaling pathways activated by NTGFs could be different in different parts of the cell. For example, NTGFs could activate receptors at the synapse linked to the MAP kinase pathway increasing glutamate uptake, or they could activate receptors on the cell body linked to the PI3 kinase system increasing cysteine uptake. Depending on the localization of the receptors expressed and the pathways linked to them, differential activation could be explained. Figure 63 summarizes the literature on EAAT3 regulation and illustrates a probable mechanism by which IGF-1 stimulation increases EAAT3-mediated cysteine transport (Sakamoto and Holman, 2008; Alesutan et al., 2010; Sopjani et al., 2010; Almilaji et al., 2012). Further studies are needed to determine the potential role of the MAP kinase pathway in EAAT3 regulation. Some such studies are proposed in the future directions section of this thesis.
Figure 63: A proposed mechanism for NTGF stimulated cysteine uptake. NTGF stimulation activates the Akt/PI3K pathway by phosphorylating the insulin receptor substrate-1 (IRS-1). Phosphorylation of IRS-1 recruits PI3 kinase to the membrane to phosphorylate PIP2 to PIP3, PIP3 activates PIP3 dependent kinase (PDK) which phosphorylates Akt. Akt can also be phosphorylated by the mammalian target of rapamycin complex-2 (mTORC2). Phosphorylated Akt, phosphorylates and inactivates Akt substrate 160 (AS160). AS160 is a GTPase activating protein which when active reduces the activity of Rab1. Rab1 directs trafficking of EAAT3 through interaction of GTRAP3-18. Activation of this pathway leads to activated Rab1 and trafficking of EAAT3 to the cell surface.

The link between NTGFs and redox is more than just coincidental. NTGFs stimulate neurons to grow axons and dendrites and form new synapses. However, these synapses, dendrites and axons come at a metabolic price. The neuron must make ATP to maintain the ion gradient needed for each action potential sent along its axon, and that
ATP production produces ROS. It would therefore make sense for NTGFs to also stimulate a system to counteract the increased formation of ROS produced by their trophic action. In the unique redox environment of the brain, the evolution of a divergent pathway which both causes an increase in ROS production and an increase in the transport of antioxidant precursors seems logical. This tightly linked control of antioxidant and neuronal activity might be a way of ensuring a narrow window of redox potential in the brain. Other research has shown that EAAT3 transcription is linked to redox through the Keap1-Nrf2 pathway (Escartin et al., 2011). When redox-sensitive cysteine residues on Keap1 become oxidized, Nrf2 disassociates and causes an increase in the transcription of EAAT3, but not the other EAATs. Additionally, the activation of Nrf2 alone does not cause an increase in the surface expression of EAAT3 (Escartin et al., 2011). The role of EAAT3 in regulating redox in the human brain is undeniable, and the regulation by NTGFs seems to link both ROS production and mitigation.
C. Methylation and Development/Differentiation

Epigenetics allows a single genome, present in all cells, to provide a unique pattern of stable gene expression for different cell types. Epigenetics encompasses modifications to both DNA and histones. 80% of epigenetic DNA modification is CpG methylation, but other modifications occur, such as hydroxymethylation. Histones can be methylated or acetylated at various amino acid residues on their exposed carboxy tail regions (Martin and Zhang, 2007). Epigenetic modifications cause chromatin remodeling by changing the way DNA interacts with itself and with accessory proteins. This remodeling can open up the DNA, making it more accessible and can recruit additional proteins such as transcription factors to increase transcription, or it can condense the DNA, decreasing chances of transcription (Michalowsky and Jones, 1989).

Epigenetics is particularly important in early development. DNA methylation is very low in germ line stem cells, and increases as sperm and egg mature (Strachan and Read, 1999). Upon fertilization, the paternal and maternal genomes are rapidly demethylated. This demethylation coincides with the fusion of ovum and sperm. The sperm is rich in the antioxidant metal selenium, needed in many of the reducing reactions discussed (Olson et al., 2005; Shalini and Bansal, 2005). The ovum is rich in sulfur metabolites (Berendt et al., 2009). When sperm and ovum fuse, the redox environment changes rapidly, and this change may play a role in the epigenetic reprogramming associated with fusion. After implantation, developing germ line cell genomes are maintained at low levels of methylation. However, the genomes of differentiating cells are rapidly methylated during differentiation (Fig. 64) (Strachan and Read, 1999; Armstrong, 2012).
Figure 64: Changes in DNA methylation during mammalian development. Summary figure of changes in DNA methylation during development and differentiation. Figure from (Strachan and Read, 1999).

DNA methylation was once thought to be static and unchanging, however, it is now known that DNA methylation is a very dynamic process, closely associated with cell differentiation and neuronal plasticity (Michalowsky and Jones, 1989; Siegmund et al., 2007). DNA methylation is carried out by a class of enzymes called DNA methyltransferases DNMTs. DNMT1 is responsible for maintaining DNA methylation, as the cell divides, DNMT1 methylates the daughter strand of DNA according to the methylation of the mother strand (Golshani et al., 2005). DNMT2 primarily methylates RNA, and DNMT3 is responsible for de novo methylation of DNA (Siegmund et al., 2007). Research has shown that redox can guide differentiation (Prozorovski et al., 2008). It has been shown that activation of the redox sensitive histone deacetylase Sirt1 under oxidizing conditions causes NPCs to differentiate to astroglia, but under reducing
conditions they differentiate to neurons (Prozorovski et al., 2008). Additionally, it has been shown that cobalamin can cause changes in cellular differentiation through its effects on methionine synthase and methylation (Battaglia-Hsu et al., 2009).

It was previously shown by our lab that IGF-1 stimulation increases methionine synthase activity (Waly et al., 2004). My work shows that IGF-1 increases methionine synthase activity through the use of redox. My work further shows that impaired cysteine uptake decreases GSH synthesis, which triggers an increase in transsulfuration, as shown by increased levels in cystathionine (Figs. 24 and 36). The negative shift in the GSH/GSSG ratio leads to the inhibition of methionine synthase, shown by elevated levels of homocysteine and decreased levels of methionine (Figs 39, 41 and 43). This inhibition leads to an increase in SAH, a potent inhibitor of SAM-dependent methylation reactions, sometimes resulting in accumulation of SAM (Figs 39, 41 and 43). The decrease in methionine synthase activity, reflected by an increase in homocysteine, may at least in part be attributed to a GSH requirement for synthesis of methylcobalamin. Cobalamin is the cofactor of methionine synthase which accepts the methyl-group from 5-methyltetrahydrofolate (5-MTHF) and subsequently transfers it to homocysteine to form methionine. During oxidative stress, it is more likely that Cbl(I), the oxidation state of cobalamin that readily forms methylcobalamin, will be oxidized to Cbl(II), which cannot be methylated, thus inhibiting methionine synthase activity. However, upon IGF-1 stimulation, cysteine uptake is increased, the redox potential is shifted to a more reducing state, and GSH is available for the glutathionylation of Cbl(II). Glutathionylation of Cbl(II) reduces it, and, upon binding to methionine synthase, it is remethylated, and methionine synthase activity is restored. The oxidation of cobalamin provides a redox-
sensitive switch for controlling the methylation cycle, and one which is very powerful in influencing epigenetics. Other studies have shown that impaired metabolism of cobalamin affects cell cycle progression and differentiation of neurons (Battaglia-Hsu et al., 2009).
D. Redox, Epigenetics and Neurological Disorders

Problems affecting epigenetic pathways have been shown to cause developmental disorders including Rett, Angelman, Prader-Willi and Fragile X syndromes (Wan et al., 1999; Carrel et al., 2002; Jacquemont et al., 2011). Additionally, dysfunctional redox regulation is associated with neurological disorders such as autism, schizophrenia and Alzheimer’s disease (Do et al., 2000; Deth et al., 2008; Zawia et al., 2009). The link between redox and methylation this thesis describes may provide some insight into how these diseases may share common problems in epigenetic regulation.

Rett syndrome is caused by mutations in the methyl CpG binding protein 2 (MECP2) protein. MECP2 binds to methylated DNA and acts as a transcriptional suppressor or activator, when MECP2 is mutated these functions are diminished and information written to the genome as DNA methylation is lost (Wan et al., 1999). Angelman and Prader-Willi syndromes arise from improper methyl-imprinting of DNA. Angelman syndrome is caused by imprinting of the paternal genome on chromosome 15 at q 11-13, and deletions and mutations in the active maternal genome (White et al., 2006). Prader-Willi syndrome is very similar, but the maternal genome is imprinted on chromosome 15 at q 11-13 and there are mutations or deletions in the paternal genome (Cavaillé et al., 2000). Interestingly, some success has been gained by treating Prader-Willi syndrome with growth factors (Carrel et al., 2002). Fragile X syndrome is caused by expansion of a CGG repeat in the 5′ UTR of the fragile X mental retardation 1 (FMR1) gene. This mutation causes hypermethylation and silencing of FMR1 (Jacquemont et al., 2011). Rett, Angelman, Prader-Willi and Fragile X syndromes are debilitating developmental disorders with distinct epigenetic causes.
Numerous studies have linked autism to problems with redox, methylation and epigenetics. Studies of autistic children have shown low plasma levels of both GSH and SAM (James et al., 2004). Multiple groups have reported on the link between a methylenetetrahydrofolate reductase (MTHFR) polymorphism and autism (Boris et al., 2004; Pașca et al., 2008; Banerjee et al., 2009; Goin-Kochel et al., 2009). MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate to MTHF, the methyl donor for methylation of homocysteine to methionine by methionine synthase. Additionally, children with autism have higher levels of autoantibodies against the folate receptor, and suffer from cerebral folate deficiency (Ramaekers et al., 2007). It has also been reported that IGF-1 is decreased in the cerebrospinal fluid (CSF) of autistic subjects, and Phosphatase and tensin homolog (PTEN) and tuberous sclerosis complex (TSC) genes of the Akt pathway are associated with autism (Baker et al., 1998; Vanhala et al., 2001; Redfern et al., 2010). Additionally DNA hypomethylation in the parents of autistic children has been found (Jill James et al., 2008). These findings directly implicate the redox/methylation system this thesis outlines (Fig. 62).

These findings, along with other studies from our lab, support a redox/methylation hypothesis of autism (Deth et al., 2008). Autism has been shown to be linked by problems in the methylation cycle, redox homeostasis and IGF-1 signaling. My work shows the interconnected relationship among these pathways, which may illuminate better understanding of autism etiology. The redox and methylation problems in autism might explain many of the similar developmental problems children with autism share with those with Rett, Angelman, Prader-Willi and Fragile X syndromes.
Environmental factors such as heavy metals can derail the redox/methylation balance. Mercury, for example, binds with extremely high affinity to selenium, which is used to reduce glutathione reductase. Without sufficient selenium, especially in the brain where selenium is essential for redox balance in the absence of excess GSH, the redox balance could be shifted by mercury. Indeed, the unique redox features of the brain make it exceptionally vulnerable to mercury. In children carrying certain genetic or non genetic predispositions to autism, such as impaired methylation capacity secondary to low cerebral folate, or polymorphism in MTHFR, the mercury-induced shift in redox could cause drastic epigenetic consequences, precipitating an autistic disorder.

Additionally, schizophrenia has also been associated with problems in redox and methylation. Studies that GSH and the oxidized cysteine metabolite taurine are both decreased in the CSF of schizophrenic patients (Do et al., 1995, 2000). Also, global DNA methylation is decreased in peripheral leukocytes in schizophrenic patients, and hypomethylation of the COMT promoter is a major risk factor associated with schizophrenia (Abdolmaleky et al., 2006; Shimabukuro et al., 2007).

These observations taken together show a link between redox and methylation, that when disturbed is associated with neurological disorders.
E. Aging and Methylation

Aging is associated with increased levels of oxidative stress and changes in DNA methylation (Finkel and Holbrook, 2000; Fusco et al., 2007). Multiple studies have shown both an age associated decrease and increase in DNA methylation (Siegmund et al., 2007; Heyn et al., 2012; Numata et al., 2012). Depending on the methods used, the direction of overall DNA methylation change with age is a matter of debate, however, the dynamic regulation of DNA methylation over the lifespan is clear. DNA methylation corresponds with a decrease in antioxidant capacity with age, as shown by a decreases in the level of GSH in the plasma (Seshadri et al., 2002; Isobe et al., 2005; Serot et al., 2005). These studies correlate nicely with the antioxidant dependent changes in DNA methylation found in SH-SY5Y cells under both oxidizing and reducing conditions (Figs. 38, 40, 42 and 44). Additionally, these decreases in GSH and DNA methylation are associated with cognitive decline in age matched comparisons (Fusco et al., 2007; Chan et al., 2008). The physiological changes observed in the brain over time suggest that oxidative stress accumulates during aging and methylation activity is altered. It has been shown that cobalamin supplementation reduces cognitive decline in the elderly (Weir and Scott, 1999; Ellinson et al., 2004; Morris et al., 2007). The cobalamin supplementation supports the activity of methionine synthase during age related oxidative stress. The beneficial effects of cobalamin may be attributed to increasing the activity of methionine synthase, and increasing the methylation capacity in the brain.

Plasma levels of IGF-1 decline with age, and this decline is associated with increased methylation of the IGF-1 promoter (Rozing et al., 2009; Numata et al., 2012). In mice and C. elegans, the IGF-1 receptor regulates lifespan and resistance to oxidative
stress (Lin et al., 2001b; Holzenberger et al., 2003). Heterozygous knockout of the IGF-1 receptor, in both species, increases lifespan by decreasing metabolism.

It seems that as one ages, the body must throttle back metabolic expenditures needed to form new synapses and memory, and attempt to maintain those synapses that carry the lessons of life. In other words, early on in life, when it is important to learn as much as possible from our environment, making new synapses and axons, and firing neurons is essential, but as one ages, and has learned to survive, the need for new memory is decreased, but the maintenance of previously acquired memories is essential. As described earlier, to maintain synapses, cells are kept out of the cell cycle by keeping the brain at a more oxidized potential. The decrease in IGF-1 seems to accomplish these goals, however at the expense of increased sensitivity to oxidative damage, and decreased methylation capacity. This hypothesis is supported by my findings which show that SH-SY5Y cells are sensitive to redox changes mediated by IGF-1 stimulated cysteine uptake, and those changes in redox state correspond to changes in methylation capacity (Figs. 24, 36 and 38).

In the few cases where cell do divide in the brain, the cells contain high levels of selenium, capable of keeping those neuronal stem cells reduced (Scharpf et al., 2007; Gleason et al., 2008). The relationship between aging and the reduction in antioxidant capacity could be an evolutionary adaptation to maintain synapses over long periods of time, it does, however, make the brain more susceptible to oxidative stress.
F. Alzheimer’s disease

Alzheimer’s disease has been associated with decreased methylation capacity and oxidative stress in many studies. AD has been associated with the 2756 C>G polymorphism in methionine synthase in several studies (Beyer et al., 2003; Bosco et al., 2004). Similarly, genetic variants of methylenetetrahydrofolate reductase (MTHFR) has been linked to AD in multiple studies (Wang et al., 2005; Bertram et al., 2007; Kageyama et al., 2008; Bi et al., 2009). Additionally, plasma levels of homocysteine are elevated in AD (Clarke et al., 1998; Seshadri et al., 2002; Serot et al., 2005), and the rate of cognitive decline is correlated homocysteine levels (Oulhaj et al., 2010). These findings serve to link AD and methionine synthase activity, although the functional significance of this link remains largely obscure. The observed polymorphisms in methionine synthase and MTHFR, the enzyme that provides the methyl donor to methionine synthase, along with the altered methionine metabolism and DNA methylation clearly implicate impaired methylation with AD. AD has also been associated with decreased levels of SAM in the CSF and brains of AD subjects, by as much as 85% (Bottiglieri et al., 1990; Bottiglieri and Hyland, 1994; Morrison et al., 1996). Studies have also shown aberrant DNA methylation patterns in AD patients (Bollati et al., 2011; Fleming et al., 2011).

The effects of Aβ conditioned media on thiols and thioethers in SH-SY5Y cells agreed with these studies (Fig. 52). In the SH-SY5Y cell culture system, Aβ oligomers increased homocysteine, and decreased the methylation capacity (Fig 52 and Table 4). The decrease in SAM levels in the brains of AD patients theoretically fits with our redox-methylation hypothesis, however in the experiments using soluble oligomers of Aβ, we found SAM to be elevated (Fig. 47). However SAM maybe elevated under these
conditions because of the, more than, 2 fold increase in SAH (Fig. 47). SAH is a potent inhibitor of methylation reactions, and could cause this accumulation of SAM. My studies also showed abhorrent DNA methylation and gene transcription associated with the administration of Aβ oligomers. Although these studies have yet to be replicated by other labs, one study has shown that the administration of SAM to SK-N-BE neuroblastoma cells in culture changes the expression of many genes including APP and Presenilin 1 (Cavallaro et al., 2006).

Other studies have shown that the plasma levels of cobalamin and folate are lower in AD patients (McCaddon et al., 2002; Stanger et al., 2009). The deficit in these essential cofactors for methionine synthase further links AD to impaired methionine synthase activity. Cobalamin and folate supplementation has been used in multiple studies as a treatment for AD with promising results (Ellinson et al., 2004; Stanger et al., 2009; Smith et al., 2010). In the British study, cobalamin supplementation slowed the rate of brain atrophy, and improved cognitive measures in AD patients.

The link between transsulfuration, methionine synthase, and methylation has been used to explain deregulated DNA methylation and the hypomethylation of protein phosphatase 2A (PP2A) (Vafai and Stock, 2002; Sontag et al., 2007). PP2A, in its active, methylated state, dephosphorylates tau. Hyperphosphorylated tau is the primary component of paired helical formations or neurofibrillary tangles. However, the pathway this thesis lays out further links oxidative stress, impaired DNA and protein methylation and Aβ with AD (Fig 65).
Figure 65: Aβ effects on transsulfuration and methylation. Aβ oligomers inhibit EAAT3 mediated cysteine uptake, decreasing GSH synthesis. Decreased GSH synthesis and Aβ produced ROS shifts redox status to a more oxidizing potential. This shift decreases methionine synthase activity decreasing the probability of protein and DNA methylation. DNA methylation leads to changes in gene transcription. Protein methylation and changed gene transcription cause changes in protein function and phenotype.

This thesis has shown that soluble oligomers of Aβ inhibit the transport of cysteine into neurons (Fig 45). This inhibition causes oxidative stress, elevated homocysteine, and a decrease in methylation capacity, precisely the changes observed in the blood and brains of AD patients (Fig 47). Furthermore, this work has shown that
soluble oligomers of Aβ cause changes DNA methylation and changes gene transcription (Figs 53 and 55). These actions provide a mechanistic link between Aβ and the major features of AD.

Potential pharmacological interventions to alleviate the oxidative stress caused by Aβ oligomer inhibition of EAAT3-mediated cysteine uptake could fall into two categories. The first could involve increasing available cysteine in the brain. N-acetylcysteine, a membrane permeable form of cysteine, has been given to probable AD patients in a double-blind clinical trial with favorable and significant results in some cognitive tasks (Adair et al., 2001). However, N-acetylcysteine only slightly slowed the progression of Alzheimer’s disease and most measures in the study showed no change. The second pharmacological intervention would be to target the IGF-1 redox pathway described in this thesis. Meta-analysis of studies looking at Alzheimer’s disease in clinical trials where IGF-1 was given has shown conflicting results concerning the efficacy of IGF-1 in treating Alzheimer’s disease (Cole and Frautschy, 2007). However, these studies are inconclusive at best, and suffer from small numbers of patients enrolled. Additionally, giving IGF-1 would have numerous side effects. As an alternative approach, a small molecule could be developed to target the GTRAP3-18 EAAT3 interaction, or to inhibit AS160, the GTPase activating protein that decreases Ras1 activity. Such a molecule would be able to increase EAAT3 surface expression and decrease oxidative stress.

This thesis illustrates the link between the pathogenic protein of Alzheimer’s disease and many of the molecular observations associated with Alzheimer’s disease.
This data along with data presented linking methylation deficiency and oxidative stress to autism, schizophrenia, Rett, Angelman, Prader-Willi and Fragile X syndromes show that impaired redox and methylation pathways are common and fundamental to neurological disorders.
V. Future Directions

The biggest limitation of this thesis was the use of a cell culture system that does not resemble brain-like conditions in a number of aspects. Two important approaches to address this limitation would be the use of a low cysteine, high selenium defined cell culture media, and the use of rat primary cortical neurons. A custom defined media would allow for the control of growth factors present in the media, and the low cysteine, high selenium containing culture media would resemble the redox components of the CSF more closely. This culture media would allow for a better understanding of which growth factors are responsible for the regulation of EAAT3 mediated cysteine uptake. Also, a more brain-like culture media might yield more information of how the extracellular brain environment influences the tightly controlled redox state of neurons. The use of primary cortical neurons would address a few key issues. First, immortalized cell lines are not perfect models of neurons. SH-SY5Y cells are a neuroblastoma-derived cell line. The cancerous nature of this cell lines make the cells genetically unstable and SH-SY5Y cells have some genetic abnormalities including a large replication of the 1q region of chromosome one and many copy number variants of genes. Additionally, a primary neuron culture wouldn’t divide, removing the effect of cell division from DNA methylation. It is beneficial to try and work with cells that are as close to human neurons as possible, and both rat primary neurons and SH-SY5Y cells have their limitations. A possible alternative could be the use of a human stem cell line differentiated to neurons.

Further experiments also should be pursued in the future. Better understanding of the mechanism of EAAT3 regulation is essential, if pharmacological tools to modulate its
activity are to be developed. Specifically, long-term time course experiments should be completed to see if the MAP kinase pathway is involved in long-term regulation of EAAT3. Also the interaction of the PI3 kinase and MAP kinase pathways in the regulation of EAAT3 should be sorted out. Long-term time course experiments using inhibitors of various kinases in the two pathways will provide good data to figure that out. The role of mTOR was left largely unstudied in this thesis, and its role in EAAT3 regulation definitely needs to be addressed.

Currently, Malav Trivedi, another Ph.D. student in our lab is treating SH-SY5Y cells with morphine, an opiate which inhibits EAAT3 cysteine uptake. He is planning to isolate the DNA from these cells and have their methylome read by microarray. These studies, along with parallel experiments to measure intracellular thiols and thioethers, gene expression and global DNA methylation, will help clarify some of the findings of my thesis in greater detail. The comparison of intracellular thiols and thioethers, gene expression, and site specific DNA methylation will help show the effects of redox on both redox regulated genes, and will identify genes that are strongly influenced by redox through associated changes in methylation capacity. These proposed and current studies will provide a much better understanding of how EAAT3 mediated cysteine uptake affects redox, methylation and gene transcription.
VI. REFERENCES


Castagna, A., Le Grazie, C., Accordini, A., Giuliodori, P., Cavalli, G., Bottiglieri, T., and Lazzarin, A. Cerebrospinal fluid S-adenosylmethionine (SAMe) and glutathione concentrations in HIV infection: effect of parenteral treatment with SAMe. Neurology 45,


by B Vitamins Slows the Rate of Accelerated Brain Atrophy in Mild Cognitive Impairment: A Randomized Controlled Trial. PLoS ONE 5, e12244.


### VII. Appendix

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAT2A</strong></td>
<td>Methionine Adenosyl transferase</td>
</tr>
<tr>
<td><strong>CBS</strong></td>
<td>Cystathionine beta Synthase</td>
</tr>
<tr>
<td><strong>GCLC</strong></td>
<td>Glutamate Cysteine ligase Catalytic</td>
</tr>
<tr>
<td><strong>MS</strong></td>
<td>Methionine Synthase</td>
</tr>
<tr>
<td><strong>CGL</strong></td>
<td>Cystathionine gamma lyase</td>
</tr>
<tr>
<td><strong>EAAT3</strong></td>
<td>Excitatory amino acid transporter 3</td>
</tr>
<tr>
<td><strong>LINE 1</strong></td>
<td>Long interspersed Nucleotide elements</td>
</tr>
<tr>
<td><strong>COMT</strong></td>
<td>Catechol ortho methyl transferase</td>
</tr>
<tr>
<td><strong>DRD4</strong></td>
<td>Dopamine Receptor 4</td>
</tr>
<tr>
<td><strong>GSS</strong></td>
<td>Glutathione Synthetase</td>
</tr>
<tr>
<td><strong>GSR</strong></td>
<td>Glutathione Reductase</td>
</tr>
<tr>
<td><strong>GLCLM</strong></td>
<td>Glutamate Cysteine ligase Modulatory</td>
</tr>
<tr>
<td><strong>DNMT3</strong></td>
<td>DNA methyl transferase 3</td>
</tr>
<tr>
<td><strong>SAHH</strong></td>
<td>S Adenosyl homocysteinase</td>
</tr>
<tr>
<td><strong>Forward Primer , Tm(°C)</strong></td>
<td>Reverse Primer , Tm(°C)</td>
</tr>
<tr>
<td>agggaattgccctaaaggagaa (60.03)</td>
<td>atttttcgtccctga (59.98)</td>
</tr>
<tr>
<td>tctgtatccacagagaagatg (59.94)</td>
<td>tttggtagttgccttcag (60.04)</td>
</tr>
<tr>
<td>agagaagggggaaaggacaa (60.5)</td>
<td>gtagaacccaggacacgccta (60.11)</td>
</tr>
<tr>
<td>tggagagcgcgtgtaatgccttg (60.01)</td>
<td>cccagcttcataccagacca (59.99)</td>
</tr>
<tr>
<td>gattcgaagccctgctgag (60.10)</td>
<td>acttccacctggaggtgtg (60.2)</td>
</tr>
<tr>
<td>ttacattgcctgccttg (59.9)</td>
<td>gcagttgtgcctaacctg (60.1)</td>
</tr>
<tr>
<td>ccaaatcattggttaactcc (59.9)</td>
<td>aacatttcctccctgaga (60.1)</td>
</tr>
<tr>
<td>acatggtcttcctcgacca (59.97)</td>
<td>tcaacgttgcagccctg (60.06)</td>
</tr>
<tr>
<td>gcctcatctctcactgacca (60.66)</td>
<td>agcacaggagagatgagc (60.33)</td>
</tr>
<tr>
<td>gcctctcatctctcactgga (60.03)</td>
<td>aaagatgccccagctctg (59.6)</td>
</tr>
<tr>
<td>tggagagcgcgtgtaatgccttg (60.01)</td>
<td>cccagcttcataccagacca (59.99)</td>
</tr>
<tr>
<td>tcacacctgtctccaccagca (60.11)</td>
<td>ctcagcctccagatacgt (60.15)</td>
</tr>
<tr>
<td>gcctcaaatctctctg (59.93)</td>
<td>cagcagctgcagctctg (60.01)</td>
</tr>
<tr>
<td>agggcaacatctttgtcacc (59.97)</td>
<td>ccaacctgcttgc (60.1)</td>
</tr>
</tbody>
</table>