Down-Regulation and Alternative Splicing of Methionine Synthase as an Adaptive Response to Oxidative Stress in Aging and Neurological Disorders

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List of Abbreviations

AD: Alzheimer’s disease
Cbl: cobalamin
CBS: cystathionine β-synthase
CSF: cerebrospinal fluid
EAAT: excitatory amino acid transporter
GSH: glutathione (reduced)
GSSG: glutathione disulfide (oxidized)
HCY: homocysteine
MeCbl: methylcob(III)alamin
MethylTHF: 5-methyltetrahydrofolate
MS: methionine synthase
MTHFR: methylenetetrahydrofolate reductase
MTRR: methionine synthase reductase
NAC: N-acetyl-cysteine
PLM: phospholipid methylation
ROS: reactive oxygen species
SAM: S-adenosylmethionine
SAH: S-adenosylhomocysteine
SOD: superoxide dismutase
TNFα: tumor necrosis factor-alpha
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Abstract

The folate and cobalamin-dependent enzyme methionine synthase converts homocysteine to methionine, but oxidation of its cobalamin cofactor halts activity, leading to increased levels of homocysteine. Homocysteine can be converted to cystathionine through the transsulfuration pathway, via the enzyme cystathionine β-synthase, resulting in glutathione synthesis. The enzymes methionine synthase and cystathionine β-synthase flank homocysteine, and their relative enzymatic activities determine the proportion of homocysteine that can enter the methylation or transsulfuration pathways. Prevailing redox conditions may adjust this flux by modulating the enzyme via multiple mechanisms.

Glutathione is the principal intracellular antioxidant, and adequate levels of its reduced form are essential for survival in an oxidative metabolic environment. The thiol-containing amino acid cysteine is rate-limiting for glutathione synthesis and can be provided through either cellular uptake or conversion through transsulfuration.

Methionine synthase possesses a linker domain termed ‘cap’ that is responsible for covering the susceptible cobalamin from oxidation, and loss of this domain may lead to enzyme inactivation. Oxidative stress, associated with lower glutathione levels, is an important contributor to neurodevelopmental and neurodegenerative disorders such as autism, Alzheimer’s disease, and schizophrenia.

I used qRT-PCR to evaluate the level of methionine synthase mRNA in post-mortem human cortex and found a significant decrease with age, from 28 weeks of fetal gestation to greater than 80 years. Domain-specific PCR showed that the cap/cobalamin ratio is significantly decreased in subjects over the age of 60, implying age-dependent alternative splicing of the cap domain. Further studies revealed the deletion of exons 19 and 20 of the cap domain, which
would reduce the domain size and possibly favor inactivation of MS, leading to increased flux of homocysteine into the transsulfuration pathway and glutathione formation.

A comparison study between autistic subjects and age-matched controls, age 4 to 30 years, revealed significantly lower levels of methionine synthase mRNA in autistic subjects. The greatest decrease occurred in the youngest subjects, when levels in control subjects were normally at their highest, implying an adaptive response to elevated levels of oxidative stress. Thus, autism likely results from a disruption of both normal redox regulation and methylation status.

Exon-specific studies probing folate-binding and cap domain exons in SH-SY5Y human neuroblastoma cells highlighted a combinatorial approach to MS composition. It is likely that different isoforms are generated, depending on the cellular redox status, as an adaptive response to oxidative stress. Non-neuronal cell lines HEK, HepG2 and LN-18 do not show similar splicing modifications, suggesting that redox-dependent splicing is not a feature of all cell types and is more prominent in neuronal cells.

Tumor necrosis factor-alpha, a pro-inflammatory cytokine, is able to induce neuroinflammation, which is a common underlying problem in autism. Tumor necrosis factor-alpha treatment significantly reduced both cobalamin-binding and cap domain mRNA in SH-SY5Y human neuroblastoma cells, while increasing homocysteine levels. Additionally, cysteine and glutathione levels were increased, despite lower cysteine uptake, indicating an increase in transsulfuration. Methionine synthase activity was inhibited by tumor necrosis factor-alpha, which, when coupled with the mRNA data, indicates transcriptional changes in methionine synthase that can affect cellular redox and methylation activity.
Alternative splicing of the cap domain may reflect previously unrecognized alterations in methionine synthase in response to oxidative stress and aging. The resultant link between redox status and methylation activity is likely to be an important factor in neurodevelopmental, neuropsychiatric and neurodegenerative disorders. These findings represent an important adaptive response to counter progressive oxidation with aging and further indicate that autism may be a neurometabolic redox disorder in which lower levels of methionine synthase activity play an important role.
I. INTRODUCTION

A. Statement of Problem

This thesis investigates how changes in mRNA status of MS can be utilized to respond to oxidative stress in the brain.

Ever since oxygen appeared in the atmosphere about 2.5 billion years ago, organisms have been evolving ways in which to survive the oxidative environment. As oxygen built up in the atmosphere, selection favored those that could survive in the increasingly oxidative environment. Cyanobacteria are the only bacteria that are able to produce oxygen as a by-product of metabolism and most likely evolved from organisms that metabolized sulfur, a key component of the amino acid cysteine, which is rate-limiting in GSH formation. Cyanobacteria survived the oxidative environment because they expressed super oxide dismutase (SOD), catalase, and glutathione (GSH), all of which are important antioxidants. These adaptive responses to oxidation have most likely shaped the evolution of metabolic pathways.

The central nervous system (CNS), particularly the brain, is extremely susceptible to oxidative stress due to its high oxygen consumption and the post-mitotic nature of cells. Manifestations of this susceptibility are largely regarded as a main cause of functional impairment in the aging brain of both vertebrates and invertebrates.

Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the body’s ability to detoxify these toxic intermediates. ROS are free radicals or molecules that contain oxygen atoms and can produce free radicals. They are also highly reactive due to unpaired electrons in the outer valence shell. ROS are generated in vivo mainly through aerobic respiration and oxygen metabolism, although they can also be produced by a number of other processes including cytochrome P450 metabolism of xenobiotic compounds. Cells depend
heavily on antioxidant defenses such as SOD, catalase, and/or small molecules like GSH in order to maintain their redox equilibrium balance and help keep ROS in check.

Methionine synthase (MS), a folate and cobalamin-dependent enzyme, converts homocysteine (HCY) to methionine. However, oxidation of the cofactor cobalamin halts enzyme activity and promotes synthesis of GSH via transsulfuration. Lower MS activity also inhibits methylation of DNA and histones, affecting epigenetic regulation of gene expression, which has been linked to developmental and neurodegenerative disorders.

Systemic oxidative stress, as detected in plasma and blood cells, is a feature of autism and other neurological disorders, such as Alzheimer’s disease (AD)\textsuperscript{7-9}. Indeed the hallmark feature for many neurological diseases is oxidative stress\textsuperscript{10}. GSH levels are also decreased in cerebrospinal fluid (CSF) and post-mortem cortex of schizophrenic patients\textsuperscript{11-13}. Additionally, animal studies utilizing mice,\textit{Drosophila} and\textit{C. Elegans} support the free radical theory of aging\textsuperscript{14-16}, which states that accumulation of free radical damage contributes to the aging process. Neurodegenerative disorders are associated with elevated levels of oxidative stress and low levels of GSH, and the increased risk of neurodegenerative disorders with advanced age may reflect gradual exhaustion of adaptive measures to combat oxidative stress\textsuperscript{17,18}.

Much like the adaptive responses that fueled the evolution of cyanobacteria, MS shows adaptations to oxidative stress across the lifespan that are important for maintaining redox equilibrium. During times of oxidative stress, MS enzyme activity may be regulated in a multitude of ways: oxidation of the cofactor cobalamin, alternative splicing or exon skipping, and/or decreased transcription. These adaptations likely elevate levels of GSH to ameliorate oxidative stress. My results have shown an age-dependent loss of the cap domain from MS mRNA isolated from the cortex of elderly controls (age >60) in contrast to the presence of cap
domain in younger control subjects (age <20). These changes may represent adaptive responses to counter progressive oxidation with aging. Abnormalities affecting redox regulation may contribute to neurodevelopmental, neuropsychiatric and neurodegenerative disorders across the lifespan.

B. Methionine Synthase: Structure and Function

MS, a folate and cobalamin-dependent enzyme that catalyzes the remethylation of HCY to methionine, is structurally organized into five domains, four of which bind HCY, methylfolate, cobalamin, and S-adenosylmethionine (SAM)\(^{19-21}\) (Fig. 1). A fifth domain, termed cap, functions as a linker domain between the folate- and cobalamin-binding domains, and partially covers and protects the cobalamin from oxidation by limiting access of ROS and electrophiles from the surrounding redox environment\(^{22}\).

The five domains of MS span 33 exons in total and consist of 1265 amino acids. The total molecular weight for MS is ~140 kDa.
Methionine is subsequently adenosylated to SAM via the enzyme methionine adenosyltransferase (MAT). SAM serves as the methyl donor in many methyltransferase reactions, such as methylation of membrane phospholipids, nucleic acids, proteins and biogenic amines. S-adenosylhomocysteine (SAH) is formed by the demethylation of SAM\(^\text{25}\). SAH is further hydrolyzed to HCY and adenosine, via the enzyme SAH hydrolase, and this reaction is reversible with thermodynamics favoring SAH formation (Fig. 10, black text). HCY can then either be remethylated to methionine or converted to cystathionine via the transsulfuration pathway.

Cobalamin is an essential cofactor for MS activity and its oxidation status plays a central role in regulation of the enzyme. During primary enzyme turnover, 5-methyltetrahydrofolate (methylTHF) transfers a methyl group to MS-bound cob(I)alamin (Cbl(I)) forming
methylcob(III)alamin (MeCbl)\textsuperscript{23} (Fig. 2). MeCbl subsequently converts HCY to methionine by donating its methyl group to the sulfur atom of HCY\textsuperscript{20} (Fig. 3). The cobalamin-binding domain interacts with both the HCY- and folate-binding domains. MeCbl then returns to Cbl(I), ready to accept a new methyl group\textsuperscript{24}.

![Figure 2: Enzymatic reaction catalyzed by MS and its subsequent reactivation. Figure from Wilson et al.\textsuperscript{26}]

![Figure 3: Models of the cobalamin-binding domain complex with both the HCY- and folate-binding domains. The cobalamin-binding domain (red) interacts with HCY-binding domain (left, green) or the folate-binding domain (right, gold). The sulfur atom of HCY can be seen as the methyl receiver (right, yellow molecule). The large displacement of the cobalamin-binding domain is illustrated in this figure from Evans et al.\textsuperscript{24}]

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In order for the reaction to continue, methylTHF needs to remethylate Cbl(I) to MeCbl; however, Cbl(I) is highly unstable and easily oxidized to the inactive form of cob(II)alamin (Cbl(II)), due to the surrounding redox environment. A reduction in methylTHF availability, which would increase the duration of the Cbl(I) state, increases the chance for oxidation of Cbl(I) to Cbl(II)\textsuperscript{26}. Cobalamin oxidation results in MS inactivation and therefore halts methylation of HCY to methionine.

In the absence of methylTHF, SAM can reactivate MS after the oxidation of cobalamin, by donating a methyl group to Cbl(II) to form MeCbl, in conjunction with electron donation by methionine synthase reductase (MTRR)\textsuperscript{27} (Fig. 2). SAM-dependent reactivation is only favored when cobalamin is present in the Cbl(II) state. Additionally, glutathionylcobalamin (GSCbl) can be converted to MeCbl, using SAM-dependent methylation, which reactivates MS by exchanging for Cbl(II). GSCbl can also exchange for Cbl(II) and be remethylated by the SAM-binding domain of MS (Fig. 10, green text)\textsuperscript{20,28}.

In times of oxidative stress, MS can be inhibited such that HCY is not converted to methionine but is shunted into the transsulfuration pathway to ameliorate oxidative stress\textsuperscript{29}. There are several possible mechanisms that can inhibit or lower MS activity: oxidation of the cofactor cobalamin, alternative splicing of the cap domain and lowering transcription levels.

C. Folate-Binding Domain Structure

The folate-binding domain consists of exons 12-18 and is ~1,000 base pairs (bp) in length. At the protein level, it has 8 helices and ~333 amino acids and is connected to the HCY domain via a small hairpin loop, which allows the two domains to function side-by-side (Fig. 4). A recent investigation of full-length cDNA transcripts from a fetal human cortex sample
revealed skipping of exons 16-18, which correspond to the folate-binding domain of MS\textsuperscript{30}. A lack of these exons could distort the binding of methylTHF to the last barrel sheet (Fig. 5).

**Figure 4:** Ribbon drawing of the HCY- and folate-binding domains of *T. maritima*. The HCY-binding domain is shown in green; the linker domain in gray; the folate-binding domain in yellow. Both substrates are bound to the domains (ball-and-stick models). Figure from Evans et al.\textsuperscript{24}.

**Figure 5:** Ribbon drawing detailing exon-specific splicing of folate-binding domain exons in MS. Exons 16-18 are shown in blue (left) and exons 12-15 are shown in yellow. MethylTHF is shown in red. The HCY-binding domain is shown in white. Deletion of exons 16-18 results in a major truncation of the folate-binding domain, altering protein structure and possibly decreasing methylTHF-binding affinity (right). Structures from *E. coli* and *T. maritima* (PDB codes 1Q8J, 1K98 and 1MSK, respectively)\textsuperscript{22-24}. 

D. Cap Domain Activation

During the vulnerable Cbl(I) interval, the cap domain hovers above the cobalamin until the folate-binding domain is ready to bring the next methyl group into close proximity. The cap domain within MS serves as a protective cover to the cobalamin; however, in times of oxidative stress and increased GSH need, my results indicate that a portion of the cap domain can be deleted, facilitating oxidation of the cobalamin. Splicing of the cap domain may alter the protein structure in such a way that the vulnerable cobalamin is more exposed to the redox environment of the cell.

The upper face of cobalamin is shielded by the 4-helix cap domain, which positions itself on top of the cobalamin. In the absence of substrate, there is evidence that a significant portion of the enzyme exists in the resting state (cap on). During the activation period, the cap domain rotates in order to expose the cobalamin for methyl group transfer. Residues between the cobalamin-binding domain and the cap domain allow for conformational changes that facilitate movement of the cap domain (Fig. 6).

Since MS is not active if cobalamin is oxidized, the cap domain can be considered protective and thus sustains enzyme activity in a threatening redox environment. When MS is inactive, HCY is diverted towards the transsulfuration pathway and GSH synthesis. It is in this way that cobalamin and the cap domain (MS) combine to form a variable redox sensor of the cellular environment.

The cap domain consists of exons 19-21 and is ~348 bp (116 amino acids). Exons 19-21 are located about midpoint in the mRNA and partial deletion of this domain alters the protein structure such that cobalamin is no longer protected (Fig. 7).
Figure 6: Ribbon drawing of MS cap domain activation. (a) Activation state: Cbl(I) receives a methyl group, cap domain (yellow) is displaced. Cobalamin-binding domain (B12) is shown in red; cobalamin in orange; SAM domain in blue. Numbered residues contribute to the molecular conformation and cap domain movement. (b) Resting state: cap rotates to cover the cobalamin to prevent oxidation as indicated by the red arrow. Cobalamin molecule is shown bound to the cobalamin-binding domain in gray; position of cap domain over cobalamin is shown in gray; cap domain original conformation is shown in yellow. Illustration from Bandarian et al.32.

Figure 7: Ribbon drawing detailing exon-specific splicing of cap domain exons in MS. Commonly spliced exons 19 and 20 are shown in pink. Exon 21 is shown in yellow. Cobalamin-binding domain is shown in white, with cobalamin in orange. Deletion of exons 19 and 20 results in a major deletion of the cap domain, altering protein structure and decreasing cobalamin coverage. Structures from E. coli and T. maritima (PDB codes 1Q8J, 1K98 and 1MSK, respectively)32-34.
E. Transsulfuration Pathway

Since intracellular levels of GSH are very high, 1-10 mM, a large part of cellular metabolism focuses on maintaining these levels, through the transsulfuration pathway. HCY is converted to cystathionine in the first step of the transsulfuration pathway²⁹ (Fig. 10, red text). Levels of cystathionine are much higher in the brain as compared to other organs and higher in humans as compared to lower primates (Fig. 8)³¹,³². Higher cystathionine levels primarily reflect lower conversion of cystathionine to cysteine by cystathionine γ-lyase (CGL), which limits cysteine availability for GSH synthesis. However, transsulfuration activity in brain is restricted by low activity of CGL³³, although a recent study demonstrated that this pathway still makes a significant contribution to GSH synthesis²⁹. HCY accumulates when MS is inactive, further contributing to increased levels of cystathionine, especially during periods of oxidative stress. These observations suggest a relationship between evolution and decreased MS activity in the brain.

Figure 8: Levels of cystathionine are higher in the human brain as compared to other organs. Cystathionine levels are also higher as compared to lower mammals and birds. Figure from Tallan et al.³¹.
HCY is converted to cystathionine by the enzyme cystathionine $\beta$-synthase (CBS), through the addition of serine (Fig. 9). MS and CBS flank HCY, and their relative enzymatic activities, which are subject to regulation, provide crucial redox-dependent regulation of the fate of HCY$^{34}$. CBS is a tetrameric enzyme containing a redox-sensitive heme group, which consists of an iron molecule within a porphyrin ring. When iron (Fe) exists in its ferrous (Fe$^{2+}$) state, CBS activity is lower, whereas if the heme group is oxidized to its ferric state (Fe$^{3+}$) under conditions of oxidative stress, CBS activity is increased. In addition, deletion of the heme group has been linked to a loss of redox sensitivity further making the case for the hypothesis that the heme group acts as a redox sensor$^{35}$. Thus, increased enzymatic activity of CBS is important for augmenting levels of cysteine through the transsulfuration pathway.

**Figure 9: The enzymes MS and CBS represent a branch point for HCY.** HCY can be converted to methionine via MS or converted to cystathionine via CBS, and these enzymes act as redox sensors for GSH synthesis. HCY is used to generate cysteine when levels are low. GSH is then synthesized in a two-step reaction.
CBS activity is also negatively regulated by its carboxy-terminal domain and binding of SAM to this domain removes inhibition of the enzyme. Proteolytic cleavage of the SAM-binding domain during oxidative stress increases enzyme activity to its highest level. Together, MS and CBS regulate the balance between methylation and redox buffering and in this way, CBS can be thought of as an additional redox sensor.

The enzyme CGL plays an important role in transsulfuration by breaking down cystathionine into cysteine and alpha-ketobutyrate. Cysteine is rate-limiting for GSH synthesis, and although cysteine can be imported into neuronal cells by the excitatory amino acid transporter (EAAT3), transsulfuration is an additional source of cysteine for GSH synthesis (Figs. 9 and 10). Inhibition of CGL results in lower levels of GSH and mitochondrial insult, highlighting the functional importance of the enzyme within the transsulfuration pathway.

GSH synthesis utilizes two enzymes to conjugate three amino acids: cysteine, glutamate and glycine. The enzyme glutamate cysteine ligase (GCL) catalyzes the ATP-dependent condensation of cysteine and glutamate to form γ-glutamylcysteine. In the next step, glutathione synthase catalyzes the ATP-dependent condensation of γ-glutamylcysteine and glycine to form GSH.

GCL is composed of two subunits: glutamate cysteine ligase catalytic subunit (GCLC) and glutamate cysteine ligase modifier subunit (GCLM). The GCLC subunit contains the main catalytic activity, while the GLCM subunit enhances the catalytic efficiency of the GCLC subunit. As one regulatory mechanism of GSH synthesis, GSH acts as its own feedback inhibitor by inhibiting the GCLC subunit. A knockout of the GCLC subunit in homozygous mice is fatal, since GSH is the main antioxidant and decreased amounts do not provide the brain with...
adequate protection when under stress. This indicates the important role of GSH in cellular redox homeostasis.\textsuperscript{39}

**Figure 10: Sulfur metabolism pathway in neuronal cells.** Red text highlights the transsulfuration pathway, ending with the formation of GSH. Blue text features dopamine-stimulated PLM, utilizing MS. Orange text shows cysteine generation from astrocyte-derived GSH, followed by its uptake into neurons via the EAAT3 transporter (yellow). Regeneration of MeCbl through the addition of SAM and GSCbl is shown in green. Black text indicates folate-mediated one-carbon metabolism.

In neurons, cysteine uptake is provided through EAAT3 (Fig. 10, orange text; Fig. 11). EAATs transport both glutamate and cysteine, and EAAT3 (EAAC1 in mice) is the primary transporter in human neurons.\textsuperscript{40} Astrocytes are a primary source of GSH within the brain and transport GSH out of the cell via multi-drug resistance protein (MRP1). Once GSH is in the extracellular space, it is first cleaved to Cys-Gly by the ectoenzyme \(\gamma\)-glutamyltranspeptidase (\(\gamma\)GT) and then to cysteine by a dipeptidase. Neurons can then take up this free cysteine via the EAAT3 transporter.\textsuperscript{41-43}
Recent studies have shown a protective role for the EAAT3 transporter in neurons against oxidative stress\textsuperscript{44-46}. EAAT inhibitors reduce GSH levels in cultured cortical neurons and knockdown of EAAT reduces cysteine uptake and GSH synthesis. EAAC1-deficient mice have reduced GSH levels and show brain atrophy in response to aging, indicating a role for EAAT3 in maintaining proper redox status, through formation of GSH via cysteine uptake\textsuperscript{46}.

\textbf{Figure 11: PI3K regulates EAAT3 expression in neurons.} (1) Akt via PI3K can directly stimulate transporter activity. (2) Akt can indirectly stimulate EAAT3 activity via protein partners involved in transport and (3) Akt can regulate gene expression of EAAT3 and/or the cytosolic pool of transporters. Figure from Nieoullon et al.\textsuperscript{47}. 

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Cell surface expression and activity of the EAAT3 transporter is regulated by phosphoinositide kinase (PI3K). This process has been found in glioma cells, and EAAT3 expression and activity can be increased through stimulation of Akt via PI3K\textsuperscript{48,49} (Fig. 11). This modulating signaling process may be adaptive in times of toxicity, such that cysteine levels require augmentation to prevent cellular death\textsuperscript{47}.

F. Oxidative Stress and Redox Status

Oxidative stress is classically described as an imbalance between production of ROS and the ability to detoxify or repair the ensuing damage\textsuperscript{50,51}. Oxidative stress is imposed on cells as a result of one of three factors: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage.

The main source of ROS \textit{in vivo} is through aerobic respiration; however, ROS can be produced by peroxisomal β-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, and tissue specific enzymes\textsuperscript{6}. Examples include the hydroxyl radical, superoxide, hydrogen peroxide, and peroxynitrite. ROS can be detoxified by cellular enzymes such as superoxide dismutase (SOD) and catalase, or by small molecules like GSH (Fig. 12). The main cellular damage results from ROS-induced alteration of macromolecules, such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA\textsuperscript{6}. Lipid peroxidation can cause altered membrane permeability and leakage, leading to cellular death. Additionally, oxidative stress and ROS have been implicated in disease states such as AD, Parkinson’s disease (PD), cancer, and aging.

Detoxification of ROS can be accomplished in part by GSH, a major antioxidant in cells. GSH is important in the detoxification and elimination process of free radicals and a reduction in
its levels can lead to an accumulation of ROS, resulting in elevated levels of oxidative stress. Adaptive changes in sulfur metabolism, in the form of GSH production, are essential for survival in an oxidative environment, and the ability to increase GSH levels in response to oxidation is a fundamental process in living cells.

**Figure 12: Oxidative stress in cells.** Oxidative stress is imposed on cells because of one of three factors: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage. ROS include the hydroxyl radical, superoxide, hydrogen peroxide, and peroxynitrite. Oxidative damage can occur in DNA, proteins and lipids. Figure from Sigma-Aldrich.

Glutathione peroxidases (GPx) are enzymes involved in the detoxification of peroxides. For example, GPx catalyzes the conversion of hydrogen peroxide to water, utilizing two molecules of GSH (Fig. 12). GSH is able to reduce disulfide bonds by acting as an electron donor, resulting in conversion to its oxidized form, glutathione disulfide (GSSG). The enzyme
glutathione reductase converts GSSG back to its reduced form (GSH). Interestingly, glutathione reductase is conserved between kingdoms and is present in bacteria, yeast, plants and animals\textsuperscript{55}. In healthy cells and tissues, most of the total glutathione pool exists in the reduced form (90%) versus the oxidized form (10%). Thus, the ratio of GSH to GSSG in cells is an indicator of cellular redox potential\textsuperscript{56}.

In times of oxidative stress, one essential way to elevate GSH levels is to augment cysteine levels, which can be accomplished by two mechanisms: transport of cysteine from outside the cell or by intracellular conversion of HCY to cysteine. The thiol amino acid HCY is formed by the methionine cycle, and subsequently can be converted to cysteine via the transsulfuration pathway.

### G. Autism

Autism is a neurodevelopmental disorder, categorized by developmental problems, repetitive behaviors and lack of communication, which usually manifests itself before the age of three\textsuperscript{57}. Autistic children often develop normal developmental skills at first, but then regress to an isolated state. Around 1.5 million Americans live with an autism spectrum disorder (Centers for Disease Control and Prevention). Cases of autism have increased in the last 20 years due to broader diagnosis capabilities; however, increased diagnosis alone cannot account for the increase in autism. Therefore, there must be additional genetic and environmental factors. Siblings of autistic children are more likely to develop the disorder than those that do not have an autistic sibling, and the concordance rate among monozygotic twins is around 90\%\textsuperscript{58,59}. Gene mutations and elevated metabolites can also play a role in autism. The enzyme methylenetetrahydrofolate reductase (MTHFR) provides methylTHF for the conversion of HCY
to methionine via MS, and polymorphisms in MTHFR have been reported to be increased in autism\textsuperscript{60,61}. Mutations in this gene are also associated with schizophrenia. This polymorphism results in methylTHF deficiencies and as a result, a decrease in methylation capabilities.

Multiple studies provide evidence for oxidative stress in autism\textsuperscript{62}. Plasma levels of GSH are abnormally low in autistic children\textsuperscript{8,62-64}, coinciding with elevated plasma levels of inflammatory cytokines and increased biomarkers for lipid peroxidation\textsuperscript{7,65-68}. There is also evidence for a decreased capacity to methylate DNA in autism, as seen by altered methionine cycle metabolites and chronic elevation of SAH in human lymphoblasts\textsuperscript{63,69}. Moreover, inflammation-associated cytokines are significantly elevated in postmortem brain samples from autistic subjects\textsuperscript{70}, raising the possibility that cortical MS status might be altered by oxidative stress in autism. Together this data leads to the conclusion that autistic children are more vulnerable to oxidative stress and have an altered methylation cycle (Table 1).

<table>
<thead>
<tr>
<th>Table 1: Autistic children are vulnerable to oxidative stress as measured in plasma metabolites. Autistic children have large decreases in cysteine and GSH as compared to controls, implying an altered methylation cycle and transsulfuration pathway. Table from James et al.\textsuperscript{62}</th>
<th>Control Children</th>
<th>Autistic Children</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine (μmol/L)</td>
<td>30.6 ± 6.5</td>
<td>19.3 ± 9.7</td>
<td>0.001</td>
</tr>
<tr>
<td>SAM (nmol/L)</td>
<td>90.0 ± 16.2</td>
<td>75.8 ± 16.2</td>
<td>0.01</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>20.1 ± 4.3</td>
<td>26.1 ± 5.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>6.3 ± 1.2</td>
<td>5.4 ± 0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Adenosine (μmol/L)</td>
<td>0.28 ± 0.16</td>
<td>0.39 ± 0.19</td>
<td>0.05</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>210 ± 18.5</td>
<td>163 ± 14.6</td>
<td>↓25%</td>
</tr>
<tr>
<td>Total glutathione (μmol/L)</td>
<td>7.9 ± 1.8</td>
<td>4.1 ± 0.5</td>
<td>↓45%</td>
</tr>
<tr>
<td>Oxidized Glutathione (nmol/L)</td>
<td>0.3 ± 0.1</td>
<td>0.55 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>GSH/GSSG Ratio</td>
<td>25.5 ± 8.9</td>
<td>8.6 ± 3.5</td>
<td>↓3-fold</td>
</tr>
</tbody>
</table>
There are two consequences to having an altered methionine cycle: (1) a decrease in methylation capacity, due to lower SAM levels and (2) lower antioxidant capabilities, due to lower GSH levels. Methylation is required for normal development and impairments in methylation capabilities may play a role in autism.

The prevalence of polymorphisms involving methylation in autism has also been investigated. These genetic risk factors include transcobalamin II, reduced folate carrier, catecholamine-O-methyltransferase (COMT), MTHFR and glutathione-S-transferase M1 and single nucleotide polymorphisms (SNPs) affecting these proteins occur with higher frequency in autistic subjects. Parents of autistic children not only carry these risk genes, but also exhibit metabolic patterns indicative of oxidative stress and impaired methylation.

H. Alzheimer’s Disease (AD)

AD is characterized by relatively late onset, 65 years or older, and loss of short-term memory, with around 5.3 million Americans living with the disease (Alzheimer’s Association, 2010). β-amyloid (Aβ) plaques, along with neurofibrillary tau tangles, are two major features of AD pathology in the cerebral cortex. The tau protein becomes hyperphosphorylated in AD, leading to formation of tau tangles. Tau proteins are responsible for stabilizing microtubule networks, which make up the cytoskeleton of cells. The pairing of tau leads to disintegration of the cytoskeleton and interferes with the transport of proteins, nutrients and molecules.

Aβ is formed by the cleavage of the amyloid precursor protein (APP), which is a transmembrane protein of unknown function. APP can be processed by α-, β-, and γ-secretases, and APP formation requires sequential cleavage using β-secretase, followed by γ-secretase. The first cleavage releases a soluble fragment, while the second cleavage releases the Aβ. Cleavage
via γ-secretases generates peptides between 39-42 amino acids long, with Aβ40 being the most common and Aβ42 being the most susceptible to conformational changes, which leads to plaque generation\textsuperscript{74}.

Most cases of AD do not have a genetic inheritance; however, some cases of AD are caused by sporadic mutations. Mutations within APP and presenilin, the sub-component of γ-secretase, are associated with increased production of Aβ42 and early-onset familial AD\textsuperscript{75}. Additionally, inheritance of the apolipoprotein E4 (ApoE4) allele has been reported to be a risk factor in 50\% of late onset cases\textsuperscript{76}. Between 40 and 80\% of AD patients possess at least one ApoE4 allele\textsuperscript{77}. The MS polymorphism A2756G, located on the carboxy-terminal portion of the SAM domain, is also a risk factor for AD\textsuperscript{78}. Additional studies have found elevated plasma levels of HCY, which further establishes and implicates a decrease in MS activity, in AD patients\textsuperscript{79-81}. The increase in HCY was age-dependent in AD patients. Lastly, 4-Hydroxynonenal (4-HNE), a lipid peroxidation metabolite and indicator of oxidative stress, was elevated in the blood serum of AD patients, compared to age-matched elderly controls\textsuperscript{9} (Fig. 13). Increased CSF levels of HCY suggest broad involvement of oxidative stress and impaired methylation in neurological and neuropsychiatric disorders\textsuperscript{82}.
I. Aging and Oxidative Stress

The free radical theory of aging, put forth by Denham Harman in the 1950’s, suggests that accumulation of free radicals and reactive oxygen species (ROS) outweighs the cell’s antioxidant capabilities and contributes to the process of aging. It has been well documented that levels of oxidative stress increase as a natural part of the aging process and, in addition, are a hallmark feature of autism and neurological disorders.

Plasma thiols, including HCY and GSH were measured via HPLC in 41 healthy individuals ranging from 21 to 92 years of age. With aging, there was a trend for elevated levels of HCY and decreased levels of GSH, suggesting a dysfunction within the transsulfuration pathway. A growing body of literature implicates ROS buildup as a primary factor in aging.

In a study using male F344 rats and C57BL/6 mice, multiple tissues, including brain and kidney, showed elevated levels of oxidative damage, as measured by 8-OHdG, over the lifespan. In all animals tested, levels of ROS increased as a function of the animal’s age with...
data points taken at 6, 18 and 24 months (Fig. 14). From that same study, a group of rats maintained on a calorie-restricted diet showed lower levels of oxidative stress than their non-calorie-restricted cohorts. Studies in non-human primates have also shown age-related increases in oxidative stress and other associated aging pathologies\textsuperscript{91-93}.

![Figure 14: Age-dependent increases in oxidative stress in rat brain and kidneys.](image)

**Figure 14: Age-dependent increases in oxidative stress in rat brain and kidneys.** Open diamonds indicate rats fed a normal diet; closed squares indicate rats fed a calorie-restricted diet. Figure from Hamilton et al.\textsuperscript{90}.

There are also studies that have investigated the use of antioxidants on the aging process. In one study, transgenic catalase-expressing mice showed an increase in life-span by about 20%, and levels of oxidative damage, as measured by 8-hydroxydeoxyguanosine (8-OHdG), and hydrogen peroxide ($\text{H}_2\text{O}_2$) were attenuated in comparison to their littermate controls\textsuperscript{14}.

A *C. Elegans* mutant for increased lifespan, *age-1*, was shown to have elevated levels of SOD, as well as enhanced resistance to $\text{H}_2\text{O}_2$\textsuperscript{16}. The *C. Elegans* mutant *mev-1*, which normally encodes cytochrome b of the mitochondrial transport chain, has higher levels of ROS, since mutations within this complex alter electron flow within the mitochondrial transport chain. The *mev-1* mutant was hypersensitive to oxidative stress and increases in oxygen markedly decreased the life expectancy of these mutants\textsuperscript{94}. The common theme amongst these animal models is
elevated levels of oxidative stress with advancing age and the ability to combat that detriment with antioxidant therapies. This evidence illuminates an oxidative stress-induced aging hypothesis that can be seen from invertebrates through humans\textsuperscript{95}.

Elevated levels of ROS can create a redox imbalance, stimulating a series of events that may lead to cellular death and dysfunction and contribute to overall aging. Lower levels of MS or decreased activity of the enzyme may contribute to an increase in GSH synthesis. Lack of the circadian rhythm protein \textit{Bmal-1} increases ROS and leads to premature aging in mice\textsuperscript{96}. Kondratov et al. found that continuous administration of the antioxidant N-acetyl-cysteine (NAC) increased the lifespan of \textit{Bmal-1} knockout mice (Fig. 15). NAC provides cysteine to the cell, which is critical for the formation of GSH.

![Figure 15: Continuous administration of NAC increases lifespan of \textit{Bmal-1/-} mice. Kaplan-Meyer survival curves were obtained for WT mice raised on regular (closed circles) or NAC-supplemented (open circles) water; and \textit{Bmal-1/-} mice raised on regular (closed triangles) or NAC-supplemented (open triangles) water. NAC significantly increased lifespan of \textit{Bmal-1/-} mice (\(p = 0.022\)). Figure from Kondratov et al.\textsuperscript{96}.](image)

\textbf{J. D4 Receptor-Mediated Methylation and Schizophrenia}

The D4 dopamine receptor possesses the unique activity of phospholipid methylation (PLM)\textsuperscript{97}. PLM, stimulated by dopamine, can increase membrane fluidity by transferring methyl
groups to the phospholipid phosphatidylethanolamine (PE), resulting in its conversion to methylated derivatives. D4 receptor stimulation also increases MS activity in SH-SY5Y cells\textsuperscript{35,98}. Within the D4 receptor, Met313 can be adenosylated, creating a methyl-donating SAM residue at this location at the inner membrane surface\textsuperscript{99} (Fig. 16). Phospholipids can be methylated either by SAM or by the S-adenosylated D4 receptor, and PLM shares the same MS-dependent methylation pathway (Fig. 10, blue text; Fig. 17). However, whereas the methionine cycle uses methionine as its methyl donor, dopamine-stimulated PLM requires methylTHF and MS activity. Therefore, a reduction in MS activity because of oxidative stress would decrease the process of dopamine-stimulated PLM. Dopamine-stimulated PLM allows for 20-50 methylations/sec/receptor, which allows dopamine to have local effects on membrane-bound proteins\textsuperscript{100}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{d4-receptor-structure.png}
\caption{D4 dopamine receptor structure. The D4 dopamine G-protein-coupled receptor is able to participate in the methylation cycle due to conformation-dependent adenosylation of the methionine residue at position 313 (MET 313), located in helix 6\textsuperscript{100}.}
\end{figure}
Figure 17: D4 receptor-mediated phospholipid methylation (PLM). D4 participates in the normal methylation pathway as previously described. D4SAM donates its methyl group to the phospholipid membrane, affecting membranes and proteins.

Over 2 million Americans are affected with schizophrenia, a chronic psychiatric disease, which accounts for about 1% of the population (National Institute of Mental Health). Initial symptoms, the most predominant being hallucinations and delusions, are usually present during the patient’s early 20’s. The dopamine receptor antagonist, clozapine, has been shown to bind preferentially to the D4 receptor\textsuperscript{101}. In addition, D4 receptors are elevated in the brain tissue of schizophrenic patients\textsuperscript{102}.

Additionally, it has been shown that GSH levels are decreased in the CSF, prefrontal cortex in vivo, as well as in post-mortem striatum tissue, from schizophrenic patients\textsuperscript{11-13}. Activity of the enzyme CGL is decreased in schizophrenic patients, implicating dysregulation in the transsulfuration pathway\textsuperscript{103,104}.

Offspring exposed to the Dutch Hunger Winter or Chinese famine, during early gestation, are associated with an increased risk in neural tube defects (NTD) and schizophrenia\textsuperscript{105-108}. Folate is most prevalent in leafy green vegetables and fortified cereals and grains, and intake of this important vitamin can be compromised during famines. NTD are associated with a lack of
folate, highlighting a possible role of folate dysregulation in schizophrenia. Additionally, polymorphisms in MTHFR, C677T and A1298C, are implicated in birth defects (spina bifida) and folate deficiency (NTD), implicating a potential glitch in the methylation/transsulfuration pathways in these disorders.

Studies have shown that folate-dependent metabolism of HCY is decreased in schizophrenic patients, with lower levels of plasma folate\textsuperscript{105,106}. This evidence, coupled with the D4 receptor’s ability to perform PLM, illuminates an oxidative stress hypothesis for schizophrenia. It is hypothesized that alterations in membrane fluid properties, through dopamine PLM, may also alter the activity of membrane proteins and, as a result, shift synchronized firing of neural networks, contributing to the etiology of schizophrenia\textsuperscript{109}. Other methylation-related processes, such as epigenetic regulation of gene expression, may also play a role.

Lower levels of GSH predispose the nervous system to oxidative stress and an inability to maintain redox status. As previously mentioned, elevated levels of oxidative stress metabolites have been found in autistic and neurodegenerative disease patients, reinforcing the hypothesis that MS status is altered in neurological disorders.

K. Methylation Status and Gene Transcription/Silencing

An important aspect of the methionine cycle is its ability to methylate cellular targets such as DNA via DNA methyltransferases (DNMTs), with an ultimate effect on gene transcription. SAM is the major methyl donor in the aforementioned reactions. The methyl group attached to the sulfur atom of SAM is reactive and easily donated to an acceptor during a methylation reaction. Inactivated MS is unable to methylate HCY to methionine; thus, HCY accumulates and since the reaction between HCY and SAH is reversible, levels of SAH
accumulate as levels of SAM decrease. Elevated SAH levels inhibit DNA methylation, which normally leads to gene inactivation\textsuperscript{110}. Methylation plays an important role in many cellular processes, one being development during the embryonic period. Normal development depends on epigenetic regulation to turn genes on through hypomethylation and turn genes off through hypermethylation.

Methylation, which allows for tissue specific gene expression, X chromosome inactivation in females, as well as promoting chromosomal stability, most often occurs at CpG sites (cytosine-phosphate-guanine), meaning that a guanosine follows a cytosine in the linear DNA sequence. DNMTs are able to use a donated methyl group from SAM and transfer it to DNA, methylating a cytosine to 5-methylcytosine. Mammals methylate between 70\%-80\% of CpG cytosines\textsuperscript{111}. Large regions of CpG sites (around 300-3,000 bp) are termed CpG islands and 70\% of the time occur in or around the human promoters of genes\textsuperscript{112,113}. Changes in methylation patterns can therefore alter gene transcription.

There is evidence for a decreased capacity to methylate DNA in autism as seen by altered methionine cycle metabolites, and chronic elevation of SAH in human lymphoblasts reduces methylation\textsuperscript{63}. In addition, autistic children have a decrease in the GSH/GSSG ratio, implying a vulnerability to oxidative stress. Elevated levels of oxidative stress cause metabolic pathways to shift in favor of GSH synthesis at the expense of methylation and chronic oxidative stress will deplete GSH and ultimately decrease its synthesis. Abnormal methylation capabilities, via a reduction in SAM levels or an elevation in SAH levels, can negatively alter patterns of gene methylation.

One study has shown low levels of SAM in AD brains versus matched controls, implying an altered methionine metabolism cycle\textsuperscript{114}. Lower levels of SAM imply decreased methylation,
which can lead to gene activation. It is possible that reduced SAM levels in AD patients cause hypomethylation, which may increase gene transcription of proteins such as tau or Aβ, key candidates for AD pathology. SAM is also involved in the regulation of acetylcholine, of which lower levels are associated with AD\textsuperscript{115}. In addition, a study conducted in mice with reduced folate levels found increased presenilin expression, γ-secretase activity and Aβ levels, all enzymes and proteins associated with AD\textsuperscript{116}. Treatment with SAM prevented the increase of the aforementioned AD-related components. This information highlights low folate levels as a potential dietary risk factor for AD and reinforces the importance of the methionine cycle in neuronal metabolism and homeostasis.

L. Exon Skipping and Alternative Splicing

Transcription is the process in eukaryotic genomes where DNA is copied using a RNA polymerase to produce a RNA complimentary strand, termed pre-mRNA. Pre-mRNA goes through the process of basic splicing in order to remove non-coding introns, and then the remaining coding exons are ligated (Fig. 18). The pre-mRNA is then translated into a protein outside the nucleus.

![Diagram of simple splicing](http://en.wikipedia.org/wiki/RNA_splicing)

**Figure 18: Diagram of simple splicing.** Pre-mRNA is spliced such that the final product contains no introns (blue) and only exons (red) (http://en.wikipedia.org/wiki/RNA_splicing).
The splicing mechanism depends on three intronic mRNA elements: the 5' GU, the 3' AG and (A) branch site. Small nuclear ribonucleoproteins (snRNPs) combine with each other and the pre-mRNA to form the spliceosome complex. It is thought that the snRNP U1 binds to the intronic 5'GU and the snRNP U2 binds to the (A) branch site (Fig. 19).

Additional snRNPs associate with each other (U4, U5, and U6) and bind to either U1 or the branch site area. A chemical attraction then causes the U456 snRNP complex to associate with U1, and U1 dissociates from the mRNA. The U456 complex then binds with the U2 snRNP causing a loop to form termed the ‘lariat’ structure. The lariat structure is then cleaved at the 3' AG site. U5 helps in ligating the separated exons together (Fig. 19).

**Figure 19:** The spliceosome complex forms a lariat structure, which is cleaved. The spliceosome consists of several proteins that are recruited to the pre-mRNA to excise introns. Introns contain a 5' GU and end with a 3' AG. Specific proteins recognize GU and (A) branch sites to form a lariat structure, which is subsequently cleaved. Figure from Cooper et al.¹¹⁷.
Alternative splicing differs from normal splicing in that not only non-coding introns are spliced out, but also exons depending on alterations in the mRNA sequence. Alternative splicing is mediated by a change in coding at the sites of normal splicing, such that the normal 5’ GU, 3′ AG and (A) branch sites are altered, and spliceosome machinery can no longer bind and carry out the normal processes. Alternative splicing exists in five basic forms: exon skipping, mutually exclusive exons, intron retention, and alternative donor and acceptor sites\textsuperscript{118-120}. The most common of these mechanisms is exon skipping, with new therapeutic techniques utilizing this method for diseases such as Duchenne muscular dystrophy (DMD).

In these methods, mRNA is engineered correctly to express the target protein through exon skipping\textsuperscript{121}. DMD arises from a lack of the protein dystrophin, which is necessary for muscle movements and function\textsuperscript{122,123}. In DMD, mutations disrupt the open-reading frame (ORF) of DMD, resulting in the absence of dystrophin and manifestation of the disease. It is proposed that diseases caused by frame-shift mutations, such as DMD, can be aided through restoration of the mRNA sequence downstream of the mutation, to its proper ORF\textsuperscript{124,125}. Targeted oligonucleotides to these regions can mask the spliceosome’s ability to target exons and initiate exon skipping of the problem area. This process allows the out-of-frame mRNA to be modified in such as way that the proper protein can be produced.

The process of alternative splicing results in the formation of new isoforms (Fig. 20). Protein A is the result of inclusion of all normally encoded exons, whereas exon 3 is alternatively spliced out of the transcript in protein B, resulting in a shortened protein (missing loop). Protein C is missing exon 4, resulting in yet another protein structure.
There are many implications of abundant mRNA editing in humans. For example, alternative splicing of the pre-mRNA can give rise to several new protein products, expanding the relatively small human genome size when compared to *Arabidopsis* and corn (Fig. 21).

**Figure 20:** Alternative splicing of a protein yields three variant proteins. Exon skipping promotes formation of proteins A, B and C. (http://commons.wikimedia.org/wiki/File:DNA_alternative_splicing.gif).

**Figure 21:** Genome sizes across species. The human genome has around 25,000 genes. Alternative splicing amplifies gene diversity. Figure adapted from web-source data (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/G/GenomeSizes.html).
M. TNFα and Inflammation

TNFα is a pro-inflammatory cytokine, able to modulate expression of various downstream transcription factors. TNFα binds to tumor necrosis factor receptor 1 (TNFR1), which is found in most tissues, including the brain. Oxidative stress is an underlying cause of inflammation, both of which are implicated in aging. In addition, TNFα levels are elevated in both post-mortem cortex and CSF of autistic subjects.

Activation of TNFR1 by TNFα is able to modulate activity of transcription factors NF-κB and AP-1, via the MAPK pathway (Fig. 22). NF-κB regulates the transcription of a wide variety of proteins involved in cell survival and proliferation, apoptosis and inflammation and there is evidence that redox alterations activate NF-κB. There are also numerous reports that identify TNFα-mediated events as beneficial. In addition, activation of Akt, via PI3K, increases the nuclear binding of NF-κB subunits.

In addition to macrophages, both neurons and astrocytes have the ability to secrete TNFα in response to cellular stimuli, such as the cytokines IL-1 and IFN-γ. These cytokines are known to be present in neurological disorders associated with inflammation, such as AD. Thus, TNFα plays a role in augmenting levels of inflammation.

Oxidative stress can lead to an increase in ROS, resulting in protein oxidation, lipid peroxidation and DNA damage. TNFα cytotoxicity is mediated through apoptosis, which is carried out by caspase signaling. Activation of caspase 3 leads to a release of cytochrome c from mitochondria, further activating apoptotic machinery (Fig. 22).
Figure 22: TNFα modulates various signaling cascades. TNFα binds to TNFR1. Downstream cascades modulate apoptosis, cell survival and proliferation, and inflammation. Transcription factors NF-κB and AP-1 can translocate to the nucleus and regulate mRNA transcription (http://en.wikipedia.org/wiki/Tumor_necrosis_factor-alpha).
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 in six-well culture dishes at 10⁶ cells/well in 2.5 mL of media and incubated for 24 hours.

HepG2 human hepatocarcinoma cells were purchased commercially from ATCC®. Cells were maintained using the above protocol, but cultured in E-MEM supplemented with 1% antibiotics and 10% FBS. Cells were grown in multiple layers in 25 cm² flasks.

LN-18 human glioblastoma cells were received from Dr. Campbell’s lab (available commercially from ATCC®). Cells were maintained using the above protocol, but cultured in D-MEM supplemented with 1% antibiotics and 10% FBS. Cells were grown as monolayers in 25 cm² flasks.

For all cell lines, experiments utilized cells between 10-80 passages, with no significant differences in results.

B. Samples

Human cortex tissue samples, including material from autistic patients, AD subjects and age-matched controls, were obtained through the Autism Tissue Program (ATP), the Australian
Brain Bank Network (ABBN) and the Stanley Medical Research Institute (SMRI). Additional samples (cDNA) were purchased from BioChain Institute, Inc.® and Invitrogen™. In total, 10 autistic samples were used with ages ranging from 4 to 30. Other experiments used a total of 41 control samples with ages from 28 weeks to 83 years. If samples were received as tissue, then RNA was isolated using the RNAqueous® -4PCR Kit from Ambion®. These samples were treated with DNase to remove any trace DNA contamination. Full sample clinical details are available in the Appendix (Table 2). The project was carried out with the approval of the Institutional Review Board of Northeastern University.

C. 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. RNA integrity was examined by running samples out on a denaturing (formaldehyde) agarose gel.

D. 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 about 20-24 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 appearance and validated in qRT-PCR studies; therefore, the
housekeeping gene GAPDH is an appropriate internal and loading control. Domain-specific primers are shown in Fig. 23.

**Figure 23: Location of MS domain primers.** Human MS contains 33 exons specifying the five domains in a sequential manner. Panel shows original primers to all domains. HCY, exons 1-11, pink; FOL, exons 12-18, green; CAP, exons 19-21, yellow; COB, exons 22-25, red; SAM, exons 26-33, blue. All primer products are listed in base pairs (bp).

E. *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.

F. 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 ΔΔCt method and normalized to GAPDH.

G. DNA Sequencing

For sequencing, PCR products were run on 1-3% agarose gels, depending on product size, for 90 minutes at 100 V and 10-18 mA. After staining, fragments were excised and purified using the MinElute® Gel Extraction Kit from Qiagen®. Samples were sequenced by GENEWIZ®. DNA template (5-20 ng) and primers (8 pmol) were pre-mixed in 0.2 mL PCR tubes, with a total volume of 12 µL. Results were displayed online and further analyzed using the online tool BLASTn from NCBI.
H. **Western Blots**

Antibodies to human MS were developed by Dr. Tapan Audyha (Vitamin Diagnostics Inc.) and were directed against amino acids 49-69 in the HCY-binding domain (R-3-8W).

Confluent 10 cm plates of SY5Y cells were washed twice with PBS and then lysed with buffer. Cells were scraped and placed in microcentrifuge tubes. These were centrifuged at 14,000 x g for 10 minutes and then the supernatant was transferred to a clean microcentrifuge tube. 100 µL of supernatant was saved in order to measure protein content in the sample and 20-25 µg of protein was loaded onto a 10% Tris-glycine gel and separated by SDS-PAGE electrophoresis. Standard methods of gel electrophoresis and western blotting were carried out. The membranes were blocked by 5% milk and probed by a polyclonal antibody generated towards an epitope in the HCY domain of MS and detected by alkaline phosphatase method. All stock reagents for gel electrophoresis and western blotting were obtained from Boston Bioproducts, Inc. Immunoreactive bands were visualized using a Kodak imager. The calculated molecular weight of MS is 140 kDa.

I. **Lowry Protein Assay**

Protein concentrations were determined by using the modified Lowry method for protein quantification using bovine serum albumin (BSA) as the standard.\(^\text{137}\)

J. **TNFα and NAC Treatments**

Cells were treated with either TNFα (30 ng/mL) or NAC (5 mM) for oxidant/antioxidant experiments. Treatments were carried out using either 6-well cell culture plates or 10 cm
standard round dishes. Short-term time-points for both TNFα and NAC included 0.5, 0.75, 1, 2, and 4 hours. Longer time-points for both treatments included 6 and 24 hours.

K. **Thiol Determination Using High Performance Liquid Chromatography (HPLC)**

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 dH$_2$O was added to each well. Cells were scraped from the dish and suspended in dH$_2$O. 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 PCA 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.

**HPLC Conditions:**

- **Column:** Agilent Eclipse XDB-C8 (3 x 150mm; 3.5 µm)
- **Guard Column:** Agilent Eclipse XDB-C8 (4.6 x 12.5mm; 5 µm)
- **Mobile Phase A:** 0% Acetonitrile, 25 mM Sodium phosphate, 1.4 mM 1-Octanesulfonic acid, adjusted to pH 2.65 with phosphoric acid
- **Mobile Phase B:** 50% Acetonitrile
- **Flow rate:** 0.6 mL/min
- **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
Temperature: 27°C
Injection volume: 10 µL

Detector and Conditions:
Detector: Electrochemical Detector, ESA CoulArray with BDD Analytical cell Model 5040
Operating Potential: 1500 mV

L. Cysteine Uptake

SH-SY5Y human neuroblastoma cells were plated in six-well standard tissue culture plates 24 hours before the assay. Confluent cells were treated with 30 ng/mL of TNFα 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 radiolabelled 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 Swanson138.
M. Methionine Synthase Activity

SH-SY5Y human neuroblastoma cells were plated in 10 cm standard tissue culture dishes 24 hours before the assay. Confluent cells were treated with 30 ng/mL of TNFα for various time-points or using standard conditions. Cells were washed 2x with ice-cold PBS and then lysed with 1500 µL of water and scraped. Samples were sonicated on ice and 100 µL from each sample was saved for the Lowry protein assay. Samples were centrifuged at 4°C and 10,000 x g for 10 minutes. The collected supernatant was used for the assay.

Assays were performed under anaerobic conditions, by bubbling nitrogen gas through stoppered vials for 1 hour. The reaction mixture for each sample contained the following: 100 mM potassium phosphate, 500 µM homocysteine, 152 µM S-adenosylmethionine, 2 mM titanium citrate, 250 µM (6R,S)-5-14 methyl-THF and 5 mM methylcobalamin in a final volume of 500 µL. The reaction was initiated by addition of the radioactive methyltetrahydrofolate (\(^{14}\text{CH}_3\)-THF), followed by an incubation of 37°C for 1 hour, under nitrogen gas. A dilute \(^{14}\text{CH}_3\)-THF solution was made by adding 1.3 µL of radioactivity to 1 mL of HBSS.

The reaction was terminated by placing samples in a 98°C water bath for 2 min. Samples were then uncapped and cooled on ice. \(^{14}\text{C}\)Methionine was separated from un-reacted \(^{14}\text{CH}_3\)-THF by passing the reaction mixture through an anion exchange column of Dowex 1-X8 (chloride form). \(^{14}\text{C}\)Methionine was then eluted from the column with 2 mL of water, and aqueous samples were collected in scintillation vials. Before measuring radioactivity, 7 mL of scintillation fluid were added to each vial. \(^{14}\text{C}\)Methionine formation was measured using a Perkin Elmer scintillation counter. Control samples, without cell extracts, were used as blanks. All reported radioactivity values were corrected for the counts observed in control assays.

MS activity was expressed as pmol / min / mg protein and was calculated as follows:


(Sample CPM-control CPM / specific activity (nmol) x (total assay volume / sample volume) x (protein value / 1000)). This protocol is based on the method developed by Banerjee et al.\cite{95,139,140}.

N. Molecular Modeling

Molecular modeling of folate- and cobalamin-binding domains was carried out using the molecular-graphics and -modeling program YASARA™. Protein data bank (PDB) files were downloaded from the RCSB Protein Data Bank at this website address: http://www.pdb.org. Specific papers that generated PDB images are referenced in the appropriate figures.

O. 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. Non-linear regressions used a two-phase exponential decay function. Comparisons between multiple groups of data were conducted using one-way analysis of variance (ANOVA) and Tukey’s post-hoc test was used to determine the differences between individual groups.
III. RESULTS

A. MS Domain Status in Cultured Cells

Original PCR investigations of MS involved the SAM-binding domain, due to data indicating a smaller molecular weight protein in SH-SY5Y cells versus HepG2 cells (Banerjee and Deth, unpublished). It was hypothesized that removal of the SAM-binding domain (either at the mRNA or protein level) could provide an adaptive response of sulfur metabolism to oxidative stress, and since MeCbl-dependent MS activity was demonstrated after removal of the SAM-binding reactivation domain, I examined the possibility that the SAM domain might be deleted at the mRNA level.

Domain-specific primers were designed to each MS domain in order to investigate the possibility that MS might be modulated at the mRNA level (Fig. 23). Full primer sequences can be found in the Appendix (Table 1).

SH-SY5Y cells showed expression of only four MS domains: homocysteine-binding (HCY), folate-binding (FOL), cobalamin-binding (COB) and S-adenosyl methionine-binding (SAM) (Fig. 24a). RT-PCR studies showed that the SAM-binding domain was present (Fig. 24a); however, there was a clear and surprising lack of cap domain PCR product (288 bp). Upon running additional PCR product in the gel, there was a weak full-length cap domain product, along with a smaller cap domain product (125 bp), suggesting possible alternative splicing (Fig. 24b). Under the same conditions, full-length cap product was robustly detected in RNA from human lymphoblasts, along with the smaller product (Fig. 24b). Sequencing of the full-length PCR product confirmed its expected composition (exons 19-21), while the smaller PCR product contained only exon 21 (which corresponds to about 125 bp).
This finding suggested cell-specific differences in SH-SY5Y MS mRNA composition and splicing, as compared to lymphoblasts. Thus exons 19 and 20 (cap domain) are absent from the majority of transcripts in SH-SY5Y cells, while present in lymphoblasts.
Figure 24: Domain-specific PCR studies in MS mRNA from SH-SY5Y cells. (a) Domain-specific RT-PCR with SH-SY5Y cell-derived RNA indicates absence of cap domain products. The expected PCR product size for HCY is 187 bp; FOL 197 bp; CAP 288 bp; COB 122 bp, and SAM 300 bp. (b) PCR products with twice as much SH-SY5Y product and human lymphoblast RNA. 288 bp product includes exons 19-21; 125 bp product includes only exon 21.
B. Cell-Specific Differences

The absence of cap domain exons in SH-SY5Y cells prompted further examination of MS domain status in various cultured cell-types, since such differences could indicate a unique redox strategy for neuronal cells versus non-neuronal cell types. Human embryonic kidney cells (HEK) and hepatocarcinoma cells (HepG2) were examined in addition to SH-SY5Y cells. Quantitative real-time PCR studies (qRT-PCR) were performed using the aforementioned cultured cell-types using primers to exons 19-20 (cap) and 24-25 (cobalamin).

Whereas SH-SY5Y cells express around 50% less cap domain (exons 19-20) than cobalamin-binding domain (exons 24-25), HepG2 cells have a cap/cobalamin ratio of around 1:1 (Fig. 25), which is the same ratio seen in lymphoblasts. HEK cells also express significantly more cap mRNA than SH-SY5Y cells and have a cap/cobalamin ratio of about 1:1.
Figure 25: SH-SY5Y cells have lower levels of MS cap domain mRNA than HEK and HepG2 cells. n=2, with 3 replicates in each qRT-PCR experiment. *= p < 0.05 compared to cobalamin (COB) for SH-SY5Y cells.
C. Characterization of MS in Human Cortex

After investigating MS domain status in cell cultures, I sought to investigate MS domain status in human cortex. Fig. 26 shows MS fingerprints from two human cortex samples: a young control (age 24) and an aged control (age 80). It was interesting to note the presence of the smaller product (125 bp) in the cap primer lane for the 24 year-old sample (Fig. 26, left). This smaller fragment, a potential splice variant of the cap domain, was also seen in SH-SY5Y cells and lymphoblasts. The expected PCR products for all five domains were visualized from a 24 year-old subject; however, the cap domain was absent from an 80 year-old subject (Fig. 26, right).
**Figure 26: Domain-specific PCR studies in MS mRNA from human cortex.** Domain-specific PCR products from 24 year-old (left) and 80 year-old subjects (right). The young control has a present cap domain and a smaller cap-derived product (125bp). The cap domain is absent in the aged control. Equal concentrations of samples were run on 10% TBE gels.
D. Age-Dependent MS mRNA Status in Human Cortex

To further explore the unexpected finding of alternative splicing, I utilized qRT-PCR to evaluate the level of cobalamin-binding and cap domain exons in cortical mRNA from subjects (n = 41) across the lifespan (28 weeks of fetal gestation to 84 years).

Using primers directed toward either the cobalamin-binding domain or the cap domain, I observed a striking age-dependent decrease in MS mRNA levels across the lifespan (Fig. 27, left and right). In both cases, qRT-PCR data was best fit to a two-component exponential decay function yielding fast and slow phase $T_{1/2}$ values of 3.4 and 29.4 yrs ($R^2= 0.91$), and 2.2 and 20.0 yrs ($R^2= 0.94$) for cobalamin-binding and cap domains, respectively. The decrease from fetal to 84 years amounted to 400-fold and 3,340-fold for cobalamin-binding and cap domains, respectively.

This remarkable decrease is likely to primarily reflect a progressive decrease in transcription, since a similar pattern was observed for both cobalamin-binding and cap domains. However, it is also possible that MS mRNA is degraded at a faster rate with increasing age, which could lead to a decrease in transcript levels.

To further evaluate age-dependent differences in MS cobalamin-binding and cap domain mRNA, we analyzed data in two categories: $\leq 20$ yrs vs. $\geq 60$ yrs. The average cobalamin-binding and cap domain mRNA values for subjects $\leq 20$ (n=9) were 163.7 $\pm$29.9 and 164.1 $\pm$ 67.0, respectively, indicating that transcripts contained both domains (Fig. 28). However, for subjects $\geq 60$ yrs (n=7) the average values for cobalamin-binding and cap domain mRNA were 1.95 $\pm$ 1.02 and 0.154 $\pm$ 0.69, respectively, indicating the presence of transcripts containing cobalamin-binding domain without cap domain. The cap/cobalamin ratio average for samples under the age of 20 was 0.9997, but for samples over the age of 60, the ratio average was much lower (0.2268)
(Fig. 28). The difference between these values was highly significant ($p < 0.0001$), indicating a greater age-dependent decrease in cap domain, as compared to cobalamin-binding domain. This lower cap/cobalamin ratio in aged samples highlights an alternative splicing mechanism for MS, in which one or more cap domain exons is deleted.

A visual comparison of PCR products from a young subject (age 4) illustrates the 1:1 cap/cobalamin ratio (Fig. 29). A sample from a middle-age subject (age 49) shows a marked decrease in both domains, suggesting a decrease in transcription. However, in a sample from an aged subject (age 84), both domains are decreased compared to both the 4 and 49 year old samples, and the ratio of cap/cobalamin is no longer 1:1. The cap domain is barely visible, in contrast to the cobalamin-binding domain.
Figure 27: Age-dependent decrease in MS cobalamin-binding and cap domain mRNA. qRT-PCR was performed on samples with ages ranging from 28 weeks to 84 years-old. Primers to the cobalamin-binding and cap domains were used. Data were fit to a two-phase exponential decay ($R^2=0.91$, left; $R^2=0.94$, right; n=41).
Figure 28: Age-dependent splicing of the cap domain in subjects >60. The bar graph represents the average ratio (cap/cobalamin) for the <20 population (n=9) and the >60 population (n=7), $p < 0.0001$. 
Figure 29: Age-dependent decreases in PCR products from probed cobalamin-binding and cap domains in normal cortex. Cap/cobalamin ratio in the youngest subject (age 4) was 1:1. The ratio decreases as subject age increases. Equal concentrations of samples were run on 10% TBE gels.
E. MS mRNA Status in Neurological Diseases

1. Autism

Oxidative stress is a hallmark feature of many neurological disorders such as autism, AD and schizophrenia. Plasma levels of GSH are decreased in autistic subjects, with the GSH/GSSG ratio being significantly reduced in conjunction with a lower SAM/SAH ratio. These traits are indicative of oxidative stress and impaired methylation, and raise the possibility that cortical MS status might be altered by oxidative stress in autism.

To investigate this possibility, we performed qRT-PCR on post-mortem cortical samples from autistic subjects and their age-matched controls, using primers directed at the cobalamin-binding and cap domains. Additional clinical details for the subjects can be found in the Appendix (Table 3).

Subjects were between the ages of 4 and 30 years-old, with n=10 for each group. Average MS mRNA levels for both cobalamin-binding and cap domains were significantly less in autistic samples compared to their paired controls (p < 0.03) (Fig. 30), indicating a decrease in MS transcription. The cap/cobalamin ratio in autistic samples was not significantly different from the control ratio, which indicates decreased transcription levels, but not alternative splicing, since both PCR products were decreased compared to controls.

Control samples showed a clear age-dependent decrease in both domains (Fig. 31); however, levels of both domains in autistic samples were decreased relative to control values, especially at early ages. Control samples were best fit to a one-phase exponential decay, $R^2=0.94$, $T_{1/2}=2.7$ years, but autistic samples were best fit by a straight line, with a slope not different from zero. Cobalamin-binding domain data showed a similar pattern to the cap domain data ($R^2 = 0.96$ for cobalamin, $T_{1/2}=2.3$ years). These results support literature reports that
autistic patients have altered methionine metabolism and elevated levels of oxidative stress, and suggest that down-regulation of MS transcription may be an adaptive response to oxidative stress.

A closer look at the paired data confirmed that the greatest deficit in autistic MS mRNA was in the youngest subjects (Fig. 32). PCR products were also visualized on a gel, further illuminating the autism-dependent decrease in cap domain at younger ages (Fig. 33). Thus, autism is associated with reduced levels of MS mRNA in post-mortem cortex, which is particularly evident at a younger age.
Figure 30: Autistic subjects have lower levels of MS mRNA than age-matched controls. MS mRNA levels from post-mortem human cortex were probed using qRT-PCR with specific primers to the cobalamin-binding and cap domains. Subjects were between 4 and 30 years-old. * = \( p < 0.03 \) compared to control for the cap primer set, ** = \( p < 0.03 \) compared to control for the cobalamin (COB) primer set.
Figure 31: Age-dependent decrease in cobalamin-binding and cap domain mRNA in autistic and control subjects. qRT-PCR was performed on samples with ages ranging from 4 years-old to 30 years-old. Primers to cobalamin-binding and cap domains were used. Control data was fit to a one-phase exponential decay ($R^2=0.94$), and autistic data was fit by linear regression ($n=10$ for each group).
Figure 32: Paired analysis of cobalamin-binding and cap domain mRNA in autistic and control samples. At younger ages, autistic subjects have a greater deficit in MS mRNA than age-matched controls (n=10 for each group).
Figure 33: PCR products from samples with ages ranging from 4 to 30 years-old. Primers to the cap domain were used. Gel shows 3 age-matched pairs including both control and autistic samples. 5/4, 11/9 and 30/30 indicate control and autistic paired ages, respectively. Equal concentrations of samples were run on 10% TBE gels.
2. Alzheimer’s Disease

The age-dependent pattern of MS mRNA decline suggests a possible relationship to neurodegenerative disorders, which occur with greater frequency in old age. Thus, I examined cap domain status in cortical samples from subjects with early-stage or late-stage AD, as compared to age-matched control subjects. Cap domain transcripts were not detected in any of the samples, irrespective of AD status (Fig. 34).

In early AD brain, age 74, cap domain was missing (Fig. 34a) as seen in the fingerprint of all domains. Since the result was similar to the aged control sample (Fig. 26, right), multiple AD samples were examined and can be seen in Fig. 35c. Four samples of both early AD and late AD were used (Fig. 34c), with an average age of 82 years and 75 years, respectively. Also present on the gel were a lymphoblast reference (LYM), a positive control (PC) and a no template control (NTC). Cap domain was not expressed in any of the four samples for each sample group (early and late AD). The terms early and late refer to the advancement of the disease.

PCR, using primers directed at the cap domain, was performed on six aged controls with an average age of 73. As is seen in Fig. 34b, no cap domain was present in any of the six control samples. Thus, AD is not associated with abnormal retention of cap domain exons.
Figure 34: MS status in human cortex from AD subjects. (a) Domain-specific PCR products for RNA from subjects with early (left panel) or late stage (right panel) AD. (b,c) RT-PCR analysis of cap domain status in control subjects (b) or early (left panel) and late-stage (right panel) AD (c). Lym= lymphoblast-derived RNA; PC = cDNA positive control.
3. Schizophrenia

Autism manifests itself at a young age, whereas AD manifests itself at an elderly age. Thus, I sought to investigate MS status in a disease where diagnosis is usually in early adulthood. Schizophrenia samples and their age-matched controls were obtained from the Stanley Medical Research Institute. Primers were designed to overlap exon junctions and primer details are listed in the Appendix (Table 1).

Schizophrenic mRNA levels were decreased across most MS exons, compared to age-matched controls (Fig. 35). There was an overall trend toward a decrease in MS mRNA from post-mortem schizophrenic cortex, suggesting a decrease in transcription levels of the enzyme. More subjects need to be investigated in order to determine statistical significance.
Figure 35: Schizophrenic subjects have less MS mRNA than age-matched controls. qRT-PCR was utilized to examine exon-specific differences in post-mortem cortex from schizophrenic and control subjects. (n=2 for each group; ages 55, 57 and 54, 57, respectively).
F. MS mRNA Status in Blood

Since systemic oxidative stress, as detected in plasma and blood cells, is a feature of autism and other neurological disorders, we aimed to develop a blood-based assay for detecting alterations in MS status. Since changes in MS gene transcription and alternative splicing were found using cell culture and human cortex samples, a preliminary study was done using Chronic Fatigue Syndrome (CFS) samples provided by Vitamin Diagnostics.

RNA was successfully isolated from blood samples and utilized in qRT-PCR studies. Results from these studies showed a significantly decreased cap domain in CFS samples as compared to controls ($p < 0.05$) (Fig. 36). The difference between CFS and control samples for the cobalamin-binding domain was not statistically different. Subject ages were unknown, and if there is an age-dependent trend, as observed in the cortex, it will be important to match for age. This experiment confirmed the ability to isolate RNA from blood samples, and this technique could be useful in developing a blood-based assay for autism and other neurological disorders.
Figure 36: mRNA from human blood was probed using qRT-PCR with specific primers to the cobalamin-binding and cap domains of MS. n=10 for CFS and n=4 for control (*= p < 0.05 for CFS compared to control for the cap primer set).
G. Tissue-Specific Differences

Since cap domain alterations appeared to be a feature of cultured neuronal cells and the human brain, I sought to investigate the existence of the cap domain in other brain areas including the medulla, pons, cerebellum and hippocampus. All samples, obtained from a commercial source, were from human subjects around 60 years-old, with the exception of the medulla, which was from a 36 year-old subject. When probed with cap domain primers, a bright band was visible in the hippocampus with a much lighter band in the cortex (age 76) (Fig. 37, left), reminiscent of SH-SY5Y cells and aged brain. In addition, cap domain was decreased or missing in the medulla, pons and cerebellum (Fig. 37, left).

When samples were probed for the SAM-binding domain, bands were also markedly decreased in the pons and cerebellum indicating that the cap domain most likely is not alternatively spliced in MS (Fig. 37, right). Since SAM-binding and cap domains are both decreased in the pons and cerebellum, there are probably lower transcription levels of MS in these brain regions. SAM-binding domain is expressed within the medulla; however, the cap domain seems to be missing and possibly spliced out in the medulla.
Figure 37: SAM-binding and cap domains are differentially expressed in brain. PCR experiment and gel. Left panel, cap primers (288 bp); right panel, SAM primers (300 bp). Abbreviations: Med, medulla; Cb, cerebellum; Hp, hippocampus; Cx, cortex.
H. MS Protein Detection Using Western Blots

Western blot experiments were performed in order to investigate MS composition at the protein level. Experiments used either SH-SY5Y cell lysates or a commercially available post-mortem human tissue panel. Custom-made antibodies generated by Dr. Tapan Audhya (Vitamin Diagnostics Inc.) were used to detect MS.

Different amounts of antibodies were used in immunoprecipitation with different volumes of lysate (Fig. 38). In all cases, two bands were visualized, with calculated molecular weights of 124 kDa and 111 kDa, respectively. Since the total molecular weight for MS is 140 kDa, loss of exons 16-18 (16 kDa) would yield a molecular weight of 124 kDa, which is approximately the calculated molecular weight of the upper doublet band. The lower doublet band of approximately 111 kDa indicates an additional isoform, since it is about 10 kDa smaller than the higher molecular weight band. SH-SY5Y PCR results suggest that about 50% of MS mRNA is missing exons 19 and 20, which, taken together add up to 8.9 kDa. This is approximately the size of the difference between the first and second bands, suggesting skipping of exons 19 and 20, in addition to exons 16-18 in about 50% of the MS mRNA in SH-SY5Y cells.

A western blot was performed, using a panel of post-mortem human tissues from ProSci Inc., in order to investigate MS molecular weight in human brain. ProSci Inc. indicated that the eight different tissues came from eight different donors, who were most likely middle-aged. Different banding patterns were observed in the different tissues. Notably, two bands of different molecular weights were observed in the liver and lung tissue (Fig. 39). The upper band was calculated to be about 139 kDa, which is approximately the predicted size of the full-length protein. The lower band was calculated to be about 130 kDa. As previously mentioned, cap
domain exons 19 and 20 add up to about 9 kDa, which is approximately the difference between the two human brain isoforms. Thus, the primary form of MS in this brain sample appears to be a truncated form missing a portion of the cap domain, consistent with my qRT-PCR findings with older subjects. These results are obviously preliminary, but they suggest the value of follow-up studies with multiple-tissue samples from individuals of known age.
Figure 38: Western blot probing SH-SY5Y cells for MS. Two doublet bands at 124 kDa and 111 kDa are visualized using the R-3-8W antibody (directed against amino acids 49-69 in HCY-binding domain).
Figure 39: Western blot probing human normal tissue for MS. The top panel indicates the molecular weight of exons associated with splicing/skipping. Exons 16-18 within the folate-binding domain (green) are 16 kDa; Exons 19 and 20 within the cap domain (yellow) are 8.9 kDa. In the lower panel, two doublet bands at 139 kDa and 130 kDa are visualized in brain tissue, using the R-3-8W antibody (sequence area 49-69). The prominent form in the brain is the smaller isoform (130 kDa).
I. Exon-Specific Analysis

1. Fetal Brain

A recent sequencing investigation of full-length cDNA transcripts from a fetal human brain sample revealed another example of exon-skipping in MS. Exons 16-18, which correspond to the folate-binding domain, were deleted in this reported isoform\(^\text{30}\). To confirm this observation, we designed two “full-length” primer sets, covering exons 14-22 and 15-21, in order to examine the entire exon makeup of the folate-binding and cap domains. To further probe the composition of MS mRNA, primers spanning selected splice junctions (15-16, 17-18, etc.) were also designed (Fig. 40) and used in the following experiments.

qRT-PCR results, using cDNA derived from a commercially obtained, human fetal cortex sample (female, 28 weeks), showed significantly lower levels of mRNA for exons 16-18 as compared to mRNA levels for both cap- and cobalamin-related exons (19-20 and 24-25) \((p < 0.05)\) (Fig. 41a). Consistent with this result, three PCR products of varying length were identified and sequenced using a multiple exon-spanning primer set (exons 15-21). The major product was full-length (denoted as A in Fig. 41b), containing all seven exons, while less abundant products (B and C in Fig. 41b) lacked either exons 19 and 20 or exons 16-18, confirming that portions of the folate-binding and/or cap domains are deleted in fetal MS mRNA transcripts.

The expected size of the seven exon full-length product was \(~960\) base pairs (bp). Fragment sizes for isoforms B and C were \(~720\)bp and \(~500\)bp, confirmed by sequencing data and illustrated in Fig. 41b (lower panel). The size of the missing exons for both products B and C was approximately \(~240\)bp and \(~435\)bp, further confirming exon skipping.

It is important to note that the majority of fetal cortex transcripts contain the cap domain. A small percentage of fetal transcripts are missing exons 16-18, corresponding to the folate-
binding domain. Since this sample was from a post-mortem fetus from an unknown cause, we cannot be sure that alternative splicing is a feature of normal brain.
Figure 40: Location of exon-specific primers. Human MS contains 33 exons specifying the five domains in a sequential manner. (a) Upper panel shows original primers to all domains (grey). Domains are as follows: HCY, exons 1-11, pink; FOL, exons 12-18, green; CAP, exons 19-21, yellow; COB, exons 22-25, red; SAM, exons 26-33, blue. (b) Exon-specific primers (size in base pairs) are located in the lower enlarged panel (black bars). Full-length primers spanning exons 14-22 and 15-21 are shown in gray.
Figure 41: MS mRNA status in 28-week-old human fetal cortex. (a) qRT-PCR was carried out using fetal-derived cDNA with primers spanning exons 15-16, 17-18 (folate-binding domain), 18-19 (folate-binding/cap domains), 19-20 (cap domain), 21-22 (cap/cobalamin-binding domains) and 24-25 (cobalamin-binding domain). * indicates significant difference from 19-20, 21-22 and 24-25 values (p < 0.05). (b) PCR was carried out using fetal-derived cDNA with primers for exons 15-21. PCR products (arrows) were sequenced, indicating full-length transcripts and transcripts missing either exons 19 and 20 or 16-18, as illustrated.
2. **SH-SY5Y Cells**

Identical experiments were performed in the human neuroblastoma cell line, SH-SY5Y, to shed further light on the previous finding of a truncated cap domain. qRT-PCR was carried out using SH-SY5Y-derived cDNA (Fig. 42a). Primers for exons 15-16 did not amplify, while primers for exons 17-18, 18-19 and 19-20 amplified to an intermediate level, as compared to exons 21-22 and 24-25. Cap transcript levels were significantly lower than cobalamin transcript levels, with exons 16-18 also being significantly decreased. Subsequent PCR products were visualized and sequenced using primers for exons 14-22 (Fig. 42b). Results showed two separate isoforms, the majority containing exons 15, 19, 20 and 21 and the smaller fragment exons 15 and 21. Thus exons 16-18 are missing from all transcripts and exons 19 and 20 are absent from approximately 50% of transcripts.

Consistent with this alternative splicing pattern, previously shown western blot analysis using an antibody generated against the SAM-binding domain epitope detected dual bands of 125 and 111 kDa, both of which are smaller than the calculated MW of MS of 140 kDa (Fig. 42c). Absence of exons 16-18 (16 kDa) accounts for the higher MW band, while the additional absence of exons 19 and 20 (8.9 kDa) accounts for the lower MW band.

However, additional SH-SY5Y experiments showed that cap domain was present in most transcripts. Transcripts for cap domain exons (19-20) were not significantly different from transcripts for cobalamin-binding domain exons (24-25). Folate-binding domain exons 15-18 were all significantly decreased compared to both cap and cobalamin-binding domains ($p < 0.05$) (Fig. 43a). Most of the transcripts existed as full-length (A in Fig. 43b), with very little transcript in the truncated form. Sequencing analysis of these results showed normal expression of probed
exons 14-22 (isoform A), but a lack of exons 16-18 (isoform B) in a very small portion of transcripts (Fig. 43b).

These unexpected findings indicate that adaptive responses to redox are variable in cell culture and warrant further examination.
Figure 42: MS mRNA status in SH-SY5Y cells. (a) qRT-PCR was carried out using SY5Y-derived cDNA with primers spanning exons 15-16, 17-18 (folate-binding domain), 18-19 (folate-binding/cap domains), 19-20 (cap domain), 21-22 (cap/cobalamin-binding domains) and 24-25 (cobalamin-binding domain). * indicates significant difference from 19-20, 21-22 and 24-25 values (p < 0.05). (b) Sequencing was carried out using SY5Y-derived cDNA with primers for exons 14-22. (c) Locations of MW markers (kDa) are shown on right. Two bands are visualized at ~124 kDa and ~111 kDa.
Figure 43: Additional splicing of MS mRNA in SH-SY5Y cells. (a) qRT-PCR was carried out using SH-SY5Y cDNA with primers spanning exons 15-16, 17-18 (folate-binding domain), 18-19 (folate-binding/cap domains), 19-20 (cap domain) and 24-25 (cobalamin-binding domain). * indicates significant difference from 19-20 and 24-25 values ($p < 0.05$). (b) PCR was carried out using SH-SY5Y cDNA with primers for exons 14-22. PCR products (arrows) were sequenced, indicating full-length transcripts and transcripts missing exons 16-18, as illustrated.
3. LN-18 Cells

The human glioblastoma cell line, LN-18, was obtained and used in exon-specific qRT-PCR experiments to further investigate cell-type differences. There are two main sources of cysteine for synthesis of glutathione: conversion of HCY to cysteine via the transsulfuration pathway and transport of cysteine from outside the cell via the EAAT3 transporter. Glial cells have the unique ability of releasing cysteine into the surrounding area, and neurons take up this cysteine via the EAAT3 transporter. When probed with MS exon-specific primers, LN-18 cells showed no significant differences across the exon panel (Fig. 44a). However, MS is expressed at a lower level in glial cells vs. neurons, when values were compared between glioblastoma and neuroblastoma cell lines. PCR analysis showed all transcripts to be full-length (Fig. 44b), including exons 15-21.
Figure 44: LN-18 glioblastoma cells express full-length MS mRNA. (a) qRT-PCR was carried out using LN-18 derived cDNA with primers spanning exons 15-16, 17-18 (folate-binding domain), 18-19 (folate-binding/cap domains), 19-20 (cap domain), 21-22 (cap/cobalamin-binding domains) and 24-25 (cobalamin-binding domain). (b) PCR was carried out using LN-18 cDNA with primers for exons 15-21. PCR products were sequenced, indicating only full-length transcripts, as illustrated.
J. TNFα Treatment in SH-SY5Y Cells

TNFα was utilized to mimic inflammatory events, which are commonly associated with oxidative stress. The rationale for using TNFα is that it is a pro-inflammatory cytokine commonly known to induce cell death and inflammation. However, it has been shown that TNFα can be protective under some conditions of neurological insult. TNFα is able to modulate expression of various downstream transcription factors. TNFα binds to tumor necrosis factor receptor 1 (TNFR1), which is found in most tissues, including the brain. An underlying cause of oxidative stress is inflammation, both of which are implicated in aging. In addition, TNFα levels are elevated in both the cortex\textsuperscript{126} and cerebrospinal fluid (CSF) of autistic subjects\textsuperscript{127}.

To examine changes in MS mRNA status in response to TNFα treatment, mRNA was isolated from HepG2 and SH-SY5Y cells at each TNFα-treatment time point, and was evaluated in a qRT-PCR experiment. As seen in Fig. 45, changes in cobalamin-binding and cap domain mRNA, in HepG2 cells, were very similar, showing a marked decrease after 2 hours of TNFα treatment.

Treatment with 30 ng/mL of TNFα in SH-SY5Y cells caused only a modest 30% decrease in cap domain mRNA at the 2-hour treatment point, and this decrease was sustained at 6 and 24 hours; however, TNFα treatment caused a larger decrease of greater than 50% in cobalamin domain-containing transcripts at 1 and 2 hours, followed by partial recovery at 6 and 24 hours (Fig. 45). The cap/cobalamin ratio remained around 1.0 throughout TNFα exposure in HepG2 cells, indicating an overall decrease in both domains, consistent with a decrease in transcription. In SH-SY5Y cells, however, the cap/cobalamin ratio was lower than one, due to a decrease in cap domain mRNA, which is representative of alternative splicing.
Since important changes in the long-term TNFα experiment occurred in the first few hours, we decided to repeat the experiment with a new set of time points. In the short-term study performed, time points included 30, 45, 60 and 120 minutes. Control cells with no TNFα added were plated for each 6-well plate experiment and 30 ng/mL of TNFα was used.

TNFα treatment, as early as 30 minutes, decreased transcript levels of both cap and cobalamin mRNA, as compared to their respective controls \((p < 0.05)\) (Fig. 46). Levels of mRNA for both domains remained low for the duration of time-points, implying a lasting change in MS transcription.

Lastly, the ratio of cap to cobalamin transcripts was evaluated to identify overall transcription changes vs. alternative splicing. Fig. 47 presents the ratio differences between SH-SY5Y cells and HepG2 cells as well as ratio changes in SH-SY5Y cells treated with TNFα. The ratio of cap to cobalamin mRNA in HepG2 cells is close to 1:1. However, as previously shown, there is much less cap mRNA (~0.3 ratio) in SH-SY5Y cells, indicating alternative splicing of the cap domain. With TNFα treatment, the cap/cobalamin ratio slightly increased to ~0.6, with a significant increase at the 45-minute time point \((p < 0.05)\). This value is still lower than the cap/cobalamin ratio of 1:1 in HepG2 cells, but demonstrates the ability of TNFα to regulate MS transcription. The increase in cap/cobalamin ratio indicates a decrease in alternative splicing in response to TNFα.

Since TNFα altered mRNA levels of MS, I investigated the thiol profile for TNFα-treated cells for the same time points. TNFα treatment significantly increased levels of cysteine and GSH and significantly decreased levels of methionine at 30 minutes \((p < 0.05)\) (Fig. 48). Cystathionine was significantly decreased at 120 minutes, while there was no change in HCY levels. Both cysteine and GSH levels peaked at 30 minutes of treatment and steadily decreased.
back to baseline. Elevated levels of cysteine indicate an increase in transsulfuration activity, and indeed, GSH levels are elevated. In conjunction, methionine levels are decreased, implying a decrease in MS activity.

A cysteine uptake experiment was performed in order to take a closer look at cysteine levels in TNFα-treated cells. Cysteine uptake was decreased starting at 30 minutes, by about 70%, which lasted for the remainder of the experiment (Fig. 49). Thus, the increase in cysteine at 30 minutes cannot be due to the increased uptake, but to increased transsulfuration. It should be emphasized that this experiment measures cysteine uptake, rather than the intracellular levels of cysteine, as presented in Fig. 48.

Finally, to check MS status at the enzymatic level, a MS activity assay was performed using SH-SY5Y cells treated with 30 ng/mL of TNFα for various time-points (Fig. 50). MS activity was significantly decreased at 2 and 4 hours of TNFα treatment ($p < 0.007$ and 0.001, respectively), complementing both the qRT-PCR and HPLC data. MS activity at 6 hours was not significantly different from the control, showing that enzyme activity recovers after the initial insult.
Figure 45: Effect of long-term TNFα treatment on cobalamin-binding and cap domain mRNA status in HepG2 and SH-SY5Y cells. qRT-PCR experiments using cap and cobalamin primers. Top left, HepG2 cells; top right SH-SY5Y cells. Cells were treated with 30 ng/mL TNFα. Bottom, cap/cobalamin ratio of line graph data for both cell groups. Each data point is the mean of duplicate determinants.
Figure 46: Effect of short-term TNFα treatment on cobalamin-binding and cap domain mRNA status in SH-SY5Y cells. qRT-PCR was carried out using SY5Y-derived cDNA with primers spanning exons 19-20 (CAP) and 24-25 (COB), respectively. * indicates significant difference from control time-point for cobalamin mRNA ($p < 0.05$). #indicates significant difference from control time-point for cap mRNA ($p < 0.05$). Cells were plated in 6-well dishes and treated with 30 ng/mL of TNFα for 30, 45, 120 and 240 minutes. The experiment was carried out three times with two replicates each time.
Figure 47: TNFα treatment increases cap/cobalamin mRNA ratio in SH-SY5Y cells. qRT-PCR was carried out using SY5Y-derived cDNA with primers spanning exons 19-20 (cap) and 24-25 (cobalamin), respectively. * indicates significant difference from control time-point for SH-SY5Y cells ($p < 0.05$). Cells were plated in 6-well dishes and treated with 30 ng/mL of TNFα for 30, 45, 120 and 240 minutes. The experiment was carried out three times with two replicates each time. HepG2 cells are on the graph as a comparison to a 1:1 ratio.
Figure 48: Effect of short-term TNFα treatment on thiols in SH-SY5Y cells. Cells were plated in 6-well dishes and treated with 30 ng/mL of TNFα for 30, 45, 60, 120 and 240 minutes. After TNFα treatments, cell lysates were prepared and analyzed via HPLC.* indicates significant difference from control time-point for each thiol ($p < 0.05$). All time-points assayed for methionine were significant compared to control. The experiment was carried out three times.
Figure 49: TNFα treatment decreases cysteine uptake at 30 minutes in SH-SY5Y cells. Confluent cells were pre-treated in culture media with 30 ng/mL of TNFα for 30, 45, 60, 120 and 240 minutes. Cysteine uptake was assayed using a scintillation counter. The experiment was carried out with three replicates for each time point (*=p < 0.05).
Figure 50: TNFα treatment decreases MS activity at 2 hours in SH-SY5Y cells. Confluent cells were pre-treated in culture media with 30 ng/mL of TNFα for 0.5, 0.75, 1, 2, 4 and 6 hours. MS activity was significantly decreased at 2 and 4 hours (*=p < 0.007; **=p < 0.001) and recovered by 6 hours.
K. NAC Treatment in SH-SY5Y Cells

In further studies, SH-SY5Y cells were treated with N-acetyl-cysteine (NAC) to probe dynamic redox responses. NAC provides the amino acid cysteine for the formation of GSH to the cytosol and bypasses the requirement for cysteine uptake. NAC treatment did not significantly alter the transcription of cap or cobalamin mRNA. Cells were treated in both separate short-term (Fig. 51) and long-term (Fig. 52) experiments with no discernible changes.

However, HPLC analysis of NAC-treated cells showed an increase in cysteine over time, with a significant increase over control at 45 minutes. Cysteine remained elevated for the duration of the experiment (Fig. 53). GSH was also significantly increased at 60 minutes, slightly after the significant increase in cysteine levels. This makes sense since cysteine is required for GSH formation. Methionine and HCY levels remained steady throughout the time trials, indicating no alterations in MS activity. Cystathionine was significantly decreased at 30 minutes, but recovered to and surpassed its basal level at the 4-hour mark. This pattern may be in response to the elevated levels of cysteine from NAC treatment, causing an early negative feedback loop followed by an over-compensation.
Figure 51: Effect of short-term NAC treatment on cobalamin-binding and cap domain mRNA status in SH-SY5Y cell. qRT-PCR was carried out using SY5Y-derived cDNA with primers spanning exons 19-20 and 24-25, respectively. Cells were plated in 6-well dishes and treated with 5 mM NAC for 0.5, 0.75, 1, 2 and 4 hours. The experiment was carried out two times with two replicates each time.
Figure 52: Effect of long-term NAC treatment on cobalamin-binding and cap domain mRNA status in SH-SY5Y cells. qRT-PCR was carried out using SY5Y-derived cDNA with primers spanning exons 19-20 and 24-25, respectively. Cells were plated in 6-well dishes and treated with 5 mM NAC for 1, 2, 4, 6, and 24 hours. The experiment was carried out two times with two replicates each time.
Figure 53: Effect of short-term NAC treatment on thiols in SH-SY5Y cells. Cells were plated in 6-well dishes and treated with 5 mM of NAC for 30, 45, 60, 120 and 240 minutes. After NAC treatments, cell lysates were prepared and analyzed via HPLC. * indicates significant difference from control time-point for each thiol ($p < 0.05$). The experiment was carried out two times.
IV. DISCUSSION

The enzyme MS is located at the intersection of four important metabolic pathways: transsulfuration, methylation, dopamine-stimulated phospholipid methylation and single-carbon metabolism. Thus, MS activity has an extensive influence over redox status and cellular function. MS is a hot spot for modulating metabolic activity in response to redox status, due to the vulnerability of the cofactor cob(I)alamin to oxidation. My results demonstrate an impressive and previously unappreciated age-dependent decrease in cortical levels of MS mRNA, along with age-dependent alternative splicing that removes a portion of the cap domain, rendering MS more sensitive to inactivation and favoring transsulfuration and GSH synthesis. It is likely that these changes are adaptive responses to increasing levels of oxidative stress, which are associated with aging. A further comparison between autistic subjects and age-matched controls revealed a significant decrease of MS mRNA in autism, particularly at younger ages. Therefore, my findings indicate a dynamic regulation of MS in the human brain, and they further indicate that autism may be a neurometabolic redox disorder in which lower MS activity plays an important role. Moreover, abnormalities affecting redox regulation may contribute to neurodevelopmental, neuropsychiatric and neurodegenerative disorders across the lifespan.
A. MS Domain Status in Cultured Cells

PCR experiments revealed a truncated cap domain in the human neuroblastoma cell line, SH-SY5Y. The 4-helix cap domain spans 89 amino acids and is able to shield the cobalamin from oxidation when in the resting conformation (Figs. 6 and 7). Cap domain mRNA consists of exons 19-21, and my data from SH-SY5Y cells indicates a partial deletion corresponding to exons 19-20. These exons are highlighted in Fig. 7 and deletion of these exons drastically alters the protein structure, exposing cobalamin to the oxidative environment. Moreover, a smaller isoform was observed in these cells. Sequencing confirmed presence of only exon 21, indicating that exons 19 and 20 were indeed spliced.

Alternative splicing may be an adaptive response of sulfur metabolism to oxidative stress. It is likely that cobalamin will be more readily oxidized in the absence or truncation of the cap domain, increasing the chance for MS inhibition. Decreased MS activity allows HCY to flow into the transsulfuration pathway, providing the antioxidant glutathione (GSH) with necessary cysteine.

This observation suggests that human neuronal cells may function in a unique mode of redox regulation, in order to preserve the vulnerable brain. The brain is particularly susceptible to free radical insult due to its ability to generate more free radicals per gram tissue than any other organ. As demonstrated by varying exon-skipping profiles in SH-SY5Y cells (Figs. 42 and 43) changes in redox status can dynamically modulate the composition of MS in order to favor either methylation or transsulfuration.

The cap/cobalamin ratio in other cells types (HepG2, HEK and LN-18) was not altered (Figs. 25 and 44), illuminating the fact that cap domain splicing is a particular feature of neurons. LN-18 cells are a human glioblastoma cell line. Astrocytes, a glial cell-type, have the unique
ability of releasing GSH into the intracellular space, where it is cleaved to cysteine. Neurons can then take up this cysteine via the excitatory amino acid transporter-3 (EAAT3). Therefore, astrocytes function at a more reduced state than neurons, and this interdependence between the two cell-types is an important source of cysteine to neurons.
B. MS Analysis in Human Cortex

Epigenetics, a term first coined by Conrad Waddington in the 1940’s\textsuperscript{141}, has been described as the developmental process by which genotype gives rise to phenotype. The human body has evolved many ways in which to expand the seemingly few 30,000 genes, which are expressed in all tissues. For example, alternative splicing of the pre-mRNA can give rise to several new protein products. However, not all cells express all 30,000 genes at the same time; the epigenetic code allows for certain gene expression at the appropriate time, in the appropriate tissue\textsuperscript{142}.

Epigenetic modifications include DNA and histone methylation, as well as histone acetylations. Epigenetic chromatin remodeling can also increase accessibility for the binding of transcription factors\textsuperscript{143}. The term epigenetics comes from the Greek “epi,” meaning “on top of,” implying that changes in phenotype are caused by mechanisms other than changes to the underlying DNA\textsuperscript{144,145}. DNA and histone modifications are not transient; epigenetic marks can be propagated through cell divisions and persist across generations\textsuperscript{146}.

Disruption of epigenetic networks can lead to various diseases/disorders such as cancer, mental retardation and unstable chromosomal syndromes. The role of epigenetics in cancer has been appreciated for decades and much research has centered on understanding the associated mechanisms. However, the role of epigenetics in other disorders, such as metabolic disorders, has not been investigated to the same extent. Data now suggests that maternal nutrition and metabolic disturbances play a large role in epigenetic effects in fetal development\textsuperscript{147}.

Epigenetics manifests itself as early as fertilization in the zygote when both the paternal and maternal genomes are rapidly demethylated in order to make a clean slate for de novo methylation. After implantation, the majority of the genome becomes hypermethylated, while
extra-embryonic tissues remain hypomethylated (Fig. 54). De novo methylation signals the structure and function of tissues by turning gene expression on and off. Using the Dutch famine as an example (discussed on p. 26), altered folate nutrition can have detrimental effects on the developing fetus. Less folate in the diet may compromise the methylation capabilities of the fetus, and this detriment would prevent the proper de novo methylation pattern from occurring. Therefore, it is likely that nutrition exerts its effects on methylation early on in development, later leading to altered neural networks and synapse formation.

![Figure 54: Changes in DNA methylation during mammalian development.](image)

Figure 54: Changes in DNA methylation during mammalian development. Figure from Strachan and Read.

Somatic cells maintain a high level of methylation after de novo methylation, which is preserved though the process of maintenance methylation (Fig. 54). Maintenance methylation, via DNA methyltransferase (DNMT), is necessary to preserve DNA methylation after every
cellular DNA replication cycle. Otherwise, DNA replication would produce unmethylated daughter strands, leading to passive demethylation over time.

Hypermethylation plays a large role in gene regulation by silencing tissue-specific genes in non-expressing cells, as well as suppressing retrotransposons. Retrotransposons are genetic elements that have been inserted into the genome, and transcription of retrotransposons may lead to aging, cancer and genome instability. DNA methylation is necessary to prevent the expression of viral genes, and loss of methylation can lead to the expression of inserted viral genes.

Epigenetics also plays a role in genetic imprinting, where alleles from either the mother or father are inherited with corresponding epigenetic modifications. Genomic imprinting is associated with neurodevelopmental disorders such as Angelman and Prader-Willi syndromes. Additionally, genetic defects involving methylation are linked to autism, fragile-X and Rett syndromes.

Autism subjects have been shown to have a decreased methylation capacity due to lower levels of the main methyl donor SAM, in plasma metabolites. High levels of MS mRNA are most likely important for normal brain development during formation of neural networks and during high periods of gene transcription, which demand high levels of methylation capabilities. In contrast, my results show lower levels of MS mRNA in autistic subjects, particularly at the youngest ages. Lower levels of MS mRNA reflect an increased need for antioxidants and highlight the role of oxidative stress in autistic subjects. Autistic children have been shown to have elevated levels of systemic oxidative stress as well as neuroinflammation in postmortem brain, thus decreased levels of MS mRNA are likely an adaptive response to...
neuroinflammation and oxidative stress. When taking all of these findings into consideration, autism likely results from a disruption of both normal redox regulation and methylation status.

Growth factors, such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-1) are critical for the developing brain in a variety of processes. PDGF is involved in embryonic development, cell proliferation, cell migration and angiogenesis\(^{157}\) and IGF-1 is the primary mediator of growth hormone effects in young children\(^{158}\). It has also been shown that IGF-1 stimulation increases MS activity and DNA methylation\(^9\). PDGF, via PI3K, increases cell surface expression of the murine form of EAAT3 (EAAC1)\(^{48,49}\), which provides cysteine to the cell for GSH formation. It has also been reported that IGF-1 is decreased in the cerebrospinal fluid (CSF) of autistic subjects, compared to age-matched controls\(^{159,160}\). In these studies, there was a significant correlation between both CSF and age with IGF-1 concentration in autistic and control subjects.

IGF-1 stimulation of MS activity increases methylation capacity, and since IGF-1 is elevated during development, young children have an increased methylation capacity. There is also an overall decrease in genomic methylation with age\(^{161-163}\), and adult mouse brains have lower levels of DNMT, the enzyme responsible for maintaining the DNA methylation state\(^{164}\). A decrease in methylation, due to polymorphisms in MS (A2756G) and MTHFR (C677T and A1298C), is linked to an increase of childhood leukemias\(^{165,166}\).

Methylation is also important in the process of myelination of neurons. Myelin is a protective sheath, composed mainly of lipids, that wraps around neuron axons in order to insulate and increase the propagation speed of electrical impulses. A decrease in the ability to methylate prevents neuron myelination as well as remyelination after an insult. Glycolipids and sphingomyelin are the prominent lipids within the myelin sheath and sphingomyelin participates
in metabolism at the plasma membrane\textsuperscript{167}. Studies have shown that cobalamin deficiencies and impairments in SAM-mediated methylation lead to demyelination in mammalian systems\textsuperscript{168-170}. Thus, it is likely that a decrease in SAM-dependent methylation decreases the formation of sphingomyelin and glycolipids due to an inability to methylate lipid components.

Decreased myelination, as a result of less methylation, can lead to inadequate synaptic pruning. Pruning serves to reduce the less meaningful neuron connections while assembling more efficient synaptic networks. A lack of pruning can lead to misdirected connections and thick, tangled, clusters of neurons. Therefore, pruning is a critical process in development, which is mediated, in part, by methylation.

In light of these findings, it is likely that the transsulfuration pathway is less active in younger years, since the need for methylation capacity is greater than the need for antioxidant resources. However, the EAAT3 transporter is able to participate in cysteine uptake for GSH synthesis in times of need. Indeed, astrocytes can provide EAAT3 with cysteine during inflammation or oxidative stress\textsuperscript{171,172}.

Decreased IGF-1 in autistic subjects could favor the transsulfuration pathway if there are not appropriate EAAT3 and cysteine levels in the developing brain. Activation of the transsulfuration pathway increases GSH levels, but also decreases the methylation capacity via a decrease in MS activity. Thus, impairment in growth factors may contribute to the root cause of autism.

CNS neurons are non-dividing and are generally not replaceable after being lost. Remaining a non-dividing cell is critical for retaining “neuron memories” between synapses and networks, and division would erase all of this stored information. Therefore, it is beneficial to keep neurons in a quiescent state. This is likely accomplished by directing cells to enter the $G_0$
phase of the cell cycle and remain there until stimulated to divide. A lack of growth factors and/or oxidative stress is utilized to keep cells in this phase of the cell cycle\textsuperscript{173}. Therefore, a partial block of the transsulfuration pathway provides a mechanism in which steady levels of oxidative stress can be maintained.

There are specific requirements for cell division such as growth factors, nutrients and various other appropriate conditions. It is possible that since autistic subjects with lower levels of IGF-1 also have lower levels of other growth factors. A lack of growth factors during fetal and early childhood development could alter neuron growth and interfere with appropriate neural networks and connections.

The D4 dopamine receptor has the unique ability to transfer folate-derived methyl groups to the phospholipid membrane and it has been demonstrated that MS activity is an absolute requirement for this process\textsuperscript{97}, making it highly sensitive to oxidative stress. Decreased MS activity can change the fluid membrane properties of the membrane. Computational models have shown that D4 phospholipid methylation has the ability to shift synchronization of brain activity during attention to gamma frequency (30-80 Hz), and individuals expressing the seven-repeat variant of the receptor show enhanced gamma frequency activity during attention-related tasks\textsuperscript{174}. The seven-repeat variant is a major risk factor for attention-deficit hyperactivity disorder (ADHD)\textsuperscript{175,176}, and the 4:1 prevalence of ADHD in males vs. females mirrors that of autism\textsuperscript{176,177}, although the frequency of the seven-repeat variant is not higher in autistic subjects\textsuperscript{178}. Decreased MS activity prevents the actions of dopamine-stimulated PLM, and decreased methylation capabilities and impaired neuronal synchrony is an important feature of autism\textsuperscript{179-181}. Methylcobalamin treatments have also been utilized in autistic children to help normalize plasma levels of sulfur metabolites and improve neurological function\textsuperscript{63,182}. 

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Therefore, a lack of growth factors and changes in cellular redox status can alter and/or impair the synchronization of neural networks.

IGF-1 also plays a significant role in the aging process, with an age-dependent decline in IGF-1 levels observed in human CSF\textsuperscript{183-185}. Oxidative stress increases with age and my results show an age-dependent change in MS mRNA levels, in which levels are highest during the early years of life (Fig. 27). As previously described, MS can be inhibited by oxidative stress and may alter its status in order to positively change the redox status of the environment. The progressive decrease in MS mRNA levels indicates that more HCY will be directed toward antioxidant GSH synthesis with advancing age.

The impressive decrease in MS mRNA is likely to reflect a decrease in transcription, since both the cobalamin-binding and cap domains exhibit a similar pattern. However, it is possible that MS mRNA is degraded at a faster rate with increasing age, which could also lead to a decrease in transcript levels. In subjects over the age of 60, the cap/cobalamin ratio was significantly less than 1:1, indicating additional age-dependent alternative splicing (Fig. 28). This process decreases the number of cap-containing transcripts and serves to decrease MS activity and increase HCY diversion to GSH synthesis, during the aging process.

I investigated MS status in various brain tissues including cortex, hippocampus, pons, medulla and cerebellum, and although my results are only from one experiment, the evolutionary aspects of brain metabolism are intriguing and merit further investigation.

Evolutionarily speaking, the hindbrain, which contains the pons, medulla and cerebellum is the oldest part of the brain\textsuperscript{186}. These structures can be found in reptiles and serve to facilitate basic and vital functions such as breathing, heart-rate and blood-pressure. Levels of MS SAM-binding and cap domain mRNA were decreased in both the pons and cerebellum, indicating
decreased transcription of the enzyme (Fig. 37). These brain regions are “older” and the lack of MS in these areas most likely reflects the possibility that “older brain” may not use methylation and/or redox pathways in the same manner as “newer brain”. The forebrain contains the hippocampus and cortex, which both show a lack of cap domain (Fig. 37). This could indicate that the adaptive response of splicing is more prominent in evolutionarily advanced tissues, due to the increased production of ROS due to oxygen consumption³.
C. TNFα Treatment Modulates MS

TNFα experiments highlight an important function of the inflammatory pathway. The pro-inflammatory cytokine TNFα was utilized to mimic inflammatory events, which are commonly associated with oxidative stress. In my studies, TNFα elicited a quick and transiently enhanced response of the transsulfuration pathway, which was associated with decreased transcription of MS mRNA (Figs. 45-48) TNFα treatment in SH-SY5Y cells also inhibited cysteine uptake at as early as 30 minutes (Fig. 49). Thus increased neuroinflammation and oxidative stress, as seen in aging and autism, switch the primary cysteine source for GSH synthesis from EAAT3 transport to transsulfuration, which is consistent with an age-dependent decrease in growth factors \(^{183-185}\), which can decrease activity of the EAAT3 transporter \(^{48,49}\).

TNFα also caused a decrease in MS enzyme activity (Fig. 50), as well as an increase in the antioxidant GSH (Fig. 48). With less HCY being converted to methionine, a higher proportion is free to enter the transsulfuration pathway and increase GSH levels. Therefore, during an inflammatory event, TNFα is able to modulate MS transcription. However, the mechanism mediating this action remains unknown.

Activation of the TNFα pathway under a toxic insult allows the cell to regulate oxidative stress and restabilize its redox status. It is well known that activation of the TNF receptor by TNFα is able to modulate activity of transcription factors NF-κB and AP-1 (via the MAPK pathway) \(^{128,129}\). NF-κB regulates the transcription of a wide variety of proteins involved in cell survival and proliferation, apoptosis and inflammation \(^{187}\). Both transcription factors NF-κB and AP-1 are redox-sensitive \(^{188-190}\). There are also numerous reports that identify TNFα-mediated events as neuroprotective in the brain \(^{131,191}\). In addition, activation of Akt, via PI3K, increases the nuclear binding of NF-κB subunits \(^{192,193}\).
The region 5' to the transcription start site of exon 1 of MS (defined as +1) was found to have multiple possible promoter sites, including an E box at -125 to -120, two CAAT boxes at -103 to -97 and -72 to -66, and a GC box at -55 to -46 (Fig. 55). The transcription factor Sp-1 has an 86.3% chance of binding to the DNA at positions -55 to -48 via a zinc-finger protein motif, which enhances transcription (red arrow, Fig. 55). Using a program designed to locate potential transcription factor binding sites, multiple potential regulatory locations were identified in the 5' region of MS exon 1\textsuperscript{194,195}.

The program identified a canonical NF-κB binding site (blue box, Fig. 55), which corresponds to one of the identified promoter sites within MS\textsuperscript{196}. The downstream target of TNFα signaling, NF-κB has an 88.5% chance of binding to the MS at positions -50 to -47, which is 5 nucleotides from a potential transcription factor initiation site. NF-κB also has a zinc-finger binding motif, making it an excellent candidate for binding at the proposed location. Since both transcription factors have a similar chance of binding to the DNA, it sets up the possibility for competition between Sp-1, which enhances transcription of DNA, and NF-κB, which is thought to have repressive actions in this situation. The literature suggests that NF-κB negatively modulates transcription, which would account for the decrease in MS transcription after TNFα treatment\textsuperscript{197-199}.

Decreased SAM levels are seen in the plasma of autistic subjects, as compared to controls, and my results show that levels of MS are decreased in these subjects. Thus, the ability to methylate is markedly decreased, leading to hypomethylation. As previously discussed, hypomethylation leads to an unwinding of the DNA, allowing transcription factors to access the template. Increases in certain pro-inflammatory cytokines, such as IL6 and TNF alpha, can lead to decreases in methylation. Thus, it is possible that NF-κB activation by TNFα is an adaptive
response to the overwhelming demethylation response from oxidative stress. It is desirable to prevent transcription of MS under inflammatory conditions, in order to augment GSH formation.

Figure 55: Nucleotide sequence of 5' region of human MS gene. Exon 1, labeled as +1, is the start of the DNA sequence (lower case). Potential transcription factor (TF) regulatory sites are boldfaced. Potential TF initiation sites are indicated by (●). Figure adapted from Chen et al.19.
D. Alzheimer’s Disease

Aging is associated with increased levels of oxidative stress\textsuperscript{50,84,85}, as well as with a decrease in plasma levels of GSH and cysteine, while cystine and HCY levels increase\textsuperscript{87,200}. The MS polymorphism, A2756G, has been linked to increased risk of sporadic AD\textsuperscript{201}, and polymorphisms in MTHFR and the reduced folate carrier (RFC) have shown association in some\textsuperscript{202-204}, but not all\textsuperscript{205,206} studies. Plasma levels of HCY are elevated in late-onset AD\textsuperscript{79-81}, and the rate of cognitive decline is correlated with HCY levels. AD is associated with epigenetic drift, affecting the methylation status of AD-risk genes such as apolipoprotein E4 (APOE4)\textsuperscript{207}. These studies suggest a broad involvement of oxidative stress and impaired methylation in neurological disorders. Thus, lower MS activity may exert an epigenetic influence over gene expression during aging via altered methylation of DNA or histones.

Cortex samples from both advanced-age normal subjects and AD subjects are lacking cap domain exons 19-21 (Fig. 34). The deletion of the entire cap domain exposes more of the cobalamin cofactor to an oxidative environment and could possibly alter the enzyme structure in response to other domains (HCY, SAM), which interact with the substrate. These structural modifications increase inactivation of MS activity, resulting in decreased methylation capacity.

My TNFα results indicate that inflammation decreases MS mRNA and increases cysteine flux through the transsulfuration pathway. A decrease in MS mRNA is associated with a decreased methylation capacity (via SAM). Hypomethylation allows for increased gene transcription and multiple studies have indentified an overall decrease in methylation in AD subjects, as well as hypomethylation of the APP promoter\textsuperscript{161,208,209}. Increased synthesis of APP leads to increased Aβ production and plaques in AD subjects.

TNFα treatment has also been found to induce expression of APP in AD subjects via the
transcription factor NF-κB\textsuperscript{210-212}. I hypothesize that increased oxidative stress, associated with aging, alters MS folate-dependent methylation and results in DNA hypomethylation in the cortex. In addition to changing redox and MS status, oxidative stress and inflammation can induce TNFα expression and signaling, further activating the transcription factor NF-κB. NF-κB initiates transcription of APP, which increases Aβ production in the cortex of AD subjects. Thus, redox status and decreased methylation associated with decreased MS activity will promote the formation of Aβ plaques in AD cortex (Fig. 56). Individuals harboring genetic risk factors affecting the efficiency of methylation (e.g. MTR or MTHFR) would be at higher risk for these pathological events.
Figure 56: Aging promotes formation of amyloid plaques. Oxidative stress increases with age and promotes TNFα production while decreasing MS activity. TNFα stimulates NFκB activity, which increases transcription of Aβ fragments, the main component of amyloid plaques. NFκB may also inhibit MS activity by blocking transcription factors from binding to MS promoters. Inhibition of MS may result in global demethylation, increasing the likelihood of transcriptional events.
E. Exon-specific Analysis

My results indicate that fetal samples express three isoforms of MS: full-length, spliced 19-20 and spliced 16-18 (Fig. 41). About 25% of transcripts are missing cap domain exons 19-20 and only a very small percentage of fetal transcripts are missing exons 16-18, corresponding to the folate-binding domain. Thus, one possible consequence of exon-skipping in the fetus would be decreased MS activity and altered methylation patterns.

My results for exon-specific analysis in SH-SY5Y cells yielded results in which variable amounts of both folate-binding and cap domains were expressed (Figs. 42 and 43). Most transcripts lacked exons 16-18, although a full-length transcript was present. Additionally, some transcripts lacked exons 16-20. It is likely that the changing redox status alters exon-skipping depending on whether transsulfuration or methylation is more important. Experiments investigating this process are outside the scope of my thesis, but deserve to be addressed in the future.

An examination of the folate-binding domain indicates that splicing of exons 16-18 may distort the structure and interfere with methylTHF binding. The additional absence of exons 19 and 20 could produce a distinctive difference in methylTHF affinity and also increase Cbl(I) vulnerability to oxidation. Splicing of exons 16-18 and 19-20 requires remodeling of the protein since the newly spliced exon “ends” need to be ligated to either the HCY- or cobalamin-binding domains, respectively. These structural modifications may decrease methylTHF affinity, thus extending the duration of the Cbl(I) state and increasing the probability of MS inactivation.
As previously mentioned, SH-SY5Y cells are tumor cell-derived and my observations may reflect tumor-specific splicing. However, similar exon-skipping phenotypes were observed in the fetal sample (spliced exons 16-18) as well as in age-dependent cortical subjects (spliced exons 19-20). Thus, alternative splicing of MS with exon skipping is a normal feature of human brain, with different patterns occurring at distinct developmental stages. These exon-specific studies highlight the combinatorial approach utilized by the brain to regulate MS. It seems likely that different isoforms are generated depending on the cellular redox condition, which can vary in response to different cellular conditions.

Alternative mRNA splicing is a process that has evolved to increase the complexity of the human genome, with the highest levels of splicing occurring in humans. Alternative splicing happens with sequentially decreasing frequency in rats, worms and plants. Alternative mRNA splicing gives rise to multiple protein products, expanding the genome and providing the cortex with greatly increasing metabolic complexity and flexibility.

The threat of oxygen to anaerobic organisms is considered to have been a driving force for evolution, and sulfur-metabolizing organisms were the best candidates for survival. Thus, it is likely that the cortex, as a highly evolved organ across species and brain regions, has evolved specialized adaptive responses to deal with oxidative stress. This strategy is evident by the remarkable evolutionary trend for higher brain levels of cystathionine.

It is unknown how alternative splicing of MS mRNA occurs, however one candidate is the RNA-specific adenosine deaminase (ADARs). ADARs edit canonical splice junctions by site-specific deamination of adenosines to inosine \((A\rightarrow I)\). ADAR activity is also increased during times of inflammation and oxidative stress, making it a possible candidate for redox-dependent splicing in the MS system. RNA editing is also prominent in the human brain, and
progressively increases during development\textsuperscript{219}, suggesting that redox status may be a driving factor for mRNA splicing. It is interesting to note that ADAR is expressed in neurons, but not astrocytes\textsuperscript{220}, and that GSH levels are lower in neurons\textsuperscript{155}. My data indicates that there is no alternative splicing in cultured glioblastoma cells (LN-18), suggesting that redox-dependent splicing is not a feature of all cell types and is more prominent in neuronal cells (Fig. 44).

The normal intron splicing process is highlighted in Fig. 57. Spliceosome machinery recognizes the 5' GU sequence and the A branch site. Interactions between spliceosome proteins loop these regions together, and the 5' site is cleaved. Lastly, the 3' AG site is cleaved and the lariat structure is discarded. This happens sequentially for the rest of the exon/introns. Normal progression of this process promotes the formation of full-length transcripts in fetal and SH-SY5Y samples.

However, modifications in the sequence (both the 3' AG and A branch site) may lead to alternative splicing of the transcript (Fig. 58). It is possible that ADARs target A branch sites within MS mRNA with site-specific deamination of adenosine to inosine (A\textrightarrow I). This modification would alter the ability of spliceosome machinery to recognize the intronic sequence, possibly promoting formation of alternative transcripts.
Figure 57: Normal splicing of MS mRNA exons 15 and 16. Splicing machinery excises intron 15 and ligates the two exons together. Splicing proteins recognize the 5' GU sequence and the A branch site. These two sections are brought together via protein interactions and the 5' site is cleaved. Lastly, the 3' AG site is cleaved and the lariat structure is discarded. This happens sequentially for the rest of the exon/introns.
Figure 58: Alternative splicing of MS exons 16-18. Folate-binding domain exons (16-18) are spliced in fetal cortex. In this proposed splicing mechanism, splicing machinery ignores intronic elements, which coordinate correct intron cleavage. This exon skipping promotes formation of a new peptide lacking exons 16-18. Fetal samples also contain full-length transcripts and transcripts lacking exons 19 and 20.
V. CONCLUSIONS

1) Exon skipping occurs in a redox-dependent manner in the folate-binding and cap domains of MS in SH-SY5Y cells. Other cell types (HEK, HepG2 and LN-18) are not involved in alternative splicing, suggesting that redox-dependent splicing is not a feature of all cell types and is more prominent in neuronal cells.

2) MS mRNA levels are lower in autistic subjects, with the greatest decrease occurring in younger subjects, when levels in control subjects are normally at their highest. Thus, autism likely results from a disruption of both normal redox regulation and methylation status.

3) TNFα treatment decreases MS mRNA levels and MS enzyme activity, indicating that inflammation and redox status play a role in modulation of the MS pathway. It is likely that redox status shifts the balance between EAAT3-mediated cysteine uptake and transsulfuration.

4) Human normal cortex shows an age-dependent decrease in both cobalamin-binding and cap domains, with alternative splicing of the cap domain occurring in subjects over the age of 60. Thus, MS plays a vital role as a sensor of redox status and a coordinator of adaptive responses, such as alternative splicing, to oxidative stress.
MS is regulated by multiple mechanisms in order to insure proper redox status in the body, and these functions address enzyme activity in both the short- and long-term. Short-term regulation of MS activity is addressed by oxidation of the cofactor cobalamin and by methylTHF availability. Modulation of mRNA, via splicing or decreased transcription, provides longer-term regulation. Exon-skipping of folate-binding and cap domain exons renders MS more sensitive to oxidative inactivation, and highlights an adaptive response to oxidative stress.

My thesis work demonstrated a remarkable age-dependent decrease in cortical levels of MS mRNA, underscoring its vital role as a sensor of redox status and a coordinator of adaptive responses to oxidative stress. Additionally, MS mRNA was decreased in autistic subjects, reflecting an adaptive response to neuroinflammation and oxidative stress.

Growth factors, such as IGF-1, stimulate cysteine uptake by promoting cell surface expression of EAAT3 transporters (Fig. 59). Due to the prevalence of growth factors in young subjects, transsulfuration is not favored, with GSH synthesis occurring via EAAT3-mediated cysteine uptake. However, oxidative stress increases with aging, thus altering redox status. This change shifts the balance between cysteine uptake and transsulfuration, with aging favoring transsulfuration. Epigenetic regulation, myelination, and network synchronization are just a few of the reactions that methylation influences. Fig. 60 summarizes my hypotheses involving cysteine influx and aging.

These findings reveal the importance of redox in the human brain and suggest that neurodevelopmental diseases such as autism are redox disorders. Furthermore, it seems likely that the same fundamental mechanisms contribute to other neurodevelopmental, neuropsychiatric and neurodegenerative disorders, as they occur across the lifespan.
Figure 59: Normal balance between cysteine uptake and transsulfuration. Growth factors stimulate cysteine uptake by promoting cell surface expression of EAAT3 transporters. In young subjects, transsulfuration (blue) is not favored, with GSH synthesis occurring via cysteine uptake (orange). However, oxidative stress increases with aging, thus altering redox status. This change shifts the balance between cysteine uptake and transsulfuration. Epigenetic regulation, myelination, and network synchronization are just a few of the reactions that methylation influences.
Figure 60: Summary figure of interplay between cysteine uptake and transsulfuration. Growth factors (triangles) bind growth factor receptors (GFRs) and stimulate Akt activity via PI3K. Downstream effects of Akt increase MS activity, increasing methylation capacity in neurons. Growth factors also stimulate cysteine uptake by promoting cell surface expression of EAAT3 transporters (pink circle). EAAT3 transporters take up astrocyte-derived cysteine (red circle) after GSH (tripeptide) cleavage in the extracellular space. Astrocytes also release TNFα, in response to oxidative stress, which binds TNFRs on neighboring neurons. TNFα stimulates NFκB activity, which increases transcription of Aβ fragments, the main component of amyloid plaques. NFκB may also inhibit MS activity by blocking transcription factors from binding to MS promoters. Inhibition of MS may result in global demethylation, increasing the likelihood of transcriptional events.
VI. REFERENCES


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194. Akiyama, Y. *TFSearch*. (Parallel Application TRC Laboratory: Japan, 1995).


204. Nishiyama, M., Kato, Y., Hashimoto, M., Yukawa, S. & Omori, K. Apolipoprotein E,


### Table 2: PCR primer sequences

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*Table 2: PCR primer sequences. Primer pairs include both forward and reverse primers, respectively (bp = base pairs).*
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Table 3: Control subject clinical details and cobalamin-binding and cap domain mRNA values. Control post-mortem cortex samples from ages 28 weeks to 84.7 years (PMI = postmortem interval).
Table 4: Clinical detail for control and autistic subjects.  

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a Autism Tissue Program identifier. All autistic samples were paired with an age- and sex-matched control.  
Mean age (± s.d.) for control subjects = 14.6 ± 9.3, for autistic subjects = 14.5 ± 9.1; paired-\( t = 0.314, 7 \text{ d.f.}, P = 0.763 \).  
Mean PMI (post-mortem interval) (± s.d.) for control subjects = 18.4 ± 7.0, for autistic subjects = 19.5 ± 5.5; paired-\( t = .775, 6 \text{ d.f.}, P = 0.468 \). Statistical results indicate no mean differences in age or PMI.  
† Autistic subjects that were not pair-matched, but were included in the mean MS mRNA analysis. Italics indicate cases of PDD-NOS (Pervasive Development Disorder Not Otherwise Specified).