Redox / methylation signaling: A novel epigenetic-based mechanism of opioid drug action

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List of Abbreviations

Aβ: amyloid β
AD: Alzheimer’s disease
BDNF: brain-derived growth factor
BSO: buthionine sulfoximine
CaMKII: calmodulin kinase II
CAT: catalase
Cbl: cobalamin
CNcbl: cyanocobalamin
CBS: cystathionine β-synthase
CREB: cAMP-regulatory element binding protein
CSF: cerebrospinal fluid
CYS: cysteine
DNMT: DNA methyl transferase enzyme
EAAT: excitatory amino acid transporter
ERK2: extracellular signal-regulated kinase 2
FMR1: Fragile X mental retardation protein type 1
G6PD: glucose-6-phosphate dehydrogenase
GCL: glutamate–cysteine ligase
GDNF: glial cell line-derived neurotrophic factor
GCS: γ-glutamylcysteine synthetase
GGT: gamma-glutamyl transferase
Glu: glutamate
Gly: glycine
GPX: glutathione peroxidase
GRX: glutaredoxin
GsCbl: glutathionylcobalamin
GSH: reduced glutathione
GSK-3β: glycogen synthase kinase-3β
GSR: glutathione reductase
GSS: glutathione synthetase
GSSG: oxidized glutathione
GST: glutathione S-transferase
GT: glutamate transporters
HCY: homocysteine
IGF-1: insulin-like growth factor-1
iPSCs: induced pluripotent stem cells
JNK: c-Jun N-terminal kinase
LINE1: long interspersed nuclear elements
LTP: long term potentiation
MAPK: mitogen-activated protein kinase
MeCbl: methylcob(III)alamin
MeCP2: methyl binding complex protein type 2
MeDIP: methylated DNA immunoprecipitation
MET: methionine
MethylTHF: 5-methyltetrahydrofolate
MOR: mu opioid receptor
MS: methionine synthase
MTHFR: methenyltetrahydrofolate reductase
mTOR: mammalian target of rapamycin
MTRR: methionine synthase reductase
NAC: N-acetyl-cysteine
NADP⁺: oxidized nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
NGF: nerve growth factor
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NMDAR: N-methyl-D-aspartate receptor
Non-LTR: non long terminal repeats
NRF2: nuclear erythroid factor type-2
ORF: open reading frame
PI3K: Phosphatidylinositol 3-kinase
PKA: protein kinase A
PLM: phospholipid methylation
POMC: pro opio melanocortin
PRDX: peroxiredoxin
PUFA: poly unsaturated fatty acid
R-S₂: disulfide
R-SH₂: reduced thiol
RNS: reactive nitrogen species
ROS: reactive oxygen species
SAM: S-adenosylmethionine
SAH: S-adenosylhomocysteine
SOD: superoxide dismutase
TNFα: tumor necrosis factor-alpha
UTR: untranslated region of DNA
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Abstract

Drugs of abuse affect the capacity for attention and awareness and produce altered states of consciousness, implying that they act upon the molecular mechanisms, which support psycho-behavioral status and reactivity. Since attention is closely linked to learning and memory, it is not surprising that frequent use of some drugs results in persistent behavioral changes at times, which can result in withdrawal syndrome and observed symptoms, indicating that neuronal systems have inherent modes of plasticity, supporting durable adaptation to repeated drug use. Mounting evidence indicates that epigenetic changes, specifically alterations in the patterns of DNA and histone methylation, are a central mechanism for learning and memory.\textsuperscript{1,2} Epigenetic changes can produce long-lasting alterations in gene expression and behavior that are linked to the initiating/precipitating event.\textsuperscript{2} Thus, an action on methylation-related metabolic pathways could help explain at least some aspects of the acute and longer-term effects of drugs of abuse. This thesis project investigates the effect of morphine and other opiates on pathways of sulfur metabolism which support and control methylation activity.

Building upon substantial preliminary data, I investigated the acute and long-term influence of selected opioid drugs on redox and methylation status (including DNA methylation status) in cultured neuronal SH-SY5Y cells. I also characterized mu opiate receptor (MOR) involvement and identified downstream signaling pathways (including different G-proteins and protein kinases) involved in mediating the cellular effects of opiates at several time points. Removal of opiate drugs after a prolonged exposure (i.e. \textit{in vitro} “washout”) also altered neuronal-cell redox status and methylation capacity in ways distinct from the initial opiate exposure. Opiate-induced changes in DNA methylation levels
were observed, accompanied by genome-wide changes in mRNA levels, the latter characterized using a microarray expression assay. mRNA levels of key enzymes and transporters comprising the relevant sulfur and methionine metabolism pathways were also altered under the influence of opiate drugs. Opiate drugs significantly altered the DNA methylation status and mRNA levels of Long Interspersed Nuclear Elements (LINEs) after acute (4-hr) and longer-term (24-hr) treatment periods; the methylation status of these retrotransposon elements serves as a surrogate for global DNA methylation. The redox/methylation metabolic interventions N-acetylcysteine (NAC) and methylcobalamin (MeCbl) abrogated the effects of morphine exposure on neuronal-cell redox and methylation status.

Taken together, these findings advance our understanding of the mechanism by which opioids influence neuronal-cell redox and methylation status and the molecular machinery responsible for regulating methylation reactions, including DNA and histone methylation, which modulate gene transcription via their epigenetic effects. The aggregate data provide some insight towards a novel perspective for the origin of addiction, tolerance and withdrawal phenomena associated with drugs of abuse that may aid in the development of new treatment approaches for these conditions.
I. INTRODUCTION

A. Statement of Problem

Canonically, opiates influence cells by binding to a G protein-coupled receptor (GPCR), initiating intracellular signaling cascades such as Phosphoinositide-3 kinase (PI3K), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) or Extracellular receptor kinase (ERK) pathways.

Many of these signaling pathways achieve their effects through regulation of transcription factors, resulting in altered gene transcription. It is increasingly appreciated that cell redox status can modulate gene transcription, since it is a major determinant of DNA and histone methylation. Cellular redox status is primarily regulated by the availability of reduced glutathione (GSH), which donates electrons for neutralization of reactive oxygen species (ROS) or the repair of oxidized proteins or lipids.

Cysteine, the rate-limiting precursor for synthesis of GSH in the cytoplasm, is made available by two pathways: extracellular uptake or metabolism of homocysteine via the transsulfuration pathway. However, transsulfuration is limited in adult cortical neurons, emphasizing the importance of cysteine uptake through excitatory amino acid transporter 3 (EAAT3), which accounts for about 90% of cysteine uptake in this cell type. Thus, EAAT3 activity is critical to regulation of redox status in mature cortical neurons. It has previously been shown that opiates can induce oxidative stress, causing a pro-oxidant shift in cell redox status, subsequently leading to a decrease in more than
200 methylation reactions, including DNA and histone methylation. These changes in methylation allow opiates to broadly affect almost every aspect of cellular metabolism via the pervasive influence of methylation reactions, including epigenetic and gene-transcription changes.

As summarized in Fig. 1, this thesis research is formulated from a central proposition that opiates alter GSH-related redox poise of neuronal cells in ways that affect the metabolic underpinning of DNA methylation and consequent gene transcription. The results define the effects exerted by opiates on the redox state of a cultured human neuroblastoma cell line, SH-SY5Y, a well-accepted in vitro model of neuronal differentiation and function. Further, the consequent influence of opiate-induced cellular redox changes on global epigenetic and transcription status is also determined. The relationship between opiate-induced neuronal redox changes and changes in global cell epigenetic and transcription status is specifically characterized for retrotransposon elements. Lastly, Redox and metabolic interventions are identified that are able to modulate the redox-based epigenetic effects of opiate drugs in ways that point to tenable therapeutic strategies for treating drug addiction and withdrawal phenomena.
Figure 1: Proposed opioid redox-based epigenetic signaling pathway in the brain. Opiate-induced oxidative stress alters EAAT3-mediated cysteine uptake, further affecting GSH synthesis and redox homeostasis in neuronal cells. The consequent changes in methionine synthase (MS) activity affect levels of SAM and the probability of DNA methylation. Changes in DNA methylation lead to changes in gene transcription. The transcriptional effects may express themselves in terms of neuronal plasticity and behavior associated with the psycho-behavioral phenomena of opiates and other drugs having abuse potential.

B. Evolution of an Oxygen-based Redox System

Molecular oxygen, whether as a gas or dissolved in aqueous media, has played an important role in evolution. The availability of oxygen as the terminal electron acceptor in the series of mitochondrial redox reactions collectively termed the mitochondrial electron-transport chain supports high-efficiency energy production through oxidative phosphorylation and ATP synthesis. A small fraction of electrons do not participate in the complete reduction of oxygen to water, generating partially reduced species of
oxygen, including free radicals and ROS. For example, approximately 2% – 4% of the oxygen consumed by mitochondria ends up as the superoxide anion free radical (O$_2^-$).\textsuperscript{4} ROS can be neutralized by chemical antioxidants and redox-active metabolic enzymes. Low levels of ROS play important physiological roles in synaptic plasticity, cognitive performance and memory formation,\textsuperscript{5} whereas ROS levels in excess of the antioxidant capacity to prevent their causing cellular damage, creates a state of oxidative stress that has been linked to neurotoxicity and neurodegeneration.\textsuperscript{6} Hence, an imbalance in physiological equilibrium between endogenous antioxidant defenses and ROS level can compromise neuronal function and, potentially cognitive performance, as conceptualized in Fig. 2. This equilibrium is termed as redox status and it can be conceptualized to drive memory formation, cognition and neurodevelopment. A better knowledge of how redox equilibrium is maintained in brain, and how it can be utilized as a signaling mechanism (i.e. redox signaling), would improve our understanding of a wide range of brain disorders, including neurodevelopmental, neuropsychiatric and neurodegenerative disorders, but also symptoms associated with drug abuse.
Physiological concentrations of ROS, controlled by the level of (chemical or enzymatic) ROS dismutation/inactivation as well as antioxidants, and can drive processes like normal long-term potentiation (LTP) and memory formation, but uncontrolled pathophysiological concentrations in excess of antioxidants might contribute to memory impairment. Hence, antioxidant status may play a role in regulating cognitive performance.

C. Oxidative Stress and Redox Status

The condition of oxidative stress is established by an imbalance between tissue ROS level/production and the ability of tissue antioxidant defenses to detoxify ROS and keep these tissue-damaging species at levels within a physiological range vs. pathological, tissue-damaging levels. When the latter occurs (i.e. ROS in excess of tissue ability to detoxify them), cell damage ensues. The damage is a consequence of the oxidative stress which, if not adequately remediated by tissue-repair mechanisms (not by detoxifying ROS), can support cell injury/death.

While the main source of ROS in vivo is aerobic respiration, they can also be produced by peroxisomal β-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, and stimulation of phagocytosis by pathogens or...
lipopolysaccharides, as well as other tissue-specific enzymes. Lastly, another major source is the pro-inflammatory ROS burst associated with activated macrophages.

Examples of ROS include the hydroxyl radical (-OH), superoxide (O$_2$-), hydrogen peroxide (H$_2$O$_2$), and peroxynitrite (ONOO-). ROS can be detoxified by cellular enzymes; such as superoxide dismutase (SOD) and catalase. ROS can be inactivated by non-enzymatic chemical reduction by reducing agents (antioxidants) such as GSH and other thiol-containing molecules, α-tocopherol (vitamin E), carotenoids, ascorbic acid, bilirubin, uric acid, and albumin. Superoxide dismutase (SOD) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide.$^7$ The SOD-catalysed dismutation of superoxide may be written with the following half-reactions:

\[
\begin{align*}
M^{(n+1)+} + \text{SOD} + \text{O}_2^- & \rightarrow M^{n+} + \text{SOD} + \text{O}_2 \\
M^{n+} - \text{SOD} + \text{O}_2^- + 2\text{H}^+ & \rightarrow M^{(n+1)+} - \text{SOD} + \text{H}_2\text{O}_2.
\end{align*}
\]

Where: $M = \text{Cu} \ (n=1) ; \text{Mn} \ (n=2) ; \text{Fe} \ (n=2) ; \text{Ni} \ (n=2)$.

Catalase, which is concentrated in peroxisomes located next to mitochondria, reacts with the hydrogen peroxide to catalyze formation of water and oxygen.

\[
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \quad \text{(catalase)}
\]

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS–SG} + 2\text{H}_2\text{O} \quad \text{(glutathione peroxidase)}
\]
Figure 3: 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 (ONOO-) which is a form of nitrosative stress. Oxidative damage can occur in DNA, proteins and lipids. GSH plays a central role in neutralizing almost all of these ROS reactions via direct and indirect pathways. Modified from Thorax et al.8

The main cellular damage caused by ROS is the oxidation of macromolecules, such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. The effect of ROS on biomolecules under physiological pH depends upon their redox potential and inherent chemical reactivity. For example, superoxide anion radical does not have the capacity to abstract allylic hydrogen from PUFAs/HUFAs and thereby initiate peroxidative membrane-lipid damage, whereas hydroxyl radical does.7
Lipid peroxidation can compromise the integrity and functioning of cellular membranes, leading to cell death. Oxidative stress and ROS have been implicated in various disease states such as Alzheimer’s disease (AD), Parkinson’s disease (PD), cancer, and aging.6

**D. Glutathione (GSH) Synthesis**

The redox state of a cell may change when the production of ROS or availability of antioxidants changes. GSH is important in the detoxification and elimination of ROS, and a reduction in cellular GSH levels can lead to ROS accumulation and oxidative stress. Adaptive changes in sulfur metabolism, in the form of regulated GSH production, are essential for survival in an oxidative environment, and the ability to maintain GSH levels in response to oxidation is a fundamental process in living cells.

GSH is a thiol peptide formed from three amino acids: glutamic acid, cysteine and glycine.9 The two-step synthesis of GSH begins with the rate-limiting conjugation of cysteine and glutamic acid by γ-glutamylcysteine ligase (GCL) to form γ-glutamylcysteine.10 GCL activity is regulated by the transcription factor Nrf-2, which binds to antioxidant response elements (AREs) in response to oxidative stress.11 GSH synthetase catalyzes conjugation of γ-glutamylcysteine with glycine, resulting in GSH formation. Energy for GSH synthesis is supplied by ATP.12 The sulphydryl group (-SH) of the cysteine residue in GSH provides the critical site for various conjugation and reduction reactions between GSH and other biomolecules.9 The oxidized dimeric form of GSH (GSSG) can be converted back to reduced-GSH through reduction by glutathione reductase. Similarly, glutathionylated proteins (GSH-bound proteins) can be reduced via
thiol-disulfide exchange by thioredoxin, with reducing equivalents provided by NADPH.\textsuperscript{12} Thioredoxin reductase uses the electronegativity of its reduced selenocysteine residue to reduce thioredoxin back to an active state.\textsuperscript{13}

E. GSH as an Antioxidant.

GSH is the most abundant thiol and most important low-molecular-weight antioxidant found in cells. The intracellular concentration of GSH in most cells averages 1-2 mM,\textsuperscript{9} and may vary from about 10 mM in hepatocytes to 0.2 mM in neurons.\textsuperscript{4,10} Hepatocytes provide much of the GSH found in plasma.\textsuperscript{12} Because of the relatively low content of GSH in neurons, replenishment of GSH by the reduction of GSSG becomes an especially important process in neurons. A sub-physiological level of GSH can lead to accumulation of ROS and oxidative stress, compromising cellular function.\textsuperscript{14} Reciprocally, increased GSH synthesis increases antioxidant preserves and promotes metabolic activity.

The sulfhydryl group (–SH) of the cysteine residue in GSH is involved in a wide array of reduction and conjugation reactions.\textsuperscript{12} Organic and inorganic free radicals, as well as peroxides, can be reduced by GSH.\textsuperscript{12} In the case of inorganic free radicals such as O$_2$\textsuperscript{−}, the free radical is enzymatically converted to H$_2$O$_2$ by SOD, and the H$_2$O$_2$ is then reduced back to its normal oxidation state (i.e. water) by GPx, using GSH as the electron donor (Fig. 3). GPx contains a selenocysteine amino acid at its active site. The selenocysteine is oxidized by peroxide to form SeOH, which is reduced by GSH to form GS-Se.\textsuperscript{15,16} Selenocysteine is then reduced by another GSH molecule to reactivate the enzyme, releasing GSSG. Glutathione reductase then converts GSSG back to GSH, using
NADPH as the reductant. Selenium is an important redox-active element because selenium is less electronegative than sulfur, which is in turn less electronegative than oxygen, meaning that selenium will reduce sulfur, which will in turn reduce oxygen. Organic peroxides are also reduced by glutathione-dependent mechanisms. Lipid peroxidation is an example of a cell-damaging reaction regulated by a GSH-dependent selenoprotein, such as glutathione peroxidase 4 (GPX4), which can reduce lipid peroxides to alcohols. Protein functions are also regulated by GSH. The role of GSH in reducing oxidized thiol-containing proteins is catalyzed by thiol-transferases.

**F. Cysteine Uptake, EAAT3 Function and Regulation.**

Extracellular cysteine uptake is mediated via several different transport proteins, including EAATs (Excitatory Amino Acid Transporters), ASC (amino acid transporter system) and the System L amino acid transporter system. However, EAAT-3 (EAAC1 in mice) is responsible for about 90% of total cysteine uptake in neuronal cells (Fig. 4), making EAAT-3 the predominant cysteine transporter in neurons. 17 Five members of the EAAT family have been identified: glutamate-aspartate transporter (GLAST or EAAT1), glutamate transporter 1 (GLT-1 or EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5. GLAST and GLT-1 are found in astrocytes, oligodendrocytes and other glial cells. 18 EAAT4 and EAAT5 are restricted to cerebellar Purkinje cells and retina. 18 EAAT3 is uniquely and exclusively localized to neurons. 19 Therefore, EAAT3 is the only transporter capable of importing cysteine into mature human neurons. EAAT3 is located on the postsynaptic surface of the neuron and can transport either cysteine or glutamate, but prefers cysteine. 11, 17 Knockdown of EAAT3 does not lead to changes in extracellular glutamate concentration but can affect intracellular cysteine concentrations, whereas knockdown
of EAAT1 and EAAT2 leads to increases in extracellular glutamate concentrations,\textsuperscript{20} indicating that the primary role of EAAT3 is cysteine uptake, not glutamate uptake. A list of all EAATs, along with their location and function is given in Table 1.\textsuperscript{10,20,21}

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

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

Figure 4: Membrane topology model of the excitatory amino acid transporter. This model of an EAAT transporter shows the 8 transmembrane domains as well as the active loop structure between transmembrane domains 7 and 8 that carries glutamate, aspartate or cysteine into the cell. This model also shows the GTRAP regulatory site on the C-terminal tail. (Adapted from Chen and Swanson)\textsuperscript{22}

Studies with EAAT3 inhibitors and EAAT3-deficient mice showed a decrease in GSH levels in neuronal cell cultures and brain, respectively, indicating that EAAT3 plays an important role in maintaining physiological GSH antioxidant levels that protect neurons against oxidative stress.
Figure 5: Several factors regulate EAAT3 expression in neurons. (1) Growth factors, acting via the PI3K pathway, can directly stimulate transporter activity by promoting their trafficking to the surface membrane. (2) Modulators of GTRAP3-18 can regulate gene expression of EAAT3 and/or the cytosolic pool of transporters. (3) Factors activating αPKC can affect transcription of EAAT3 protein. (Adapted from Nieoullon, A et al) \(^{19}\)

Under physiological conditions, EAAT3 is primarily sequestered in intracellular vesicles, with only 20% of the transporter localized at the cell surface.\(^4,14,20\) An increase in translocation of EAAT3 to the cell surface can be induced by three mechanisms: PI3K/Akt activation, PKC activation, and inhibition of glutamate transport-associated protein (GTRAP3-18) binding to EAAC1 (Fig. 5).\(^10,23,24\) Activation of PKCα causes
phosphorylation of serine 465 that precedes the increase in cell surface expression, and this activation both increases EAAC1 membrane insertion and decreases EAAC1 endocytosis. Previous studies in our laboratory have shown that neurotrophic growth factors (NTFs), including BDNF, IGF-1 and NGF, promote EAAT3 activity via the PI3K pathway. GTRAP3-18 interacts with EAAT3 at its intracellular carboxyl-terminal domain and acts as a negative regulator of EAAT3, decreasing cell-surface expression of EAAT3. In addition, knockdown of GTRAP3-18 with antisense RNA leads to an increase in EAAT3 cell-surface expression and an increase in intracellular GSH. Chronic morphine up-regulates GTRAP3-18 and decreases EAAT3 surface expression by up to 50%. Activation of the δ-opioid receptor also decreases cell-surface expression of EAAT3. Previous studies in our laboratory have characterized the presence of EAAT3 in SH-SY5Y human neuroblastoma cells. EAAT1 is also expressed in these cells, to a lesser extent than EAAT3, but studies with the specific EAAT3 blocker L-beta-threo-benzyl-aspartate (LBTBA) showed that EAAT3 is the major cysteine transporter for SH-SY5Y cells.

Isoflurane and other volatile anesthetics up-regulate EAAT3 transcription and increase glutamate uptake. This up-regulation is independent of both PKC and PI3K, however, and phosphorylation of serine 465 is critical for isoflurane-induced EAAT3 cell-surface expression; however, intravenous anesthetics have no effect on EAAT3 expression. In other studies, the transcription factor regulatory factor X1 (RFX1) was shown to increase both EAAT3 expression and activity. Another important regulator of EAAT3 level is the redox-sensitive transcription factor nuclear-erythroid factor type-2 (Nrf-2), which increases the transcription of antioxidant pathway genes in
response to oxidative stress, including those for enzymes involved in GSH synthesis and EAAT3.27,33

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

The brain exists within a closed compartment, shielded by the blood-brain barrier and surrounded by cerebrospinal fluid (CSF). However, the level of the sulfur-containing amino acid cysteine, which is rate-limiting for GSH synthesis, is remarkably lower (>10-fold) in CSF compared to plasma (Fig. 6),9,11 implying limited antioxidant resources in brain. The neuronal GSH level reflects a metabolic interdependence between astrocytes and neurons, with consequences for regulation of neuronal redox status.10,12,20 GSH released from astrocytes is hydrolyzed to cysteine, which is taken up by neurons via EAAT3 and used by neurons to synthesize GSH. This metabolic interdependence allows antioxidant resources to be stored and released in a local, site-specific manner while maintaining low GSH levels in CSF. As a result, neurons are highly dependent upon astrocyte-derived cysteine for maintenance of their redox status, and the gating of cysteine uptake by EAAT3 becomes an exceptionally powerful mechanism for regulating redox status in neurons.
Figure 6: Thiols in the brain. The human brain contains low levels of cysteine and GSH as compared to blood. GSH levels in astrocytes are 4-fold higher than in neurons. Neurons rely on cysteine from astrocyte-derived GSH to synthesize their GSH and maintain neuron redox balance.

H. Transsulphuration

Cysteine availability is the rate-limiting factor for GSH synthesis. Figure 7 illustrates the two possible pathways that can provide the necessary cysteine for GSH synthesis in mature neurons: 1) conversion of homocysteine (HCY) to cysteine; 2) transport of extracellular cysteine via EAAT3.

The two-step process involved in synthesis of cysteine from homocysteine is termed the transsulfuration pathway. In the first step, cystathionine-β-synthase (CBS) conjugates serine with HCY to form cystathionine, an intermediate between the methionine cycle and cysteine formation. SAM levels is a positive modulator of CBS, and similarly oxidative stress and reduction in GSH levels can also increase enzyme
activity. Cystathionine-gamma-lyase (CGL) then cleaves cystathionine into cysteine and alpha-ketobutyrate.

About 50% of the cysteine needed as a precursor for the production of GSH in liver is supplied by the transsulfuration pathway. However, incubation of neuronal cultures with methionine does not increase the intracellular levels of GSH, indicating that formation and cleavage of cystathionine is not a major source of cysteine in neuronal cells. Astroglial cells serve as the primary source of extracellular cysteine for neuronal uptake, releasing about 10% of their intracellular content of GSH into the extracellular space. As indicated in Fig. 7, the released GSH undergoes sequential cleavages to form cysteine, which is available for uptake by adjacent neurons. The ectoenzyme γ-glutamyl transpeptidase cleaves GSH to glutamate and dipeptide cysteinylglycine. Cysteinylglycine is then broken down to cysteine and glycine. Glial cells contain an active transsulfuration pathway and are constantly synthesizing and exporting GSH. Glial cells take up cystine and reduce it to cysteine for synthesis of GSH.
Figure 7: Sulfur metabolism pathways in neuronal cells. Red text highlights the transsulfuration pathway, ending with the formation of GSH. Blue text features dopamine-stimulated phospholipid methylation, utilizing methionine synthase (MS). Orange text shows cysteine uptake into neurons via the EAAT3 transporter, the cysteine originating from GSH released from glial cells and sequentially cleaved to its amino-acid components. Regeneration of methylcobalamin through the addition of SAM to glutathionylcobalamin is shown in green.

I. Methionine synthase acts as a redox switch

Apart from its metabolism to cysteine, homocysteine is also an important regulatory metabolite for the methylation cycle. Homocysteine is methylated to methionine by the enzyme methionine synthase (MS). MS is a folate- and cobalamin-dependent enzyme structurally organized into five domains, four of which bind HCY, methylfolate, cobalamin, and S-adenosylmethionine (SAM). A fifth domain, termed the
cap domain, links the folate- and cobalamin-binding domains and partially covers and protects cobalamin from oxidation by limiting access of ROS and electrophiles from the surrounding redox environment.

The enzyme methionine adenosyltransferase (MAT) converts methionine to SAM in an ATP-dependent manner. SAM, the methyl donor for more than 200 methylation reactions, is utilized in the methylation of membrane phospholipids, nucleic acids, proteins, biogenic amines and a wide variety of other substrates. SAH is formed after SAM donates its methyl group to other molecules, a reaction catalyzed by several hundred methyltransferase enzymes. Further, SAH is hydrolyzed to homocysteine and adenosine by the enzyme SAH hydrolase (Fig. 7). However, this hydrolysis is reversible, with SAH formation being thermodynamically favored. SAH is a potent inhibitor of methylation reactions, based upon its relatively high affinity for the SAM binding site. Thus, HCY is an important thiol metabolite, because it can be (1) remethylated to methionine to support methylation, (2) converted to cystathionine via the transsulfuration to support GSH synthesis, or (3) metabolized to SAH to inhibit methylation.

Cobalamin is an essential cofactor for methionine synthase, and its oxidation state regulates the activity of the enzyme. Figure 8 illustrates the differential oxidation state of the cobalamin cofactor during the activation and reaction of MS enzyme and also depicts the regeneration of this cofactor after its oxidation. Methylcobalamin or methylcob(III)alamin is formed when 5-methyltetrahydrofolate (MTHF) transfers a methyl group to Cob(I). Methylcobalamin subsequently donates the methyl group to the thiol group of homocysteine, forming methionine. Cob(I)alamin can then accept another
methyl group from methylTHF and continue with methylation of another molecule of homocysteine.

Figure 8: Enzymatic reaction catalyzed by methionine synthase and reactivation of cobalamin after its oxidation. (Adapted from Wilson, A. et al.)

For the MS reaction to continue, methylTHF needs to remethylate Cbl(I) to MeCbl; however, Cbl(I) is highly unstable and is easily oxidized to the inactive form, cob(II)alamin (Cbl(II)), depending upon the cellular redox environment. The chance for oxidation of Cbl(I) to Cbl(II) increases as a consequence of limited methylTHF availability, which further results in MS inactivation, halting methylation of HCY to methionine. When methylTHF is scarce or under oxidative-stress conditions, Cbl(I) is increasingly oxidized to Cbl(II). SAM can reactivate MS by donating a methyl group to Cbl(II) to form MeCbl, (Fig. 8); however, SAM-dependent reactivation is only favored when cobalamin is present in the Cbl(II) state, ensuring that methylTHF is the primary
source of methyl groups. Cbl is generally thought to remain bound to MS, but recent studies have revealed alternatively spliced forms of the cap domain of MS in human brain tissue and cultured SH-SY5Y cells that allow Cbl(II) dissociation and its replacement with MeCbl or glutathionylcobalamin (GSCbl) (Fig. 9). GSCbl can be converted to MeCbl via SAM-dependent methylation. These dissociation-based options provide alternative mechanisms for MS reactivation. During oxidative stress, MS is inhibited to prevent HCY conversion to methionine, shifting HCY to the transsulfuration pathway for synthesis of GSH so as to maintain cellular antioxidant defenses against oxidative stress. Restoration of adequate GSH levels allows GSCbl formation and MS reactivation.

Thus, there are multiple mechanisms, which regulate MS activity and help maintain redox homeostasis, namely: oxidation of the cofactor cobalamin, alternative splicing of the cap domain, and modulation of transcription levels. MS, therefore, functions as an important redox-sensitive switch that regulates the balance among the transsulfuration pathway, GSH levels, methionine levels, redox homeostasis and methylation.
Methionine synthase acts as a redox switch. Methionine synthase contains a redox-active methylcobalamin cofactor. Under oxidative stress, this cofactor becomes oxidized, limiting methionine synthase activity. Under these conditions, homocysteine can be condensed with serine to form cystathionine and then with cysteine to support GSH synthesis. Only when cellular redox state is restored does the favorable GSH / GSSG ratio allow for the glutathionylation of oxidized cobalamin and methylation of the glutathionylcobalamin to reactivate the enzyme MS.
J. Methylation, Epigenetics

Methylation reactions, including DNA and histone methylation, utilize SAM produced by the methylation cycle (Fig. 10). SAH, a product of methylation reactions, serves as a methylation inhibitor; increasing the availability of SAH and/or decreasing SAM levels leads to inhibition of methylation reactions. Since SAH is reversibly converted to HCY by SAH hydrolase (Fig. 7), increased levels of HCY also inhibit methylation. Hence, activities of the two enzymes that act on HCY (MS and CBS) exert an important regulatory influence over methylation reactions: an increase in MS activity will potentially increase DNA methylation, whereas a decrease has the potential to limit methylation reactions. Since MS activity is redox-sensitive, DNA methylation status is also highly sensitive to cellular redox status, with MS and CBS providing the crucial link.

**Figure 10: DNA Methylation.** DNA methyltransferase adds the methyl group from SAM to position 5 on the cytosine ring, converting cytosine to 5-methylcytosine. This methyltransferase reaction converts the SAM back to SAH. Usually, the methylation takes place at specific cytosine-guanine dinucleotides, clustered into “CpG islands”.

![DNA Methylation Diagram](image)
The high sensitivity of MS activity to inactivation by ROS or other electrophiles provides a mechanism for cells to maintain their redox status. Inactivation of MS by oxidative stress increases the conversion of HCY to cysteine thereby enhancing substrate availability for synthesis of GSH, which aids in restoring a cellular redox balance favorable for methylation. Changes in gene expression during periods of MS inactivation can also contribute to restoration of redox balance, illustrating the close metabolic relationship between cellular redox state and methylation levels. Thus, factors regulating sulfur metabolism and cysteine uptake can influence methylation and epigenetics.

All cells in an individual organism contain the same DNA and the explicit feat of cell type-specific differential gene expression is accomplished by an ensemble of regulatory events, collectively described as epigenetic mechanisms, including DNA methylation, histone methylation and acetylation, and which functions in concert with promoter or repressor effects of transcription factors, and other regulatory elements such as microRNAs (Fig. 11).

Methylation of DNA at CpG sites is a primary epigenetic mechanism, which can reversibly suppress gene expression. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and involves the addition of a methyl group from the methyl donor, SAM, to position 5 in the cytosine pyrimidine ring, resulting in 5-methylcytosine (5meC). Methylation of DNA in brain is catalyzed by three main enzymes; DNA methyltransferases (DNMT) type 1, 3a and 3b. A role for brain DNA methylation in adult neural plasticity is supported by the observation that DNMT
inhibitors alter behavioral effects in learning and memory paradigms. DNA demethylation is initiated by Tet-family enzymes which convert 5-methylcytosine to 5-hydroxymethyl cytosine, which is subsequently converted to cytosine via one or more mechanisms. Other DNA demethylation mechanisms include 5-methylcytosine glycolases, which convert 5-methylcytosine to cytosine. Thus levels of 5-hydroxymethylcytosine contribute to the cell levels of total DNA methylation. Several environmental stimuli induce demethylation of particular genes in the brain. For example, consolidation of memory after fear conditioning increases demethylation as well as expression of the gene reelin, which is important for synaptic plasticity.

**Figure 11: Regulation of gene transcription.** Transcription of genes is regulated by canonical transcription-factor binding and epigenetic DNA modification.
Methylation of specific cytosine-guanine dinucleotide clusters (CpG islands) interferes with transcription factor binding to target sequences through recruitment of numerous co-repressor complexes. Such repressive complexes are recruited to methylated DNA through the actions of methyl-binding domain-containing proteins such as MeCP2 and MBD1, which function to localize and stabilize further additional co-repressors [e.g. histone deacetylases (HDACs)] at gene promoters. Binding of the transcription factor CREB, for example, can be affected by DNA methylation at gene promoters, since the consensus cAMP response element (CRE) sequence contains a CpG island, which, when methylated, prevents CREB binding to its target sequences. As a primary driving force for development, epigenetic regulation is essential for X-chromosome inactivation, genetic imprinting, cellular homeostasis, silencing of DNA elements, and chromatin remodeling. These modifications are both stable and dynamic. Epigenetic patterns can be inherited across generations, but \textit{de novo} CpG methylation and demethylation is also constantly occurring, providing genomic plasticity during normal brain development across the lifespan and especially during early postnatal stages.

\textbf{K. LINE-1 Retrotransposons.}

LINE-1 (L1) retrotransposons comprise ~ 20 \% of total human genomic DNA and are considered to be an instrumental force in the evolution of genome architecture.\textsuperscript{40,41} Almost all of known L1s are molecular fossils which do not move (i.e. retrotranspose) to new genomic locations.\textsuperscript{41,42} However, studies have identified a few human-specific L1 (L1Hs) elements which are competent for retrotransposition and have been strongly implicated in the occurrence of various diseases.\textsuperscript{43–45} L1s use a ‘copy and paste’
mechanism to insert extra copies of itself throughout the genome. They influence chromosome integrity and gene expression upon reinsertion. These L1Hs elements are stratified into several subfamilies (pre-Ta, Ta-0, Ta-1, Ta1-d, Ta1-nd) according to sequence variations within their 5’ and/or 3’ untranslated regions (UTRs). Additionally, several L1Hs elements are dimorphic and are differentially present across genomes and/or are present in an individual’s genome, but absent from the haploid Human Genome Reference sequence (HGR). Approximately ~80–100 active (retrotransposition-competent) L1Hs elements are estimated to be present on average in the human genome, and only few highly active L1Hs elements (“hot” L1s) are responsible for the major retrotransposition activity reported in the HGR. These and other recent studies indicate that ongoing L1 retrotransposition can contribute to inter-individual genetic variation in humans. Additionally, studies show that L1 elements are active and ‘jumping’ during neuronal differentiation and might even contribute to the process of neuronal differentiation.
Figure 12: **L1 retrotransposition cycle.** L1 mRNA (red) is exported into the cytoplasm, translated, and L1-encoded proteins (L1 ORF1p, L1 ORF2p) bind to their own mRNA (cis preference) and form ribonucleoprotein (RNP) complexes, which are re-imported into the nucleus. Subsequently, L1 RNA is reverse-transcribed, and the cDNA is inserted into the genome by a mechanism termed target-primed reverse transcription (TPRT). Frequently, reverse transcription fails to proceed to the 5' end, resulting in truncated non-functional L1 de novo insertions. (Adapted from Schuman et al.)

It is also believed that newly inserted somatic L1s can generate ‘genomic plasticity’ in neurons by causing a variation in genomic DNA sequences and by altering the transcriptome of individual cells, causing intra-individual variations or mosaicism. Thus, L1-induced variation could affect neuronal plasticity and behavior. Studies also show that LINE-1 expression increases in nucleus accumbens with cocaine administration and might lead to global patterns of genomic destabilization, exemplifying the ability of some drugs of abuse to alter LINE1 expression patterns.
Changes in global DNA methylation are expected to alter the methylation state of LINE-1. This can influence the role of LINE-1, which is transcriptionally silenced mainly due to CpG methylation. Any change in the levels of methylation, especially at the promoter region, could affect the retrotransposition activity of these elements, further affecting genomic plasticity and transcription status in neurons. However, these functional consequences are not yet elucidated.

L. Opiate drugs and opioid receptor pharmacology.

Opiate compounds are the most potent analgesics, and opiate drugs have been used for centuries. Opiates occur as natural alkaloids in the opium poppy plant and belong to the large biosynthetic group of benzylisoquinoline alkaloids. Morphine, codeine, and thebaine are the major psychoactive opiates. Clinical studies identify morphine as the gold standard, or benchmark, for relieving severe acute and chronic pain and suffering. Despite their effectiveness in treating acute pain, serious complications are prevalent with long-term opiate use, including tolerance, physical dependence, and mainly opiate addiction, and even acute opioid use (e.g., post-surgery) may be associated with quite unpleasant side-effects (nausea, emesis). The medical use of morphine and other opiates for chronic pain disorders has steadily risen, in the absence of superior treatment options. In one study, former opiate addicts showed a strong preference for heroin and morphine in comparison to other opioids like hydromorphone, fentanyl, oxycodone, and pethidine/meperidine, which might suggest that heroin and morphine are particularly susceptible to abuse and addiction; however, more evidence is needed to strongly support this conclusion. Heroin is metabolized to
morphine before binding to opioid receptors in the brain and spinal cord, where morphine then causes its subjective effects.

Endogenous opioids include endorphins, enkephalins, dynorphins, and even morphine itself. Morphine is reported to act in a similar manner to endorphins, which are responsible for analgesia (reducing pain), causing sleepiness and feelings of pleasure and are generally released in response to pain, strenuous exercise, orgasm, or excitement.⁵⁸ [Met⁵]-enkephalin, an endogenous opioid peptide, plays an important role in cell proliferation and tissue organization during development and is also a neuromodulator in the nervous system.⁵⁹ [Met⁵]-enkephalin is also termed as opioid growth factor (OGF) and is one of the two forms of enkephalin, the other being [leu]-enkephalin.⁶⁰ Due to their high affinity and selectivity for δ-opioid receptors, the enkephalins are considered to be the primary endogenous ligand for the δ-opioid receptor.⁶¹ [Met⁵]-enkephalin is found mainly in the adrenal medulla and throughout the central nervous system (CNS), including the striatum, cerebral cortex, olfactory tubercle, hippocampus, septum, thalamus, and periaqueductal gray.⁶¹

Morphine interacts predominantly with the μ-opioid receptor (μ-OR).⁶² Pharmacological evidence and receptor binding studies have proposed several μ opioid receptor subtypes transcribed from various alternatively spliced variants of the μ opioid receptor (Oprm) gene.⁶³ μ-receptors are discretely distributed in the human brain, and high receptor densities have been characterized in the posterior amygdala, hypothalamus, thalamus, nucleus caudatus, putamen, and certain cortical areas.⁶³
Opioid receptors belong to the superfamily of G protein-coupled receptors (GPCRs) (Fig. 13) (class A) and have the characteristic structure of seven transmembrane domains, with an extracellular amino-terminus, multiple glycosylation sites, third intracellular loop with multiple amphipathic α-helixes, and a fourth intracellular loop formed by putative palmitoylation sites in the carboxyl tails.

Figure 13: Two-dimensional schematic of a generic class-A GPCR set in a lipid raft. The seven GPCR transmembrane helices with their interconnecting extracellular and intracellular loops are shown. The binding sites for various protein kinases are also depicted. The box at the bottom indicates the consensus sequences and important peptide motifs characteristic of class-A GPCRs such as the opioid receptors. (Adapted from Chen, Y. et al)
Specifically, μ-opioid receptors are prototypical “Gi/Go-coupled” receptors because their signals are efficiently blocked by pertussis toxin (PTX); a bacterial toxin produced by *Bordetella pertussis* that ADP-ribosylates and inactivates the α-subunits of Gi/Go proteins (Gαi/o subunits). Pharmacological studies have consistently reported a number of different signaling proteins that respond to morphine-induced activation of μ-opioid receptors (Fig. 14), including adenylyl cyclase, N-type and L-type Ca^2+^ channels, phospholipase C, inward rectifying K^+^ channels, mitogen-activated protein kinases ERK1 and ERK2, protein kinase A (PKA), calcium/calmodulin-dependent kinase II (CaMKII), nitric oxide synthase (NOS), N-methyl-D-aspartate acid glutamate receptors (NMDAR), and regulators of G-signaling (RGS) proteins. However, the precise relationship between one signaling pathway and the consequent observed pharmacological effects has yet to be completely characterized. Additionally, the μ-OR can also undergo phosphorylation and subsequently couple to beta-arrestins, which have both regulatory and signaling functions. Studies suggest that ligands with the greatest addictive potential, such as morphine, promote interactions with Gi more strongly than interactions with arrestins.
Figure 14: Potential cellular responses and transcriptional regulation initiated by activation of opioid receptor signaling. Arrows indicate activation of the signaling pathway; the "T" signs indicate inhibition of the signaling pathway. AC, adenylyl cyclase; Akt, also called protein kinase B (PKB); cAMP, cyclic AMP; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C. Gαi/o inhibits adenylate cyclase, whereas morphine and opioids have also been shown to activate adenylate cyclase and trigger downstream signaling.

Canonically, morphine is associated with activation of the μ-opioid receptor and inhibition of adenylyl cyclase activity via the Gi/o pathway; however, several recent studies have also reported an increase in adenylyl cyclase activity consequent to μ-opioid receptor activation. Involvement of the G-protein βγ complex (Gβγ) in stimulating adenylyl cyclase, and direct coupling between opioid receptors with Gs, have been proposed to account for this stimulation. Receptor binding assays have established that the μ-opioid receptor family has several alternatively spliced variants with minor differences in μ-opioid ligand affinities and ligand selectivity. However, adenylyl cyclase activation and [35S]-GTPγS binding assays have revealed major
differences in both potency and efficacy among these different μ-opioid receptor variants. Naloxone and naltrexone are general opioid receptor antagonists, whereas CTAP and CTOP are μ-opioid receptor-specific antagonists.

With multiple effectors being regulated by opioid receptors, the molecular and structural basis for pharmacology of opioid agonists is very complicated and incompletely characterized. To answer some of these issues, the structure of the μ-OR has recently been characterized, which can be used as a platform for further investigation and drug discovery purposes. A 2.8 Å crystal structure of the mouse μ-OR in complex with an irreversible morphinan antagonist is shown below. It is noteworthy to mention that the μ-OR structure in the crystallized complex reflects an inactive receptor form/topology, which may differ from the receptor’s structure in an agonist-activated conformational ensemble.

Compared to the buried binding pocket observed in most class-A G-protein-coupled receptor structure, the morphinan ligand binds deeply within a large solvent-exposed pocket of the μ-OR. Interestingly, the μ-OR crystallized as a two-fold symmetrical dimer through a four-helix bundle motif formed by transmembrane segments 5 and 6 (Fig. 15). Experimental evidence for both homo- and heterodimers (or oligomers) involving the μ-OR and δ-opioid receptors has been reported. Additionally, studies indicate hetero-oligomerization between μ-OR and non-opioid receptors, for example, the α2a adrenergic receptor, which also modulates μ-OR structure and signaling.
Figure 15: The opiate receptor dimer and a four-helix bundle dimer interface. 

a, The schematic depicts the four-helix bundle between dimerized receptors at the interface of TM5-TM6. 
b, As viewed from the extracellular surface, a tight association is seen in the binding pocket between the ligand (green sticks) and residues involved directly or indirectly in forming the dimeric interface (blue spheres). 
c, The four-helix bundle is expanded and shown in detail with interacting residues within 4.2 Å shown as sticks. TM; transmembrane helix. (Adapted from Manglik, A. et al)
M. Opiate tolerance and withdrawal.

Tolerance to the analgesic effects of morphine develops rapidly. Several different hypotheses have been put forth to explain how tolerance develops, including: (i) phosphorylation of the $\mu$-OR (inducing a receptor conformational change), (ii) functional decoupling from G-proteins (leading to receptor desensitization) (iii) $\mu$-opioid down-regulation and/or receptor internalization, and (iv) up-regulation of the cAMP pathway (a counter-regulatory mechanism to opioid effects). Counter-regulatory pathways, which are responsible for opioid tolerance, might also be mediated by cholecystokinin (CCK), for CCK-antagonist drugs like proglumide slow the development of morphine tolerance. NMDA antagonists such as ketamine, or dextromethorphan, as well as opioid receptor antagonists, may inhibit the development of tolerance to morphine.

The prototypical opioid withdrawal syndrome is induced by cessation of dosing after repetitive or sustained opioid administration. It is believed that withdrawal from morphine and other opioids proceeds through a number of stages. However, the intensity and length of withdrawal for each opioid is different. Weak opioids and mixed agonist-antagonists may induce acute withdrawal syndromes that do not reach an extreme level. Early withdrawal symptoms include watery eyes, insomnia, diarrhea, runny nose, yawning, dysphoria, sweating and, in some cases, a strong drug craving. However, as the withdrawal period progresses, severe headache, restlessness, irritability, loss of appetite, body aches, severe abdominal pain, nausea and vomiting, tremors, and even stronger and more intense drug craving appears.
N. Opiate-induced changes in cellular redox status.

Although pharmacological actions and downstream signaling pathways consequent to μ-OR activation have been described, the nature of the biochemical and metabolic changes contributing to the development of opiate chronic toxicity, drug dependence, and withdrawal syndrome are less known. Opioids can induce untoward effects due to biochemical alterations in target cells. Studies have reported heroin and morphine-induced depletion of the antioxidant GSH in the peripheral tissues, and recent reports also document pro-oxidant effects of morphine in the central nervous system (CNS). Several studies report a decrease in antioxidant defenses in animals and humans after the administration of heroin or morphine as well as during withdrawal from these agents. Intracerebroventricular administration of morphine is associated with significantly decreased GSH levels in cerebrospinal fluid samples taken from patients, rendering the central nervous system vulnerable to damage from oxidative stress.

Neural membranes are composed of phospholipids, glycolipids, cholesterol, and proteins, and morphine is reported to induce lipid peroxidation (compromising neuronal membrane integrity) and oxidative DNA damage, leading to neuronal apoptosis, neurotoxicity and neurodegeneration. Production of peroxynitrite (ONOO⁻) or other ROS in mitochondria can mediate these toxic effects of chronic morphine via c-Jun N terminal kinase activation.

Hence, morphine administration can induce ROS production and deplete levels of GSH antioxidant, consequently inducing neuronal adaptations to maintain redox
homeostasis. The failure of these compensatory mechanisms can contribute to opiate addiction and withdrawal phenomena as well as neurodegeneration and neurotoxicity.

**O. Opiates alter epigenetic status.**

Chromatin remodeling is important for mediating aberrant transcriptional responses in limbic brain regions, which contributes to various psychiatric disorders, including depression, schizophrenia and drug addiction.\(^{100-102}\) For example, repeated exposure to drugs of abuse induces long-lasting gene expression changes in the key brain reward region, the nucleus accumbens (NAc).\(^{53}\) Chromatin-based mechanisms are thought to mediate these transcriptional changes, contributing to behavioral abnormalities associated with drug addiction.\(^{102-104}\)

Cocaine and related stimulants can affect the enzymes that catalyze the addition or removal of post-translational modifications on histone tails\(^{53,105}\) and a similar modulatory effect on histone acetylation is also exerted by opiates.\(^{106-109}\) Histone and DNA methylation regulates normal cognitive function, and dysregulation of histone/DNA methylation has been implicated in the development of several psychiatric disorders.\(^{100,110}\) Dimethylation of histone H3 at lysine 9 (H3K9me2) across the entire genome is catalyzed by enzyme G9a, which is the core subunit of a multimeric repressive histone lysine methyltransferase (KMT) complex.\(^{111,112}\) This complex, including G9a, plays a crucial role in regulating H3K9me2 in cocaine-induced transcriptional and behavioral plasticity changes as well as the consequent regulation of susceptibility to chronic stress by prior cocaine exposure.\(^{105}\) Similarly, chronic morphine down-regulates H3K9me2 in NAc across several different classes of repetitive elements, including LINE-
However, the functional implications of this repressive histone methylation under the influence of opiates are not yet characterized. The regulation of G9a/H3K9me2 in NAc by chronic morphine as well as cocaine treatment indicates an integral role for G9a as part of dynamic repressive machinery in neurons for maintaining normal patterns of transcription and preventing aberrant gene expression under the influence of drugs or in response to negative environmental stimuli. The pathological down-regulation of this repressive machinery after chronic exposure to morphine or other emotional/environmental stimuli can lead to aberrant transcriptional control that contributes to abnormal behavioral adaptations.

**P. Opiates alter gene expression and induce neuronal plasticity**

Opiate drugs induce neuronal restructuring in the brain's limbic regions, which may contribute to long-term behavioral plasticity driving drug-addiction. For example, morphine and cocaine alter the density of dendritic spines on medium spiny neurons (MSNs) in the nucleus accumbens (NAc). Although these structural changes are well-known in NAc medium spiny neurons, the underlying molecular mechanisms have not been identified (Fig. 16). Opiates and some other drugs of abuse, such as methamphetamine, can induce alterations in cytoskeleton regulatory genes in NAc, such as Homer 1 and PSD95,\(^ {114}\) (scaffolding proteins in the postsynaptic cytoskeleton), and transcriptional regulators like ΔFosB and CREB.\(^ {115,116}\) Despite strong evidence indicating that CREB induction in NAc is involved in mediating tolerance and dependence to morphine as well as cocaine reward (reviewed in detail by Carlezon et al\(^ {117}\)), only a few studies have examined the importance of CREB in mediating structural changes following exposure to drugs of abuse. Additionally, in several other brain areas, CREB
induces spinogenesis\textsuperscript{118,119} mediated through transcriptional targets such as myocyte enhancing factor 2C (MEF2C) and brain-derived neurotrophic factor (BDNF), both of which are also involved in addiction-related plasticity (Fig. 16).\textsuperscript{120–122} NFκB is also involved in mediating the structural plasticity effects of chronic morphine and other drugs of abuse such as cocaine. Structural plasticity is also mediated via CREB-induced microRNA, such as mir132,\textsuperscript{123} which induces neurite outgrowth of hippocampal neurons in culture. MicroRNAs are key transcriptional regulators, along with epigenetic mechanisms, which together or individually can mediate cellular and structural plasticity and might contribute to drug-addiction phenomenon. However, results from few studies argue that this drug-induced spine plasticity is an epiphenomenon unrelated to drug tolerance and sensitization.\textsuperscript{123}
Figure 16: Signaling pathways involved in addiction-related cytoskeleton reorganization. Transcription factors, such as nuclear factor kappaB (NFκB), ΔFosB, cyclic AMP response element binding protein (CREB), and myocyte enhancing factor-2 (MEF2), play a role in regulating dendritic spines, and can be activated by a variety of signaling pathways. Structural plasticity induced by morphine can therefore result from manipulation of several signaling pathways that impinge upon actin assembly processes, with some of the changes mediated via altered gene expression. The net effect of morphine-induced activation of these fundamental signaling pathways is sensitized behavioral responses, although each pathway in isolation may produce distinct effects on addiction-like behavior and synaptic plasticity. (Modified from Russo, J. et al.)
Studies of VTA and LC demonstrate complex and important changes in cellular, synaptic, and structural plasticity that are involved in mediating chronic opiate effects on catecholamine neurons and other neuronal types in these regions, influencing drug reward and dependence. While these cellular and structural plasticity changes have been characterized, the biochemical/physiological changes underlying these differences in plasticity that occur with chronic opiate administration in VTA across multiple neuronal types still remain to be elucidated.
II. SPECIFIC AIMS

Opioids and other drugs of abuse commonly act on the molecular mechanisms that support an individual’s capacity for attention and awareness, thereby altering an individual’s state of consciousness and responsiveness. Learning and memory are closely associated with attention, and hence, frequent use of opioid class of drugs of abuse elicits persistent behavioral changes, including withdrawal syndromes. Mounting evidence indicates that epigenetic changes, specifically alterations in the patterns of DNA and histone methylation, can produce long-lasting alterations in gene expression and behavior, affecting learning and memory.2,125,126 Thus, the activities of methylation-related metabolic pathways in neuronal cells could help explain at least some molecular aspects of the acute and longer-term effects of the opioid class of drugs of abuse.

Cellular redox status influences methylation through the folate- and vitamin B12-dependent enzyme methionine synthase (MS), whose activity controls the SAM / SAH ratio in a cell.127–129 Under oxidative conditions, MS is inhibited, diverting its substrate HCY to the transsulfuration pathway for GSH synthesis, which regulates the redox status in the cell. However, transsulfuration activity is limited in adult cortical neurons, and about 90% of intracellular cysteine is transported into neuron by excitatory amino acid transporter 3 (EAAT3).17 Thus EAAT3 plays an extremely important role in maintaining GSH based redox status, and consequently, changes in EAAT3 activity, affects more than 200 known methylation reactions, including DNA and histone methylation.
This thesis project will investigate the effect of selected opioid drugs of abuse on EAAT3 activity and pathways of sulfur metabolism which support and control methylation activity (especially DNA methylation) in neuronal cells, which regulates gene transcription and can affect protein synthesis and cellular homeostasis.

**SPECIFIC AIM #1: To characterize time- and concentration-dependent changes in cysteine uptake, GSH / GSSG-based redox status, and SAM / SAH-based methylation status in neuronal cells treated with various opioid drugs.**

Epigenetic changes and neuronal redox adaptations are hypothesized to play a central role in the acute and long-term effects of opioid drugs, including morphine.\(^\text{124,130}\) However, drug-exposure parameters (i.e. duration and dose) eliciting these changes have not yet been defined in neuronal cells. This aim will identify the time course and concentration-response function of opioid drugs of abuse which lead to neuronal redox adaptations *in vitro*. To investigate these actions, cysteine uptake will be measured in a cultured neuronal cell line after treatment with opioid drugs at selected doses and for various exposure times. Comparisons will be made against saline-treated controls. The drug effects on the intracellular thiol and thioester metabolites and global DNA methylation levels will be assessed under treatment conditions that elicit a significant change in neuronal cysteine uptake. Redox and methylation status in cultured neuronal cells will be represented by GSH / GSSG and SAM / SAH ratios, respectively. These measurements would allow correlation of the effects of one of the representatives of the opioid class of drugs of abuse on changes in neuronal redox status with the changes observed in methylation capacity (SAM / SAH ratio), represented by DNA methylation as a prototype.
SPECIFIC AIM #2: To assess the mechanisms involved in mediating the effects of opioids on cysteine transport through EAAT3.

Chronic morphine is reported to activate multiple signaling pathways which involve several protein kinases; e.g., MAPKK, ERK, PI3K and PKA. Morphine treatment is also reported to activate the ubiquitin proteosome pathway through induction of Nedd4 and PTEN, which further down-regulates EAAC1 (rat homolog of EAAT3) in vivo. In addition, this effect was blocked by naltrexone administration, which confirms the involvement of opioid receptor. Also, the δ-opioid receptor is reported to suppress the cell-surface expression of EAAT3. Thus, different opioid receptors interact with EAAT3 and affect its activity. This aim will identify the exact mechanism and receptor subtype responsible for the effect of opioids on cysteine transport and subsequent changes observed under Specific Aim #1. To identify the involvement of particular receptor subtype, cysteine uptake, thiol and DNA methylation measurements will be performed with various opioid agonists in presence of μ-, and δ-opioid receptor antagonists. Morphine and other opioids act on different opioid receptors, which are coupled with different G protein downstream signaling pathways. Pertussis toxin (PTX) and cholera toxin (CTX) inhibit G proteins Gαi/o and Gαs, respectively. Thus, the involvement of specific G proteins and further activation of specific receptor subtypes in mediating the effects of opioids would be investigated by treating cells with opioid agonists in presence of these toxins and specific antagonists. In addition, downstream signaling pathways, e.g., mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) are reported to regulate the activity of EAAT3 with different temporal dynamics. This aim would also
investigate the implications of these pathways for the effects of morphine observed under Specific Aim #1.

**SPECIFIC AIM # 3: To assess changes in mRNA levels of selected redox- and methylation-related genes in response to exposure to various opioid drugs of abuse.**

Changes in redox status might mediate changes in DNA methylation caused by opioids and are likely to induce changes in the expression of genes coding for the transporters and enzymes whose activities affect methylation pathways, perhaps as part of a compensatory mechanism for restoring cellular redox equilibrium. A custom-designed qRT-PCR array will be used to assess the effects of opioids on mRNA transcript levels of EAAT3, MS, CBS, CGL, cystathionine-γ-lyase, γ-glutamylcysteine ligase (catalytic and modulatory subunits), methionine adenosyl transferase II, SAH hydrolase, DNMTs 1 and 3, Brain-derived neurotrophic factor (BDNF), μ-OR and LINE-1 will also be assessed. I will also investigate the opioid-induced changes in CpG island methylation status and determine whether any changes in methylation status are correlated with changes in their mRNA expression levels.

**SPECIFIC AIM #4: To characterize changes in cysteine uptake and the status of redox and methylation in neuronal cell culture upon in vitro drug “withdrawal.”**

Changes in neuronal redox status and DNA methylation levels represent adaptive responses to sustained presence of opioids, and a washout period/drug removal can be hypothesized to lead to reciprocal metabolic adaptive response. To test this hypothesis, cells will be treated for a sustained period of time with a single appropriate
concentration of the opioids, which will then be washed out. Cysteine uptake, thiol metabolites, redox and methylation status, and DNA methylation levels will then be measured at selected time-points after washout.

**SPECIFIC AIM #5: To evaluate the ability of redox and methylation modulators to alter the response to various opioid drugs of abuse.**

This aim will focus on testing whether the redox modulators that strengthen neuronal ability to maintain GSH levels and methylation capacity, are also able to alter the effects induced by opioids. \(N\)-acetylcysteine (NAC), which bypasses EAAT3 as an extracellular cysteine source, and methylcobalamin (MeCbl), which supports MS activity, will be used for this purpose. Cells will be treated with these agents prior to opioid exposure, and cysteine uptake, low-molecular-weight thiol measurements, and DNA methylation assays will then be performed.
III. Materials and Methods

A. Materials

Minimum essential medium, alpha-modification (α-MEM), trypsin, Hank’s balanced salt solution (HBSS) and penicillin/streptomycin antibiotic solution were purchased from Mediatech (Manassas, VA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). \[^{35}\text{S}\]-cysteine was purchased from American (St. Louis, MO). SH-SY5Y human neuroblastoma cells were from ATCC® (Manassas, VA).

B. Cell Culture

SH-SY5Y cells were grown as monolayers in 10-cm tissue culture dishes containing 10 mL of alpha-modified minimum essential medium (α-MEM) supplemented with 1% penicillin-streptomycin-fungizone and 10% fetal bovine serum (FBS) in an incubator chamber under 5% CO\(_2\) - 95% air at 37°C. For most experiments, cells were plated and incubated for 48 hours prior to use. For cell lines, experiments utilized cells between 5-30 passages, with no significant differences in results. Additionally, cells are reported to undergo apoptosis after 4-7 days post-plating. Since some of the experiments were performed for up to 96 hours after the time of plating the cells in presence or absence of drugs, there is a chance of apoptosis or necrosis, which can lead to cell death. Hence, a viability assay was performed to avoid any confounding effect due to cell death in the long-term studies. A trypan blue exclusion assay was performed to measure the levels of cell death for the long-term time points in presence or absence of drugs. Trypan blue is a dye taken up by cells that are either apoptotic or necrotic. On average, about 5-7% dead cells were observed after about 96 hours, and the presence of drugs did not induce further cell death. Additionally, the protein measurements were
done for each experiment, and the experimental results were normalized against the protein levels during each assay. Hence, the apoptotic effects were normalized and cell death as a confounder was avoided.

C. Cysteine Uptake

Cysteine uptake was evaluated using the method developed by Chen and Swanson.22 In brief, SH-SY5Y human neuroblastoma cells were plated in six-well standard tissue culture plates containing 2 mL of media for 48 hours before the assay. Following the incubation, opioid drugs were added at different time points for time-dependent studies. For dose-dependent studies, the cells were treated with opioids for 30 min. Opioid receptor blockers, namely naltrexone, CTAP and naltrindole, were added first, followed by treatment with respective opioids. In some studies cells were pre-treated and incubated with pertussis toxin and cholera toxin for 24 hours, followed by treatment with opioids for different time points. For all experiments, at the end of incubation with opioids, the medium was aspirated, and the cells were washed with 600 μL 37°C Hanks Buffered Salt Solution (HBSS). The HBSS was aspirated, replaced with 600 μL of 37°C HBSS containing radiolabelled cysteine \([^{35}S]\) cysteine, (1 μCi/1 mL), 10 μM unlabeled cysteine and 100 μM DTT, and incubated for 5 minutes. The \([^{35}S]\) cysteine/HBSS mixture was aspirated, and cells were washed twice with ice-cold HBSS. Cells were then lysed with 600 μL 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. Cell-associated radioactivity was normalized against protein
content. Protein concentration was determined by a modified Lowry method using bovine serum albumin (BSA) standard.

**D. Isolation of intracellular thiol metabolites:**

SH-SY5Y neuroblastoma cells were grown to confluence in α-MEM as above, and morphine or other drugs were incubated with the cells for specified periods of time, similar to cysteine uptake studies. Media were aspirated, and the cells were washed 2X with 1 mL of ice cold HBSS. The HBSS was aspirated, and 0.6 mL ice-cold dH2O was added to the cells. Cells were collected by scraping into dH2O. The cell suspension was sonicated for 15 seconds on ice, and 100 μL was used to determine protein content. The remaining sonicate was added to a microcentrifuge tube, and an equal volume of 0.4 N perchloric acid was added, followed by incubation on ice for 5 min. Samples were centrifuged at 7500 g, and the supernatant was transferred to new microcentrifuge tubes. 100 μL of sample was added to a conical microautosampler vial and kept at 4°C in the autosampler cooling tray. 10 μL of this sample was injected into the HPLC system.

**E. HPLC measurement of intracellular thiols:**

Separation of redox and methylation-pathway metabolites was accomplished using an Agilent Eclipse XDB-C8 analytical column (3 x 150 mm; 3.5 μm) and an Agilent Eclipse XDB-C8 (4.6 x 12.5 mm; 5 μm) guard column, as described previously. Two mobile phases were used. Mobile Phase A was 0% acetonitrile, 25 mM sodium phosphate, 1.4 mM 1-octanesulfonic acid, adjusted to pH 2.65 with phosphoric acid. Mobile Phase B was 50% acetonitrile. The flow rate was initially set at 0.6 mL/min, and a step gradient was utilized: 0-9 min 0% B, 9-19 min 50% B, 19-30 min 50% B. The
column was then equilibrated with 5% B for 12 min prior to the next run. Temperature was maintained at 27 °C. The electrochemical detector was an ESA CoulArray with BDD Analytical cell (Model 5040), and the operating potential was set at 1500 mV. Sample concentrations were determined from the peak areas of metabolites using standard calibration curves and ESA-supplied HPLC software. Sample concentrations were normalized against protein content. All analytical samples were within the linear response range of the detector.

**F. DNA Isolation**

Cellular DNA for the analysis of DNA methylation was harvested and isolated using the FitAmp™ Blood and Cultured Cell DNA Extraction Kit from EPIGENTEK® (Refer to manufacturer’s instructions for full protocol). Isolated DNA was quantified using a ND-1000 NanoDrop spectrophotometer. Only 1 μL of DNA was needed for quantification. The contaminating RNA was removed by treatment with RNase treatment.

**G. DNA Methylation Analysis**

DNA isolated from cultured SH-SY5Y cells after various pretreatments was used for this purpose. Any contaminating RNA was removed by RNase treatment. Assessment of global DNA methylation status was accomplished using the MethylFlash® Methylated DNA Quantification Kit from EPIGENTEK® as described previously. The methylated fraction of DNA was identified using 5-methylcytosine monoclonal antibodies and quantified by an ELISA-like reaction. The levels of methylated DNA were calculated using
the OD intensity on a microplate reader at 450 nm. The results were compared against a standard curve prepared with different concentrations of 5-MeC.

**H. Validation of global DNA methylation protocol.**

As cells divide, their DNA is demethylated, and then remethylated. To confirm that DNA methylation is in steady state during the experimental conditions for our DNA methylation studies, cells cultures were grown to confluence for different time points. DNA methylation was then measured after 24, 48, 72 and 96 hours (Fig. 17). Global DNA methylation levels were quantified using anti-5-methylcytosine monoclonal antibodies in an ELISA-like reaction. No changes in DNA methylation were observed 24-96 hours after growth.

**Figure 17: DNA methylation over 96 hours in SH-SY5Y cells.** Quantification of 5-methylcytosine using ELISA in human neuroblastoma cell line. DNA was isolated after 24, 48 and 72 hours. n=3. Asterisks (*) show a significant difference (p<0.05) from control.
I. Site-specific CpG methylation: Fragmentation and Methylated Binding Domain protein (MBD) capture

SH-SY5Y cells were treated with morphine for 4 or 24 hrs and DNA was isolated. Fragmentation was performed on Covaris S2 with following settings: duty cycle 10%, intensity 200 cycles per burst during 190 sec, to obtain fragments with an average length of 200 base pairs. The power mode was frequency sweeping, temperature 6-8°C, water level 12. A maximum of 3 μg was loaded in 130 μL Tris-EDTA buffer, in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). For samples with less input DNA (down to 500 ng), the DNA was diluted 1:5 (by volume) with TE. DNA with an input of 3 μg was then analyzed on the Agilent 2100 (Agilent Technologies, Santa Clara, California, USA). DNA with an input lower than 3 μg was concentrated in a rotary evaporator to 25 μL, and the fragment distribution was checked on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode AF-100-0048, Belgium) according to the manufacturer’s protocol. Starting concentration was 200 ng. The yield was typically between 0.5 and 8 ng total captured DNA and sometimes too low to measure. Fragments were subsequently sequenced using the Illumina Genome Analyzer II. Concentrations of the fragmented and captured DNA were determined on a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany with the Quant-iT™ Picogreen® dsDNA assay kit (Invitrogen P7589, Merelbeke, Belgium) at 480/520nm. The work flow-chart is as below.
Validation of the presence of LINE-1 sequence elements in SH-SY5Y cells.

To validate the presence of LINE-1 elements in SH-SY5Y cells, PCR detection was performed followed by Sanger DNA Sequencing with LINE-1 primers. The specific primers used for the PCR reaction were; forward: 5’- CCAAATCATGGGTGAACCC-3’, and reverse: 5’- AACATTCCATGCTCATGGGT-3’. The PCR products were separated by gel electrophoresis and the isolated products were sequenced with the help of GENEWIZ facility®, Boston, MA.
Figure 18: LINE-1 PCR products. PCR was performed to characterize the LINE-1 elements in SHSY5Y cells. Products were separated using gel electrophoresis. The size of the expected product was 208 base pairs. β-actin was used as a control. M= morphine treated SH-SY5Y cells, C=control. Right panel shows the results from the Sanger sequencing analysis. Comparing this sequence using GeneBLAST tool identified it as LINE1-HS, reported to be dynamic and functionally active in Homo sapiens.

Thus, LINE1 retrotransposons were identified to be present in the SH-SY5Y cells and could be used for further analysis.

J. Analysis of the methylation levels in the repeat elements LINE-1

DNA methylation analysis of LINE1 repeats was performed at a commercial company (NXT-DX). For full details on the method of analysis, refer to the company’s protocol at: http://www.nxt-dx.com/epigenetics/genome-wide-methylation-sequencing/. In brief, all the reads of LINE1 repeats across the entire genome were mapped. The mapping was done with low stringency settings using publicly available software (bowtie, single end, allowed multiple hits=1000, mismatches in the seed=1).
The average mapped reads ratios for each group vs. control were examined, and a corresponding Chi-Square value was calculated.

**K. 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®. For a full protocol please refer to the company's website. The isolated RNA was treated with DNase, as stated in the extended RNA isolation protocol, followed by RNA quantification using a ND-1000 NanoDrop spectrophotometer.

**L. Primers**

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

**M. cDNA synthesis.**

cDNA synthesis and subsequent PCR amplification were performed using the First strand cDNA synthesis™ from Roche. The cDNA synthesis uses 1 µg RNA, 1 mM dNTP mix, 60 µM random hexamer primers, with sufficient dH2O added to achieve a final sample volume of 13 µL. Samples were denatured at 65°C for 5 minutes and then placed on ice. Transcriptor RT™ (20 units/µL), Protector RNase inhibitor™ (40 U/µL), 5x
Transcriptor Reverse Transcriptase Reaction Buffer and dH₂O in a final volume of 7 μL were used in the second part of the reaction to bring the final volume to 20 μL. This was followed by incubation in the PTC-Thermocycler (MJ Research) at 25°C for 10 min followed by 30 min at 55°C. The reverse transcriptase enzyme was inhibited by incubation at 85°C for 5 min.

N. Microarray.

For microarray hybridizations, 500 ng of total RNA from each sample was labeled with fluorescent dye (Cy3; Amersham Biosciences Corp, Piscataway, NJ) using the Low RNA Input Linear Amplification Labeling kit (Agilent technologies, Palo Alto, CA) following the manufacturer’s protocol. The amount and quality of the fluorescently labeled RNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer. According to manufacturer’s specifications, 1.6 mg of Cy3-labeled cRNA was hybridized to the Agilent Human Whole Genome Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA) for 17 hrs, prior to washing and scanning. Data were extracted from scanned images using Feature Extraction Software (Agilent Technologies, Inc., Palo Alto, CA).

O. qRT-PCR Analysis

qRT-PCR was performed on duplicate samples using the LightCycler® 480 qRT-PCR machine from Roche™. The assay was run in 96-well optical reaction plates. qRT-PCR uses 5 μL of cDNA template, 10 μM sense and antisense primers, 10 μL SYBR Green I Master® from Roche™, and dH₂O in a final volume of 20 μL. The following thermal
parameters will be used: incubation for 5 min at 95 °C, and then 45 cycles of 95 °C for 10 sec, 60 °C for 20 sec and 72 °C for 30 sec, followed by a single cycle of 95 °C for 5 sec, 1 min at 65 °C and 97 °C for the melting curve, followed by cooling at 40 °C for 90 sec. No template controls were run on each plate, and dissociation curves were generated to determine any non-specific products. Data were analyzed using the Roche absolute quantification second derivative method as well as the relative quantification method when the respective sample treatment is available and were normalized to β-actin.

P. Statistical Methods

Statistical analyses were carried out using Graph Pad Prism® version 5.01. The Student’s t-test for independent means was used to test for significant differences between control and experimental groups. Data were 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.
IV. RESULTS:

A. Regulation of EAAT3-mediated cysteine uptake by opioids.
To determine the effects of morphine on EAAT3-mediated cysteine uptake, SH-SY5Y cells were treated with increasing concentrations of morphine (0.1 nM – 10 µM) for 30 minutes, and [35S]-cysteine uptake was measured. Morphine inhibited [35S] - cysteine transport in a concentration-dependent manner with an IC50 of 2.4 nM (Fig. 19a). Pretreatment with naltrexone (a non-selective opioid antagonist) blocked morphine inhibition of [35S]-cysteine uptake, implicating the opioid receptor in the inhibition of EAAT3-mediated cysteine uptake by morphine (Fig. 19a). A dose-response curve for morphine at different time points (0.5, 1, 4, 8, 24 hrs) was measured and 1 µM morphine was used for following experiments (Fig. 19b).

Figure 19: Inhibition of cysteine uptake by morphine via μ-opioid receptors in SH-SY5Y cells. Radiolabeled cysteine uptake by SH-SY5Y human neuroblastoma cells is reduced by a 30-min exposure to morphine (0.1 nM to 10 µM) in a concentration-dependent manner with an IC50 of 2.4 nM. Pretreatment with the non-selective opiate antagonist naltrexone (1 µM) blocked the morphine-induced decrease of cysteine transport. (b) Dose response changes in cysteine uptake at different time points after morphine treatment (n = 6); Asterisks (*) indicate a significant difference (p<0.005) from control.
B. Specific receptors involved in mediating the effects of opioids on cysteine uptake.

Chronic morphine treatment is reported to decrease EAAT3 levels in neuronal cells.\textsuperscript{136} and to decrease intracellular GSH levels in mouse liver.\textsuperscript{137} To characterize the effects of morphine at different time intervals including long-term treatments, the time-dependency of the morphine-induced inhibition of cysteine uptake in SH-SY5Y cells was determined. SH-SY5Y neuroblastoma cells were treated with 1 μM morphine for times ranging from 30 min to 24 hrs, after which cysteine uptake was measured. This concentration was selected based on my preliminary studies and previous research from other laboratories which showed that 12-24 hours of morphine treatment (1 μM) induced tolerance in SH-SY5Y cells.\textsuperscript{90,138} A complex and reproducible pattern of alternating inhibition and restoration was observed, ultimately demonstrating significant net inhibition of cysteine uptake (p<0.05) (Fig. 20). Previous reports have demonstrated expression of both μ and δ opioid receptors in SH-SY5Y cells, and morphine has been reported to activate both.\textsuperscript{81,139} Hence, the involvement of specific opiate receptor subtype(s) in mediating the effects of morphine on EAAT3 was characterized.

For this purpose, SH-SY5Y cells were pretreated with either the μ-opioid receptor specific antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH\textsubscript{2} (CTAP) or the δ-opioid receptor specific antagonist naltrindole (NTI) prior to short- or long-term treatment with morphine. As shown in Fig. 20, pretreatment with CTAP blocked the effects of morphine, whereas NTI had no effect on the morphine-induced inhibition of cysteine transport at any time. Hence, the μ-opioid receptor mediates the action of morphine on the inhibition of cysteine transport via EAAT3 in SH-SY5Y cells.
Figure 20: Time-dependent effects of morphine on cysteine uptake by SH-SY5Y cells. SH-SY5Y cells treated with 1 µM morphine showed a complex pattern of inhibition and recovery/stimulation of cysteine uptake at different time points (0.5, 1, 4, 8 and 24 hrs), finally resulting in decreased cysteine uptake at 24 hrs. Pretreatment with CTAP (0.1 uM), a specific mu-opioid receptor blocker, abrogated the effects of morphine. Pretreatment with naltrindole (0.1 uM), a specific delta-opioid receptor blocker, did not alter the effects of morphine on cysteine uptake at 24 hrs. n=6. Asterisks (*) show a significant difference (p<0.05) from morphine-treated control.

Met-enkephalin is an endogenous opioid receptor agonist with 2 to 10-fold higher affinity for δ- as compared to μ-OR, and the δ-opioid receptor is reported to regulate EAAT3 membrane localization.26 To investigate the role of met-enkephalin on EAAT3-mediated cysteine uptake, SH-SY5Y cells were treated with 10 nM met-enkephalin for various times (0.5, 1, 4, 8 and 24 hrs), and radiolabelled cysteine uptake was measured. As indicated in Fig. 21, cysteine uptake was stimulated after 1, 4 and 8 hrs of incubation with met-enkephalin; however, no effect was observed at 24 hrs of met-enkephalin treatment. Involvement of δ-opioid receptor was also confirmed, since pretreatment with the δ-OR blocker naltrindole blocked the stimulatory effects of met-enkephalin, whereas pretreatment with the μ-OR blocker CTAP did not alter the met-enkephalin stimulation of EAAT3-mediated cysteine uptake. These results are consistent with other studies indicating that δ-OR co-localize with EAAT3, leading to decreased surface expression of EAAT3, and that activation of δ-OR stimulated the surface localization of EAAT3, leading to increased cysteine uptake.
**Figure 21**: Time-dependent effects of met-enkephalin on cysteine uptake by SH-SY5Y cells. Met-enkephalin (10 nM) increased cysteine uptake in SH-SY5Y cells at different times (1, 4 and 8 hrs), reverting to control levels at 12 and 24 hrs. Pretreatment with a delta-opioid-specific antagonist, naltrindole (0.1 uM), (red line) completely prevented the met-enkephalin-induced potentiation of cysteine uptake. The green line indicates pretreatment with a mu-opioid receptor blocker, CTAP (0.1 uM), which did not inhibit the effect of met-enkephalin. N=6. Asterisks (*) indicate a significant difference (p<0.005) from met-enkephalin treated samples.

C. **G proteins involved in opioid-induced effects on EAAT3-mediated cysteine transport**

Morphine has the potential to activate multiple G proteins (e.g., G\(_{i/o}\) and G\(_{s}\)) in SH-SY5Y cells via the \(\mu\)-opioid receptor\(^{81,139,140}\). Hence, we wanted to characterize the specific G-proteins involved in mediating the effects of morphine on EAAT3 via \(\mu\)-opioid receptors in SH-SY5Y cells. We used pertussis toxin (PTX) and cholera toxin (CTX) to inhibit G\(_{i/o}\) and G\(_{s}\) G-protein subtype, respectively. As shown in Fig. 22, pretreatment with PTX for 24 hrs abolished the short-term (30 min, 1, 2, and 4 hrs) effects of morphine on cysteine transport, but did not alter the longer-term (12 and 24 hr) effects. Interestingly, pretreatment with CTX for 24 hrs blocked the long-term effects, but did not affect the short-term effects of morphine on cysteine transport (Fig. 22). These results indicate that short-term effects of morphine are mediated by activation of the G\(_{i}\)-subtype of G-proteins coupled to the \(\mu\)-opioid receptor, whereas the long-term effects involve coupling of \(\mu\)-opioid receptors to G\(_{s}\). Thus, differential G-proteins are coupled to the \(\mu\)-OR in a temporal manner to induce the effects of morphine on EAAT3 in SH-SY5Y cells.
Figure 22: Involvement of G-proteins as downstream mediators of the time-dependent effects of morphine on cysteine uptake by SH-SY5Y cells.

Morphine (1 µM)-induced acute effects (0.5, 1 and 4 hrs) on cysteine uptake by SH-SY5Y human neuroblastoma cells were blocked by pretreatment with pertussis toxin, a G\textsubscript{i/o} inhibitor (PTX, 0.5 µg/mL, 24 hrs). Morphine-induced chronic effects on cysteine uptake (12 and 24 hrs) in SH-SY5Y human neuroblastoma cells were blocked by pretreatment with cholera toxin, a G\textsubscript{s} inhibitor (CTX, 0.5 µg/mL, 24 hrs). n=6. Asterisks (*) indicate a significant difference (p<0.005) from morphine treatment.

In general, activation of δ-OR is reported to result in the receptor’s coupling with Gi/o subtype of G-proteins.\textsuperscript{139} To investigate the coupling of Gi/o and δ-OR, SH-SY5Y cells were pretreated with PTX for 24 hrs followed by met-enkephalin treatment and cysteine uptake was
then measured at various time points. The stimulatory effects induced by met-enkephalin on EAAT3-mediated cysteine uptake were completely blocked by pretreatment with PTX (Fig. 23), indicating involvement of Gi/o subtype of G-proteins in mediating the effects of met-enkephalin on EAAT3-mediated cysteine uptake.

![Figure 23](image)

**Figure 23:** Involvement of G-proteins as downstream mediators of the time-dependent effects of met-enkephalin on cysteine uptake by SH-SY5Y cells. Met-enkephalin (10 nM) induced effects (1, 4 and 8 hrs) on cysteine uptake by SH-SY5Y human neuroblastoma cells were blocked by pretreatment with pertussis toxin, a G<sub>i/o</sub> inhibitor (PTX, 0.5 μg/mL, 24 hrs). n=6. Asterisks (*) indicate a significant difference (p<0.005) from met-enkephalin treatment.

**Summary:**

- **Initial effects of both morphine and met-enkephalin are inhibited by a pretreatment with PTX, suggesting that the acute effects of opioids on cysteine transport through EAAT3 are mediated via the Gi/o subtype of G-protein.**

- **Both μ- and δ-opioid receptors couple to Gi/o subtype of G-protein and mediate the effects of morphine and met-enkephalin, respectively, at earlier time points, consistent with previous reports regarding the coupling of μ and δ-opioid receptor to Gi/o G proteins in SH-SY5Y neuroblastoma cells.**
D. Involvement of protein kinases in opioid-induced effects on EAAT3-mediated cysteine transport.

Several downstream protein kinases [e.g. PKA, ERK, PI3K] can regulate the activity and surface expression of EAAT3 in SH-SY5Y cells. Studies also suggest differential activation of protein kinases by μ-OR after acute vs. chronic morphine treatment, whereas the downstream signaling pathways involved in modulating the effects of δ-OR on EAAT3 surface expression have not been investigated. To identify and characterize the downstream signaling protein kinases involved in mediating the effects of opioids on EAAT3-mediated cysteine uptake, SH-SY5Y cells were pretreated with the PKA inhibitor H-89 for 4 hours, the MAPK/ERK kinase inhibitor PD98059 for 24 hours, or the PI3K inhibitor LY294002 for 2 hours, followed by treatment with opioids. Cysteine uptake was then measured at different times after opioid exposure.

Pretreatment with LY294002 blocked the elevated cysteine uptake observed at 1 and 8 hrs of morphine treatment, suggesting a role for PI3K in mediating the stimulatory effects of morphine (Fig. 24). Similarly, H-89 (100 nM) blocked the short-term (30 min and 4 hrs) inhibitory effects of morphine (Fig. 25), whereas pretreatment with PD98059 blocked the long-term effects of morphine (24 hrs) (Fig. 26). These results indicate that PKA is involved in mediating the short-term inhibitory effects of morphine, and MAPK/ERK kinase mediates the longer-term inhibitory effects of morphine, whereas PI3K mediates the stimulatory effects of morphine on EAAT3-mediated cysteine uptake.
Figure 24: Involvement of PI3 kinase in the effects of morphine on cysteine uptake by SH-SY5Y cells.

Stimulation of cysteine uptake by SH-SY5Y human neuroblastoma cells induced by morphine (1 μM) was blocked by the PI3 kinase inhibitor, LY294002 (100 nM, 2 hrs). n=6. The dotted lines indicate the non-LY treated (black) and LY-treated (green) control levels of uptake.

Figure 25: Involvement of protein kinase A in the effects of morphine on cysteine uptake by SH-SY5Y cells. The acute effects of morphine (1 μM) on cysteine uptake by SH-SY5Y human neuroblastoma cells were blocked by the PKA inhibitor H-89 (100 nM, 4 hrs). n=6. The dotted lines indicate the non-H-89-treated (black) and H-89-treated (red) control level of uptake.
Figure 26: Involvement of MAP kinase in the effect of morphine on cysteine uptake by SH-SY5Y cells. The chronic (24-h) effect of morphine (1 μM) on cysteine uptake by SH-SY5Y human neuroblastoma cells was blocked by MAPK/ERK kinase inhibition with PD98059 (10 μM, 24 hrs) n=6. The dotted lines indicate the non-PD98059-treated (black) and PD98059-treated (blue) control level of uptake.

A similar investigation for characterizing the downstream protein kinases involved in mediating the effects of met-enkephalin on cysteine uptake via δ-OR was also performed using protein kinase inhibitors. Pretreatment with H-89 and PD98059 did not alter the effects of met-enkephalin on cysteine uptake. However, pretreatment with LY294002 blocked the stimulatory effects of met-enkephalin on EAAT3-mediated cysteine uptake at 1 and 4 hrs of met-enkephalin treatment (Figs. 27, 29), suggesting the involvement of PI3K in mediating the stimulatory effects of met-enkephalin on EAAT3 via the δ-OR (Fig.28). Interestingly, the stimulatory effects of morphine on cysteine uptake were also inhibited by pretreatment with LY294002, suggesting the general involvement of PI3K in mediating the stimulatory effect of opioids on EAAT3-mediated cysteine uptake. PI3K has been previously shown to be involved in stimulation of EAAT3-mediated cysteine uptake by growth factors like IGF-1, BDNF and...
NGF. The exact mechanism involved in the stimulatory effects induced by PI3K is not characterized, but translocation of the endosomal stores of EAAT3 transporter to the cell surface can be suggested as a tenable mechanism for the increase in the EAAT3-mediated cysteine uptake.

Figure 27: Involvement of protein kinase A in the effects of met-enkephalin on cysteine uptake by SH-SY5Y cells.
PKA inhibitor H-89 (100 nM, 4 hrs) did not affect the cysteine uptake stimulated by met-enkephalin in SH-SY5Y cells. N=6. The dotted line indicates the corresponding controls: black line, no-treatment control; green line, H-89 control. Asterisks (*) indicate a significant difference (p<0.05) from met-enkephalin-treated samples.
Figure 28: Involvement of downstream signaling kinases in the effects of met-enkephalin on cysteine uptake by SH-SY5Y cells.
Pretreatment with the PI3K inhibitor LY294002 (100 nM, 2 hrs) blocked the acute stimulatory effects of met-enkephalin (10 nM) (1 and 4 hrs) on cysteine uptake. N=6.

Figure 29: Involvement of downstream signaling kinases in the effects of met-enkephalin on cysteine uptake by SH-SY5Y cells.
Pretreatment with MAPK inhibitor PD98059 (10 μM) did not affect the met-enkephalin (10 nM)-induced stimulation of cysteine uptake. N=6.
Summary

- **Increased levels of EAAT3-mediated cysteine uptake after morphine or met-enkephalin exposure are mediated by PI3kinase.**

- **Short-term (4 hrs) inhibition of cysteine uptake after morphine exposure is induced by PKA and long-term (24 hrs) inhibition is dependent on MAPK/ERK.**

Several different downstream protein kinases are involved in mediating opioid-induced effects via μ and δ-opioid receptor on cysteine uptake in a temporally selective and complex manner. This is confirmatory of other studies which indicate coupling of opioid receptors to several different signaling cascades. Additionally, these studies also confirm the previous reports which indicate regulation of EAAT3 activity via different signaling protein kinases.

**E. Effects of opioids on intracellular thiols in cultured neuronal cells.**

In light of the observed effects of opioids on cysteine uptake by SH-SY5Y cells, we investigated whether opioids influence SH-SY5Y thiols and thioethers involved in GSH synthesis. Thiol species were quantified by electrochemical detection with reference to known external reference compounds after chromatographic separation by HPLC. Based upon data detailed above regarding the effect of opioids on EAAT3-mediated cysteine uptake by SH-SY5Y cells, as well as previous studies in our lab and others, 4 and 24 hrs after opiate exposure were considered to be appropriate times for investigating potential opiate-mediated changes in intracellular thiols (Fig. 30).
Figure 30: Morphine-induced changes in neuronal-cell thiols and thioethers.

SH-SY5Y neuroblastoma cells were treated with morphine (1 μM) for 4 and 24 hrs. The levels of thiol and thioether metabolites, analyzed by HPLC with electrochemical detection, were plotted as percent change normalized to untreated control cells. Asterisk (*) indicates comparison of morphine-treated samples vs. no-treatment control cells, p<0.05, N= 6.

Consistent with the observed inhibition of EAAT3-mediated cysteine uptake with morphine, short- (4-h) and longer-term (24-h) treatment with morphine decreased intracellular levels of cysteine (Fig. 30). Further, intracellular levels of GSH were also decreased, likely as a direct consequence of decreased intracellular cysteine concentrations, since cysteine is the rate-limiting precursor for GSH. Methionine and SAM levels in SH-SY5Y cells were also decreased after both short- and long-term morphine exposure (Fig. 30).
Figure 31: Time dependent effects of met-enkephalin on thiols and thioethers in SH-SY5Y cells.
HPLC separation and electrochemical detection of % change in thiols and thioethers normalized against the control in SH-SY5Y neuroblastoma cells after treatment for either 4 or 24 hrs with met-enkephalin (10 nM). N=4, asterisk (*) indicates statistical significance (p<0.05).

In contrast to the morphine-induced reduction of SH-SY5Y thiols, treatment with met-enkephalin for 4 hrs increased intracellular levels of cysteine, GSH, methionine and SAM (Fig. 31), whereas 24 hrs met-enkephalin treatment did not alter intracellular cysteine levels, but increased the GSH, methionine, homocysteine and SAM levels (Fig. 31). These differences in the levels of thiols under the influence of met-enkephalin are generally consistent with preceding changes observed in EAAT3-mediated cysteine uptake.
F. Effect of opioids on redox and methylation status in cultured neuronal cells.

The GSH / GSSG ratio serves as one of the main indices of cellular redox state and a decrease in this ratio is indicative of oxidative stress associated with shifts in intracellular thiol levels, especially in neurons. To evaluate the potential effect of opioids on neuronal-cell redox status, the GSH / GSSG ratio was determined after treating SH-SY5Y cells with opioids. A significant decrease in the GSH / GSSG ratio was induced by 4 and 24 hrs of morphine treatment, demonstrating a cellular shift to an oxidative stress state (Fig. 32). In contrast to the effects of morphine, 4- and 24-hour met-enkephalin treatment elevated the GSH / GSSG ratio, indicating a shift towards more reduced state (Fig. 33).

The ratio of SAM / SAH was also calculated in the same experiments. No changes were observed in the SAM / SAH ratio upon 4 hrs of morphine treatment, whereas with 24 hrs of morphine exposure, the SAM / SAH ratio decreased significantly (Fig. 32). This shift in SAM / SAH ratio indicates a reduced capacity for methylation reactions, including DNA methylation. In contrast, met-enkephalin significantly elevated the cellular SAM / SAH ratio at 4 hrs, and the ratio returned to control value at 24 hrs (Fig. 33).
Figure 32: Effect of morphine on GSH / GSSG and SAM / SAH ratios in SH-SY5Y cells. GSH / GSSG and SAM / SAH ratios from cells treated with morphine (1μM, 4 and 24 hrs), n=3. Asterisks (*) show a significant difference (p<0.05) from untreated control values.

Figure 33. Effect of met-enkephalin on the GSH / GSSG and SAM / SAH ratios in SH-SY5Y cells. Cells were treated for 4 hrs and 24 hrs with met-enkephalin (10 nM). GSH / GSSG ratios were calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in SH-SY5Y human neuroblastoma cells. n=4. Asterisks (*) indicate a significant difference (p<0.05) between met-enkephalin-treated vs. matched untreated control cells.
Thus morphine induces a significant decrease in the levels of GSH and decreases GSH / GSSG ratio, indicative of oxidative stress, whereas, met-enkephalin treatment does not induce these changes. In fact met-enkephalin treatment induces elevated levels of GSH and shifts the redox potential of the cell to a more reduced state. Similarly, morphine induces a decreased methylation capacity by decreasing the levels of SAM / SAH whereas met-enkephalin does not induce any change in the SAM / SAH ratio and does not affect the methylation capacity in the cell.

G. Involvement of different protein kinases in mediating opioid-induced changes in redox and methylation status.

As characterized above, the levels of intracellular thiols and the GSH / GSSG and SAM / SAH ratios are indirectly regulated by intracellular cysteine concentration. Several downstream signaling protein kinases were observed to be involved in mediating the effects of opioids on EAAT3-mediated cysteine uptake by SH-SY5Y neuronal cells. Hence, the potential involvement of the protein kinases in mediating the effects of opioids on the levels of intracellular thiols and cellular redox (i.e. GSH / GSSG ratio) and methylation (i.e. SAM / SAH ratio) status was characterized. For this purpose, SH-SY5Y cells were pretreated with protein kinase inhibitors PD98059 (10 μM, 24 hrs) and H-89 (100 nM, 4 hrs) in a similar manner as in the cysteine uptake experiments followed by treatment with either morphine or met-enkephalin, and intracellular thiol levels were resolved and measured using HPLC with electrochemical detection.

Pretreatment with protein kinase inhibitor H-89 blocked some of the changes observed in intracellular thiols after 4 hrs of morphine treatment. Specifically, the changes observed
after 4 hrs of morphine treatment in the intracellular levels of cysteine, cystine, GSH and cystathionine (Fig. 34) and in the GSH / GSSG and SAM / SAH ratios (Fig. 35) were no longer observed after H-89 pretreatment. These results, in combination with those of the cysteine-uptake experiments (Fig. 25), indicate that PKA is involved in mediating the effects of 4 hrs of morphine exposure on redox and methylation status in SH-SY5Y cells via EAAT3-mediated cysteine uptake. Treatment with H-89 alone was performed separately and no changes were observed. (Results are indicated in the Appendix- Fig. 2)

![Graph: Influence of PKA inhibition on morphine-induced changes in SH-SY5Y cellular thiols and thioethers.]

**Figure 34: Influence of PKA inhibition on morphine-induced changes in SH-SY5Y cellular thiols and thioethers.**

SH-SY5Y neuroblastoma cells were pretreated with or without the PKA inhibitor H-89 (100 nM, 4 hrs) followed by treatment with morphine (1μM, 24 hrs). The levels of thiol and thioether metabolites (measured via HPLC with electrochemical detection) were plotted as a percent-change normalized against no-treatment control. Asterisk (*) indicates comparison of morphine-treated samples vs. no-treatment control; (#) indicates comparison of H-89 pretreated samples vs. morphine-treated samples.
Figure 35: Effect of protein kinase inhibitors on morphine-induced changes in the redox state and methylation capacity of SH-SY5Y cells. Cellular GSH / GSSG and SAM / SAH ratios were calculated from thiol and thioether contents of SH-SY5Y cells as determined by HPLC analysis with electrochemical detection. Cells were pretreated with or without the PKA inhibitor H-89 (100 nM, 4hrs), followed by treatment with morphine (1μM, 4 hrs). n=4. Asterisks (*) indicate a significant difference (p<0.05) against matched control.

Pretreatment with the MAPK/ERK kinase inhibitor PD98059 blocked the changes observed in intracellular thiols after 24 hrs of morphine exposure; in particular the changes in intracellular levels of cysteine, GSH and SAM were blocked after pretreatment with PD98059 (Fig. 36). Effects of PD98059 alone were also characterized and no major changes were observed. (Results in Appendix, Fig. 2) Morphine-induced changes in GSH / GSSG and SAM / SAH ratios were also prevented by PD98059 (Fig. 37). These results are coherent with cysteine uptake experiments (Fig.26), providing further support for the conclusion that MAPK/ERK kinase is involved in mediating the effects of 24 hrs morphine on neuronal-cell redox and methylation status.
Figure 36: Influence of MAPK inhibition on morphine-induced changes in SH-SY5Y cellular thiols and thioethers. SH-SY5Y neuroblastoma cells were pretreated with or without a MAPK/ERK kinase inhibitor PD98059 (10 μM, 24 hrs) followed by treatment with morphine (1 μM, 24 hrs). The levels of thiol and thioether metabolites measured by HPLC with electrochemical detection were plotted as a percentage change normalized against no-treatment control. Asterisk (*) indicates comparison of morphine treated samples v/s no treatment control; # indicates comparison of PD98059 pretreated samples v/s morphine treated samples.

Figure 37: Effect of morphine on the redox state and methylation capacity of SH-SY5Y cells. GSH / GSSG and the SAM / SAH ratios were calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in SH-SY5Y human neuroblastoma cells. The cells were pretreated with or without PD98059 (10 μM, 24 hrs), followed by treatment with morphine (1μM, 24 hrs). n=4. Asterisks (*) show a significant difference (p<0.05) vs. matched control.
Since PI3K was shown to be involved in the effects of met-enkephalin on EAAT3-mediated cysteine uptake by SH-SY5Y cells (above), potential involvement of PI3K in regulating cellular thiol metabolite concentrations and the GSH / GSSG and SAM / SAH ratios was investigated. SH-SY5Y cells were treated in a similar manner to the cysteine uptake experiment: Cells were pretreated with LY294002, followed by treatment with met-enkephalin for 4 hrs, and thiol metabolite quantification was carried out. No major changes were observed under the influence of LY294002 itself (Results shown in Appendix, Fig. 2). Fig. 38 indicates that met-enkephalin-induced changes of intracellular thiol levels in SH-SY5Y cells, especially changes in intracellular cysteine, GSH and SAM, were abolished by pretreatment with LY294002, which also prevented met-enkephalin-induced alterations in GSH / GSSG and SAM / SAH ratios (Fig. 39)
Figure 38: Influence of PI3K inhibition on morphine-induced changes in SH-SY5Y cellular thiols and thioethers. SH-SY5Y neuroblastoma cells were pretreated with or without a PI3K inhibitor LY294002 (100 nM, 2 hrs) followed by treatment with met-enkephalin (10 nM, 4 hrs). The levels of thiol and thioether metabolites measured by HPLC with electrochemical detection were plotted as a percentage change normalized against the no-treatment control. Asterisk (*) indicates comparison of met-enkephalin treated samples vs. no treatment control, p<0.05, student’s t-test.
Figure 39: Effect of met-enkephalin on the redox state and methylation capacity of SH-SY5Y cells. GSH / GSSG and SAM / SAH ratios were calculated from electrochemical HPLC measurement of intracellular thiols and thioethers in the SH-SY5Y human neuroblastoma cell line. Cells were pretreated with or without LY294002 (100 nM, 2 hrs), followed by treatment with met-enkephalin for 4 hrs. n=4. Asterisks (*) indicate a significant difference (p<0.05) matched control.

Summary.

- **Protein kinase inhibitors** H-89 and PD98059 blocked the short-term and long-term inhibitory effects of morphine on cellular levels of cysteine respectively, and also the morphine-induced decreases in GSH / GSSG and SAM / SAH ratios were blocked. These results indicate that PKA is involved in mediating the short-term effects of morphine and MAPK/ERK kinase is involved in mediating the long-term effects of morphine.

- **Effects of met-enkephalin treatment** were blocked by pretreatment with LY294002, which indicates involvement of PI3K in elevated cysteine uptake.

- **These results support previous studies** indicating that PKA and MAPK/ERK are involved in mediating the acute and chronic effects of morphine.\(^{71,132}\)
H. Effect of opioids on global DNA methylation levels.

It has been hypothesized that shifts in redox status towards oxidative stress would cause a decrease in the roughly 1000 methylation reactions, including DNA methylation, carried out by the 209 methyltransferases in humans, by decreasing the availability of the methyl donor SAM.\textsuperscript{141,142} Since data presented above demonstrate that opioids alter the SAM / SAH ratio in SH-SY5Y cells, it was hypothesized that the methylation capacity in these cells would also be altered by opioid exposure. Hence cellular methylation activity in response to opioids was measured using DNA methylation as a prototype of an important, highly orchestrated methylation reaction in neuronal cells.

Along with histone methylation, transcription factors, and microRNAs, the epigenetic phenomenon of DNA methylation plays an important role in regulating gene expression. Administration of opioids (especially heroin and morphine) has recently been shown to decrease histone methylation levels in the nucleus accumbens of rodent brain.\textsuperscript{113} However, changes in DNA methylation levels after morphine exposure is not yet characterized. To investigate the effects of opioids on DNA methylation global 5-methylcytosine levels in isolated genomic DNA were measured by ELISA as a proxy for global DNA methylation. 5-MeC is the most abundant methylated form of DNA. This approach has been validated and used by our laboratory previously.\textsuperscript{27}

Global DNA methylation in SH-SY5Y neuronal cells was not influenced by 4 hrs of morphine treatment. However, global DNA methylation in the neuronal cells was significantly decreased (by about 50%) after 24 hrs of morphine treatment (Fig. 40). Quantification of DNA methylation levels at 4 hrs and 24 hrs of treatment with met-enkephalin revealed
elevated levels of global DNA methylation (Fig. 41). These respective changes in global DNA methylation levels correlate with the opioid-induced changes in the cellular SAM / SAH ratio: morphine caused a net decrease in cellular methylation capacity (Fig. 32), whereas met-enkephalin acutely increased the SAM / SAH ratio (Fig. 33). The aggregate data support the conclusion that, opioids affect DNA methylation via a redox-regulated methylation pathway, which is a consequence of changes in EAAT3-mediated cysteine transport.

Figure 40: Effect of morphine on global DNA methylation in SH-SY5Y cells. 5-methylcytosine levels were quantified using an ELISA-based assay. Cells were treated for 4 or 24 hrs with morphine (1 μM). n=6. Asterisks (*) indicate a significant difference (p<0.05) from control.
Figure 41: Effect of met-enkephalin on global DNA methylation in SH-SY5Y cells.
Cells were treated for 4 or 24 hrs with met-enkephalin (10 nM) and DNA methylation was quantified using ELISA based assay. n=6. Asterisks (*) show a significant difference (p<0.05) from control.

Data presented in previous sections of this thesis implicated distinct protein kinases (namely PKA, PI3K, and MAPK/ERK) as mediators of opioid-induced effects on cysteine uptake and GSH / GSSG and SAM / SAH ratios in SH-SY5Y neuronal cells. The correlation between the SAM / SAH ratio in these cells and the measured levels of global DNA methylation suggests the involvement of protein kinases in mediating the opioid-induced effects on global DNA methylation levels. To address this point experimentally, SH-SY5Y cells were pretreated with specific protein kinase inhibitors prior to incubation with opioids for different durations, whereupon global DNA methylation was measured. As shown in Fig. 42, the PKA inhibitor H-89 did not alter the effects of morphine on global DNA methylation at 4 hrs, whereas the MAPK/ERK inhibitor PD98059 completely blocked the effects of morphine on global DNA methylation at 24 hrs. These data implicate MAPK/ERK kinase in mediating 24 hrs the morphine-induced effects on cysteine uptake and cellular GSH / GSSG and SAM / SAH ratios as well as global DNA methylation. Additionally, PD98059 did not
alter the changes induced by 4 hrs of morphine, and similarly, H-89 did not alter the changes induced by morphine at 24 hrs.

Figure 42: Involvement of protein kinases in mediating effects of morphine on global DNA methylation in SH-SY5Y cells. Cells were treated with morphine (1 μM) for 4 hrs and 24 hrs in presence or absence of H-89 (100 nM, 4 hrs) or PD98059 (10 μM, 24 hrs), respectively (n=6). DNA was isolated and global methylation was quantified on 50ng of DNA using an anti-5-methylcytosine antibody and measured by ELISA. Asterisks (*) indicate a significant difference (p<0.05) from untreated controls.

Since PI3K was implicated in mediating the met-enkephalin effects on cysteine uptake (Fig. 28) and the GSH/GSSH and SAM / SAH ratios (Fig. 39) in SH-SY5Y neuronal cells, the potential involvement of specific protein kinases in mediating the effects of met-enkephalin on global DNA methylation was examined. For this purpose, SH-SY5Y cells
were pretreated with the PI3K inhibitor LY294002 followed by incubation with met-enkephalin, after which global DNA methylation was measured at 4 hrs. LY294002 completely blocked the met-enkephalin effect on global DNA methylation (Fig. 43), suggesting that PI3K is involved in mediating the met-enkephalin-induced changes in cysteine uptake (Fig.28), and GSH / GSSG and SAM / SAH ratios (Fig. 39) as well as global DNA methylation.

Figure 43: Involvement of PI3K in mediating the effects of met-enkephalin on global DNA methylation in SH-SY5Y cells. Cells were treated with met-enkephalin (10 nM) for 4 hrs in presence or absence of LY294002 (100 nM, 2 hrs) (n=6). Global methylation was quantified using an anti-5-methylcytosine antibody and ELISA. No significant changes were observed after 4 hrs of met-enkephalin, and, pretreatment with LY294002 also had no effects.

Additionally, no significant changes in DNA methylation were observed with H-89, LY294002 or PD98059 treatment alone. (Data provided in Appendix, Fig. 1)

100
I. Morphine alters DNA methylation in repetitive elements and their transcription to RNA

To confirm the morphine-induced changes in global DNA methylation previously detailed (Fig.40), a methylated DNA immunoprecipitation (MeDIP) assay was performed. SH-SY5Y cells were treated with morphine for 4 and 24 hrs, at which times genomic DNA was isolated, and DNA methylation was mapped across the entire genome at 270,000 CpG sites on LINE-1 specific regions. The level of methylation in morphine-treated cells was then compared to that in control cells not exposed to morphine.

A 24-hour morphine exposure induced hypo-methylation of CpG sites in LINE-1 regions, whereas a 4-hour morphine exposure induced CpG hyper-methylation in LINE-1 regions (Fig. 44A). These results correlate with temporal changes in the levels of SAM / SAH (Fig. 32), as well as changes in global DNA methylation after incubated with morphine (Fig. 40), wherein we observed decreased levels of SAM / SAH and decreased levels of global DNA methylation which would indicate global hypomethylation after morphine exposure.

We next measured changes in CpG methylation specifically in the LINE-1 promoter region in the genomic DNA of SH-SY5Y cells that had been incubated with morphine for 4 and 24 hrs. Fig. 44C shows a volcano plot for LINE-1 (transcription start site (TSS) ± 500 base pairs) demonstrating hypermethylation in the promoter region at 4 hrs of morphine exposure and slightly reduced methylation levels after 24 hrs of exposure.

CpG methylation levels in the promoter region are inversely correlated with mRNA expression levels. To determine whether the observed morphine-induced changes in LINE-1 CpG methylation in SH-SY5Y cells were associated with changes in its transcription, primers
specific for the LINE1-HS family were designed and used to quantify the respective mRNA levels using qRT-PCR. Exposure of SH-SY5Y cells to morphine for 4 hrs significantly decreased LINE-1 mRNA levels (Fig. 44B), which correlates with increased CpG methylation levels in the promoter regions of LINE-1 (Fig. 44A). A 24-hour morphine exposure induced a significant elevation of LINE-1 mRNA levels. These effects on the mRNA levels of LINE-1 were blocked by a pretreatment with specific protein-kinase inhibitors. The PKA inhibitor H-89 blocked the effects of 4hrs of morphine exposure and pretreatment with PD98059 blocked the effects of 24 hrs of morphine exposure, on LINE-1 mRNA levels.
Figure 44: Morphine induces hypomethylation in LINE-1 repetitive elements and increases LINE-1 mRNA levels in SH-SY5Y neuronal cells. (A) Cellular 5-methylcytosine levels in LINE-1 repetitive elements were measured using MBD sequencing. Four hrs of morphine treatment induced an increase in LINE-1 CpG methylation, whereas 24 hrs of morphine treatment decreased LINE-1 methylation. (B) qRT-PCR analysis of LINE-1 mRNA levels. Four hrs of morphine treatment decreased LINE-1 mRNA levels, whereas 24 hrs of morphine treatment induced a large increase in LINE-1 mRNA. Pretreatment with H-89 (100 nM, 4 hrs) or PD98059 (10 μM, 24 hrs) blocked the changes in LINE-1 mRNA levels after 4 or 24 hrs of morphine treatment, respectively. (C) A volcano plot for promoter methylation (TSS ± 500 base pairs) of LINE-1 is shown. Morphine increased promoter methylation after 4 hrs, which returned to normal levels after 24 hrs of morphine treatment. N=5. Asterisks (*) indicate a significant difference (p<0.05) from untreated controls. MBD: methylated CpG binding domain protein. TSS: Transcription start site.

Summary.

Together these results indicate that the morphine-induced changes in cysteine transport and intracellular cysteine alter cellular redox and methylation status, subsequently inducing global changes in methylation, reflected as changes in global DNA methylation, as well as time-dependent changes in the CpG methylation status of LINE-1 retrotransposons and their mRNA levels. These global epigenetic changes after morphine exposure can have a large impact on gene transcription status.
J. Morphine induced changes in transcription status of the cells.

Several modes of regulation impinge upon gene expression in eukaryotes and thereby further also influence mRNA levels. DNA methylation is a common, highly important epigenetic signaling tool that most commonly locks genes in the "off" position and down-regulates gene expression. Results presented above demonstrate that morphine induces significant changes in the global DNA methylation level in SH-SY5Y neuronal cells (Fig. 40). These results invited investigation of the consequences of these epigenetic methylation effects on genome-wide mRNA levels in SH-SY5Y cells. For this purpose, an Agilent Sureprint-3 microarray-based expression approach was utilized to measure the global transcription changes in these cultured neuronal cells as a consequence of 4 hrs and 24 hrs of morphine exposure.

![Figure 45: Effect of morphine on genome-wide mRNA levels in SH-SY5Y cells.](image)

Genome-wide microarray results were plotted after measuring the mRNA levels in SH-SY5Y cells treated with morphine for (A) 4 and (B) 24 hrs. At both times, morphine increased gene expression (i.e. differentially expressed mRNA transcripts). About 96% and 98% DETs were up-regulated after 4 and 24 hrs of morphine, respectively. DETs (differentially expressed gene-transcripts.)
Transcripts that were significantly altered under the influence of morphine as compared to untreated controls are listed in Tables 2 and 3. Interestingly, the ratio of the number of up-regulated transcripts (96-98%) to those down-regulated (2-4%) was highly significant (Fig. 45), consistent with the global DNA hypomethylation observed after incubation with morphine (Fig. 40), which would invite an increase in gene expression.

K. Functional analysis of SH-SY5Y neuronal-cell mRNA transcripts altered after morphine exposure.

To investigate the functional pathways altered by 4- and 24-hr morphine exposures in SH-SY5Y cells, Ingenuity software based pathway (IPA) analysis was performed for all the DETs identified to be differentially expressed after incubation with morphine at different time points.
**Figure 46: Pathway Analysis for DETs after 4 hrs of morphine treatment.**

Top functional pathways associated with the differentially expressed gene-transcripts (DETs) for 4 hrs of morphine exposure. The DETs were selected based on the p-value (p<0.05, vs. control). Values in the “list” column indicate the total number of DETs observed to be either up-regulated (under the red triangle) or down-regulated (under the green triangle) from a particular pathway after incubated with morphine.

The chart in Fig. 46 indicates the functional pathway-based stratification of all the differentially expressed genes in SH-SY5Y cells after 4 hrs of morphine exposure. Some 5% of the DETs were reported to be involved in the calcium signaling pathway, which is involved in mediating opioid action. 15% of the DETs were related to maintaining cellular metabolic homeostasis. Some 8% of the DETs affected by morphine are involved in the cytokine-cytokine interactions, and morphine mediated anti-inflammatory action via cytokines.
Cytokines are soluble extracellular proteins or glycoproteins that are crucial intercellular regulators and mobilizers of cells engaged in innate as well as adaptive inflammatory host defenses, cell growth, differentiation, cell death, angiogenesis, and development and repair processes aimed at the restoration of homeostasis. Cytokines are released by various cells in the body, usually in response to a stressful condition or activating stimulus, and they induce responses through binding to specific receptors on the cell surface of target cells. Cytokines can be grouped by structure into different families and their receptors can likewise be grouped. The particular SH-SY5Y cellular transcripts for the cytokine family, which are altered by morphine exposure are depicted in Fig. 47.

Some 8% of the DETs were identified as molecules regulating cell-cell adhesion. Some of these proteins are involved in mediating neuronal synapse formation and maintaining axonal junctions. Some other functionally important DETs involved in the ribosomal regulatory pathway, the spliceosomal pathway and the neurotrophin signaling pathway were also altered in SH-SY5Y cells after incubated with 4 hrs morphine exposure.
Figure 47: Cytokine-cytokine canonical pathway obtained by IPA for DETs in SH-SY5Y cells. Control cells (not treated with morphine) and cells after 4 hrs of morphine treatment are compared. Morphine-induced gene alterations are indicated in red.
Similar functional stratification of the various DETs in SH-SY5Y cells after 24 hrs of morphine exposure was also performed, and the results are summarized in Fig. 48.

Figure 48: Pathway Analysis for DETs after 24 hrs of morphine treatment.
Top functional Pathways associated with the DETs for 24 hrs of morphine exposure, analyzed by IPA. The DETs were selected based on the p-value (p<0.05, vs. control). The numbers in the column “list” indicates the total number of DETs observed to be either up-regulated (under the red triangle) or down-regulated (under the green triangle) from a particular pathway after incubated with morphine.

For approximately 20% of the DETs in SH-SY5Y cells, the effect of 24 hrs of cellular morphine exposure was mainly observed on various metabolic pathways. Interestingly, some 2% of these DETs were involved in glutathione metabolism.
Furthermore, some of the functional pathways that were affected by 4 hrs of morphine exposure were also altered after 24 hrs of morphine exposure, for example, cell-adhesion molecules, cytokine-cytokine interactions, neuroactive ligand-receptor interaction pathways.

Fig. 49 depicts the DETs related to the RNA transport mechanism altered after morphine exposure. RNA transport involves proteins and enzymes involved in dendritic or axonal mRNA transport. Local protein synthesis takes place at the level of synapse, and axonal levels of mRNA are highly critical for this protein synthesis.

**Figure 49:** RNA transport canonical pathway obtained by IPA for DETs in SH-SY5Y cells. Control cells (not treated with morphine) and cells after 24 hrs of morphine treatment are compared. Morphine-induced gene alterations are indicated in red.
DETs were also identified that regulate the actin cytoskeleton (Fig. 50). Actin is involved in axon initiation, growth, guidance and branching, in the morphogenesis of dendrites and dendritic spines, in synapse formation and stability, and in axon and dendrite retraction. Hence, actin is not only important for synaptic plasticity, but also neuronal homeostasis and cell survival. The changes in DETs after morphine exposure related to the actin cytoskeleton could have implications regarding maintenance of synaptic plasticity of the neuronal cells.
Figure 50: Actin cytoskeleton canonical pathway obtained by IPA for DETs in SH-SY5Y cells. Control cells (not treated with morphine) and cells after 24 hrs of morphine treatment are compared. Morphine-induced gene alterations are indicated in red.

Morphine exposure for 24 hrs also induced DETs responsible for the MAPK signaling pathway (Fig. 51). The (MAPK) cascade is a highly conserved module involved in various cellular functions, including cell proliferation, differentiation and migration and cellular plasticity. These results validate the earlier results presented (Fig. 26) which suggested the involvement of MAPK/ERK signaling in mediating changes in global DNA methylation levels after incubated with morphine via EAAT3 mediated cysteine uptake.
Figure 51: Canonical MAPK signaling pathway obtained by IPA for DETs in SH-SY5Y cells. Control cells (not treated with morphine) and cells after 24 hrs of morphine treatment are compared. Morphine-induced gene alterations are indicated in red.

Lastly, there were about 964 DETs for the comparison of two different time points of morphine treatment. Figure 52 indicates stratification of the 964 DETs in functional pathways. Important pathways including the cysteine-methionine metabolism pathway were changed in gene expression after incubation with morphine at different time points.
Stratification of the mRNA transcripts in SH-SY5Y cells altered by morphine indicates that 4 hrs of morphine exposure induced changes in the expression of genes important for anti-inflammatory responses and maintaining metabolic homeostasis, whereas 24 hrs of morphine exposure induced changes in genes important for signaling cascades (e.g. MAPK), synaptic protein synthesis and synaptic plasticity, and synaptic homeostasis.
L. Cluster analysis of genes regulated by morphine exposure.

In SH-SY5Y cells after 4 hrs of morphine exposure, ~1665 DETs were reported, which increased some 2-fold to 3650 DETs after 24 hrs of morphine exposure. Hence, in addition to the functional pathway analysis, it became of interest to compare the global patterns of gene expression after 4 hrs morphine versus 24 hrs morphine treatment. For this purpose, a hierarchical cluster analysis based on standard correlations was used. The regulated genes fell into several clusters. Some genes were up-regulated or down-regulated as a result of 4 and/or 24 hrs of morphine treatment. The individual DETs in SH-SY5Y cells after 4 and 24 hrs of morphine treatment (as compared to control cells not treated with morphine) are represented in Appendix Tables 2, 3. Some transcripts remained unaltered after 4 hrs of morphine treatment, but were up- or downregulated after 24 hrs of morphine exposure, whereas some did not change even after 24 hours of morphine exposure (Figs. 53, 54).
Figure 53: Hierarchical Cluster Analysis for DETs in SH-SY5Y cells after 4 hrs of cellular morphine exposure. Hierarchical cluster analysis was based on changes from triplicate-arrays using standard correlations and results colored using a heat map (red, upregulated; blue, downregulated). The figure shows genes that are significantly upregulated (bottom) or downregulated (top) by exposure to morphine. The list of genes is in the Appendix as Table 2.
Figure 54: Hierarchical Cluster Analysis for DETs in SH-SY5Y cells after 24 hrs of cellular morphine exposure. Hierarchical cluster analysis was based upon changes from triplicate arrays, using standard correlations and results are colored using a heat map (red, up-regulated; blue, down-regulated). The figure shows genes that are significantly up-regulated (bottom) or down-regulated (top) by exposure to morphine. The list of genes is in the Appendix as Table 3.
As indicated in Figs. 53 and 54, morphine induced elevated levels of large number of transcripts, and exposure to 24 hrs of morphine had a greater impact in terms of number of up-regulated transcripts as compared to 4 hrs of morphine. Many of the genes that were differentially expressed belonged to the categories of opioid signaling pathways and downstream effector proteins, including protein kinase signaling pathways (e.g., MAPK). Genes involved in cell adhesion or the actin cytoskeleton were also reported to be up-regulated, as observed even in the functional pathway analysis. Genes related to N-glycan biosynthesis were found to be down-regulated after 24 hrs of morphine exposure, N-glycan being involved in endocytosis of μ- and δ-opioid receptors. Several other altered genes were also related to ABC transporters, apoptosis and toll-like receptor signaling. Opioid activation of toll-like receptor 4 contributes to drug reinforcement via the MAPK signaling pathway. Thus, the cluster analysis showed the individual transcripts altered after morphine exposure, and illustrated that morphine elicited global changes in DNA transcription in SH-SY5Y cells via global epigenetic changes mediated by influencing cellular cysteine uptake and redox status.

M. Opioid-induced changes in genes involved in transsulfuration.

Morphine-induced global changes in mRNA expression levels in SH-SY5Y cells detailed above relate to genes involved in maintaining synaptic plasticity, neuronal structural and metabolic homoeostasis. However, few of the genes involved in glutathione metabolism as well as cysteine and methionine metabolism were observed, and transcriptional changes in the enzymes/proteins specifically related to the transsulfuration pathway were not specifically
focused upon. Hence, a qRTPCR-based approach was used for this purpose, and a customized array was developed for these genes. RNA was isolated from SH-SY5Y cells exposed to morphine exposure for either 4 or 24 hrs, and cDNA was synthesized and used for the qRTPCR assay. The data were quantified using the Δ(ΔCt) method.

Figure 55: Effect of a 4-hour morphine exposure on the expression of redox- and methylation-linked genes in SH-SY5Y cells.
SH-SY5Y neuroblastoma cells were treated with morphine in presence or absence of PKA inhibitor H-89 (100 nM, 4 hrs). mRNA was isolated and expression of mRNA levels from SH-SY5Y neuroblastoma cells were probed using qRT-PCR with primers designed for a panel of redox and methylation-linked genes. Values were normalized to β-actin levels and then to control gene expression, using the Δ(ΔCt)) method. n=4.

Morphine exposure for 4 hrs decreased the levels of LINE-1, CBS and EAAT3 mRNA in SH-SY5Y neuroblastoma cells (Fig. 55). Levels of dopamine receptor DRD4 were also significantly altered. DRD4 is closely linked to methionine cycle and its gene variants are associated with drug addiction. Smaller changes were also observed in CGL and DNMT3a. The levels of Nrf-2 mRNA were found to be significantly elevated. This indicates that
morphine treatment suppresses EAAT3 mRNA-transcription, which might contribute to the decreased EAAT3-mediated cysteine uptake after incubation with morphine (Fig. 20). Nrf-2 is antioxidant response element (ARE), and the Nrf2-Keap1 system is involved in regulating the mRNA levels of proteins involved in redox homeostasis. Hence, Nrf2 is a redox-sensitive transcription factor, and an increase in its transcription is indicative of a cellular response to oxidative stress at the mRNA level.

A parallel investigation for characterizing the potential effect of morphine at 24 hrs on mRNA levels in SH-SY5Y cells was also performed (Fig. 56). A 24-hour exposure to morphine elevated levels of LINE-1, Nrf-2, CBS, BDNF and µ-OR mRNA. Also, the mRNA level of GSH synthetase was significantly decreased along with other mRNA levels of the enzyme GCLM, both of which are involved in synthesis of GSH. The increased levels of µ-OR indicate development of tolerance at the receptor level, as previously observed. BDNF is involved in maintaining neuronal and structural plasticity and regulates EAAT-3 mediated cysteine uptake via the PI3K pathway. Additionally, a pretreatment with the PKA inhibitor H-89 and the MEKK/ERK inhibitor PD98059 blocked the effects of morphine, which supports the involvement of these cell-signaling pathways in mediating the effects of morphine at 4 and 24 hrs, even at the transcription level on SH-SY5Y neuroblastoma cells.
Figure 56: Effect of 24 hrs of morphine exposure on the expression of redox and methylation-linked genes in SH-SY5Y cells.

SH-SY5Y neuroblastoma cells were treated with morphine in presence or absence of the MEEK/ERK inhibitor PD98059 (10 μM, 24 hrs). mRNA was then isolated, and expression of mRNA levels from SH-SY5Y neuroblastoma cells was quantified. Values were normalized to β-actin levels and then to control gene expression, using the Δ(ΔCt) method. n=4.

Investigating the levels of mRNA after 4 hrs of met-enkephalin treatment revealed elevated levels of EAAT3, Nrf-2, as well as BDNF (Fig. 57), whereas mRNA levels of CGL and CBS decreased. Met-enkephalin potentiated cysteine uptake after for 4 hrs, and the increased EAAT3 mRNA levels support these results. These elevated mRNA levels of EAAT3 as well as BDNF returned to normal after 24 hrs treatment with met-enkephalin, which again corresponds to no changes observed in the EAAT3 mediated cysteine uptake.
LINE-1 mRNA levels were also decreased after 24 hours of met-enkephalin treatment, which might be a potential consequence of increased methylation under the influence of met-enkephalin as observed previously.

Figure 57: Effect of met-enkephalin on the expression of redox and methylation-linked genes in SH-SY5Y cells. SH-SY5Y neuroblastoma cells were treated with met-enkephalin for 4 and 24 hrs, at which times mRNA was isolated. mRNA levels were probed using qRT-PCR with primers designed for a panel of redox and methylation-linked genes. Values were normalized to β-actin levels and then to control gene expression, using the Δ(ΔCt) method. n=4. The Y-axis indicates fold changes in mRNA levels.
Summary.

- Thus, results from the qRTPCR assays not only indicate that opioid exposure could impact changes in genes related to glutathione, cysteine and methionine metabolism after incubation with opioids, but these results also validate the microarray analysis.
- Morphine exposure influenced important cellular functional pathways at the mRNA levels; e.g. actin cytoskeleton, cytokine-cytokine interactions, glutathione metabolism pathway etc.
- More importantly these studies illustrate that morphine elicited global changes in DNA transcription in SH-SY5Y cells via global epigenetic changes mediated by influencing cellular cysteine uptake and redox status.

N. Effects of in vitro opioid washout from SH-SY5Y cell cultures

The human SH-SY5Y neuroblastoma cell line displays morphological, neurochemical, and electrophysiological characteristics similar to sympathetic neurons. Several published studies have shown that chronic opioid exposure for 24 to 72 hrs in SH-SY5Y cells can induce tolerance that is diminished after opioid washout. Additionally, opioid washout after chronic treatment for 24-48 hrs, or treatment with an opioid-receptor blocker, has been reported to alleviate the oxidative stress associated with chronic opioid exposure. As detailed above, exposure to opioids for 24 hrs was observed to induce changes EAAT3 mediated cysteine uptake (Figs. 20, 21), intracellular levels of cysteine, GSH and SAM (Figs. 30, 31), the redox status (Fig. 32, 33), methylation capacity (Fig. 32, 33) as well as global DNA
methylation status (Fig. 40, 41). Thus, we further examined whether opioid washout from SH-SY5Y cells in culture would alter/reverse the opioid-induced effects on these cells (Fig. 58).

**Figure 58: In vitro washout regimen.** SH-SY5Y cells were treated with morphine or met-enkephalin for 4 and 24 hrs followed by three washes with HBSS. After the washes, the cells were again incubated for another 24 hrs with fresh growth media without an opioid. Cysteine uptake was performed at the end of this 24-hour incubation with the fresh media, termed the “opioid washout.”

The data in Fig. 59 demonstrate that the 24-h washout that elicited the recovery of cellular cysteine content to the control level following a 4-h morphine exposure. These elevated levels of cysteine uptake were inhibited in the presence of LBTBA (10 μM, 2 hrs), which is a specific EAAT3 blocker, indicating that the increased cysteine uptake during the washout period was due to the EAAT3.
Figure 59: *In vitro* drug washout after 4 hrs of morphine treatment. SH-SY5Y cells were treated with 1µM morphine for 4 hrs. Cells were then washed, and fresh media was added without any morphine for 24 hrs. A significantly higher cysteine uptake was observed in morphine-exposed cells that were then incubated in fresh media for 24 hrs. Pretreatment with LBTBA (10 μM, 2 hrs) blocked the stimulation of cysteine uptake induced by morphine washout. N=6, Asterisks (*) indicate a significant difference (p<0.005) from no-treatment control. “WO” stands for “washout.

Previous cysteine uptake experiments showed that 4 hrs of morphine decreased cysteine uptake (Fig. 21). A 24 hrs of washout induced elevated levels of cysteine uptake, similar to the elevated levels observed after washout following the 4 hrs of morphine exposure (Fig. 60). However, a 24 hr post-morphine washout induced elevated levels of cysteine uptake, which was higher than the level of cysteine uptake in untreated control cells.

Hence, morphine treatment significantly reduced the cysteine content of SH-SY5Y neuroblastoma cells, and this reduction was reversed at 24 hrs following morphine washout. Treatment with LBTBA (10 μM, 2 hrs) during the morphine washout period blocked this
washout restoration of intracellular cysteine levels, supporting the role of EAAT3 in mediating these changes during the washout period.

**Figure 60: In vitro drug washout after 24 hrs of pretreatment with morphine.** SH-SY5Y cells were treated with 1µM morphine for 24 hrs. Cells were washed, and fresh media was added without any morphine for 24 hrs. Significantly higher cysteine uptake was observed in morphine-exposed cells that were changed to fresh media for 24 hrs. A pretreatment with LBTBA (10 μM, 2 hrs) blocked the stimulation of cysteine uptake induced by morphine washout. N=6, Asterisks (*) show a significant difference (p<0.005) from no-treatment control.

Similar investigation for characterizing the effects of washout following the treatment with met-enkephalin was also performed (Figs. 61, 62). However, no changes were observed after 4 or 24 hrs of washout period following met-enkephalin treatment.
Figure 61: *In vitro* drug washout after 4 hour met-enkephalin treatment. SH-SY5Y cells were treated with 10 nM met-enkephalin for 4 hrs, followed by a wash and further incubation with fresh media, cysteine uptake was performed at 24 hrs. No difference was observed in met-enkephalin exposed cells that were changed to fresh media for 24 hrs. N=4.

Figure 62: *In vitro* drug washout after 24 hour met-enkephalin treatment. SH-SY5Y cells were treated with 10 nM met-enkephalin for 24 hrs, followed by a wash and further incubation with fresh media without any met-enkephalin for another 24 hrs. No difference was observed in met-enkephalin exposed cells that were changed to fresh media for 24 hrs. N=4.
Hence, significant differences were observed after washout period of 24 hrs following morphine treatment. However, no changes were observed in the met-enkephalin treated samples during the washout period. These differences in variation in different opioids may be due to differences in the underlying signaling mechanism responsible for mediating the effects of opioids.

**O. Changes in intracellular thiol metabolites after washout**

The intracellular thiols and thio-ester metabolites were also investigated to characterize the effect of *in vitro* washout on redox metabolites. The GSH / GSSG and SAM / SAH ratios were also investigated. As shown in Fig.63, a 24 hrs of washout after 24 hrs of morphine treatment increased the intracellular levels of cysteine, GSH and SAM. Homocysteine, methionine, and SAH levels also returned to normal after the washout period. Treatment with LBTBA (10 μM, 2 hrs) during the washout period blocked the washout-induced changes in intracellular thiols and thioester metabolites, implicating EAAT3 in recovery of metabolite levels following morphine washout.
SH-SY5Y neuroblastoma cells were treated with morphine for 24 hrs. Cells were washed, and fresh medium not containing morphine was added for 24 hrs. Pretreatment with LBTBA (10 μM, 2 hrs) blocked the effects induced by morphine washout. Thiol and thioether metabolites were quantified by HPLC with electrochemical detection and are plotted as a percentage change normalized against the control. N=6, (*) indicates a significant difference (p<0.005) from no-treatment control. (#) indicates a significant difference (p<0.05) between morphine 24 hour treatment vs. morphine washout. (***) indicates a significant difference (p<0.05) between morphine washout vs. morphine washout in the presence of LBTBA.

A 24-hour washout period following a 24-h morphine exposure induced a significant elevation in the GSH / GSSG and SAM / SAH ratios in SH-SY5Y neuroblastoma cells (Fig. 64). These results are in contrast to the effects observed after 24 hrs of morphine exposure (Fig. 32), which resulted in a significant decrease in the levels of GSH / GSSG as well as SAM / SAH. Hence, the washout period reversed the effects of 24 hrs of morphine on the cysteine uptake, intracellular concentrations of cysteine, GSH / GSSG and SAM / SAH ratios.
Figure 64: Effect of morphine washout on GSH / GSSG and SAM / SAH ratios. SH-SY5Y neuroblastoma cells were treated with 1 µM morphine for 24 hrs followed by washing with fresh media and 24 hrs incubation with morphine-free media. Intracellular GSH / GSSG and SAM / SAH ratios were then quantified. n=3. Asterisk (*) indicates a significant difference (p < 0.05) from untreated controls after 48 hrs.

Global DNA methylation levels were also measured following the washout period, and elevations in the levels of 5-methylcytosine were observed after the washout period of 24 hrs (Fig. 65). Thus the washout period also reversed the effects of 24 hrs of morphine exposure on global DNA methylation.
Figure 65: Effect of *in vitro* drug washout after 24 hrs of pretreatment with morphine on global DNA methylation levels. SH-SY5Y cells were treated with 1µM morphine for 24 hrs. Cells were washed, and fresh media was added without any morphine for 24 hrs. Significantly higher DNA methylation levels were observed in morphine-exposed cells that were changed to fresh media for 24 hrs. A pretreatment with LBTBA (10 µM, 2 hrs) blocked the stimulation DNA methylation induced by morphine washout. N=6, Asterisks (*) show a significant difference (p<0.05) from no-treatment control.

P. Effects of redox and methylation modulators on morphine-induced changes.

N-acetylcysteine (NAC) by-passes EAAT3 and enters cells via passive diffusion to serve as a precursor for cellular GSH synthesis after its de-acetylation. Methylcobalamin (MeCbl) maintains MS activity, and synthesis of MeCbl is GSH-dependent. Since both of these agents can modulate cellular redox status independent of the effects of EAAT3-mediated cysteine uptake, it was hypothesized that these compounds might reverse the effects of morphine on intracellular cysteine levels, and hence, maintain intracellular redox and methylation homeostasis. To investigate the role of these redox modulators on the effects of morphine, SH-SY5Y cells were treated with either NAC (5 mM) or MeCbl (1 µM) followed by incubation with morphine for 4 hrs or 24 hrs at which times cysteine uptake was measured radiochemically.
Figure 66: Effects of morphine exposure after pretreatment with redox and methylation modulators. SH-SY5Y neuroblastoma cells were treated with morphine for 4 and 24 hrs in the presence or absence of N-acetyl cysteine (NAC; 5 mM) (upper panel) or methylcobalamin (MeCbl; 1 µM) (lower panel). Cysteine uptake was then quantified radiochemically. N=6, asterisks (*) indicate a significant difference (p<0.005) from no-treatment control.
As shown in Fig. 66, a pretreatment with NAC or MeCbl did not alter the morphine-induced effects on cysteine uptake at 4 or 24 hrs. MeCbl and NAC both had no effects on cysteine uptake by themselves. This can be explained by the fact that NAC and MeCbl might not influence the levels of EAAT3-mediated cysteine uptake, but might influence downstream processes related to cysteine uptake, including GSH / GSSG, SAM / SAH ratios as well as global DNA methylation levels. To investigate the same, intracellular thiols and thioester metabolites were measured after treatment with NAC or MeCbl followed by 4 or 24 hrs of morphine exposure (Figs. 67, 68). Both NAC (Fig. 67) and MeCbl (Fig. 68) reversed the morphine-induced decreases in cellular GSH / GSSG and SAM / SAH ratios.
Figure 67: Effect of N-acetylcysteine on morphine-induced changes on the redox state and methylation capacity of SH-SY5Y cells. SH-SY5Y cells were pretreated with NAC (5 mM) followed by treatment with morphine (1µM) for 4 and 24 hrs. The cellular GSH / GSSG and SAM / SAH ratios were calculated from electrochemical HPLC measurement of intracellular thiols and thioethers. n=4. Asterisks (*) indicate a significant difference (p<0.05) matched control.
Figure 68: Effect of methylcobalamin on morphine-induced changes on the redox state and methylation capacity of SH-SY5Y cells. SH-SY5Y cells were pretreated with MeCbl (1 µM) followed by a treatment with morphine (1µM) for 4 and 24 hrs. The GSH / GSSG and SAM / SAH ratios were calculated from electrochemical HPLC measurement of intracellular thiols and thioethers. n=4. Asterisks (*) indicate a significant difference (p<0.05) vs. matched control.
To further confirm the ability of these agents to influence morphine effects, global DNA methylation was also measured after incubated with opioids in presence or absence of NAC and MeCbl. The results (Fig. 69) indicate that both NAC and MeCbl reversed the morphine-induced reduction of global DNA methylation.

**Figure 69:** Effect of N-acetylcysteine and methylcobalamin on morphine-induced reduction of global DNA methylation. SH-SY5Y cells were treated with NAC (5 mM) or MeCbl (1µM) followed by a treatment with morphine (1µM) for 4 and 24 hrs. A pretreatment with NAC as well as MeCbl abrogated the effects of morphine on global DNA methylation. N=6, Asterisks (*) indicate a significant difference (p<0.05) from no-treatment control.
These results add additional support to two major conclusions. Firstly, morphine alters the methylation status of the cells (including DNA methylation) and induces global transcriptional changes by impinging on the redox status and GSH levels, directly dependent upon EAAT3-mediated cysteine uptake. Further, agents that replenish cellular GSH levels restored redox and methylation imbalances induced by morphine. Hence, these drugs could potentially be used to rescue the neuronal cells from the global consequences of oxidative stress resulting from opioid actions and the subsequent consequences.
V. DISCUSSION

Canonically, opiate-mediated perturbations in central nervous system (CNS) function are believed to initiate processes that reinstate functional homeostasis, which leads to the development of opioid tolerance and dependence.\textsuperscript{145} Based upon this hypothesis, attempts have been made to explain the nature of relevant adaptations responsible for opioid addiction, and almost all of these studies have focused on identifying the biological basis of core features of addiction to opioid drugs, particularly tolerance, withdrawal syndrome, and compulsive use of the drug in the face of known harm of opioids. With repeated administration of opioid drugs, adaptive mechanisms are initiated that result in short-term, as well as protracted changes in the functioning of opioid-sensitive neurons and neural networks.\textsuperscript{87,145} These changes are mediated centrally by binding to a G protein-coupled receptor, initiating multiple secondary messenger pathways, associated with effector coupling, receptor trafficking and nuclear signaling. Almost all of these signaling pathways achieve their effects by impinging upon common transcription factors, resulting in altered transcription of genes.\textsuperscript{87,90}

These effects are critical for understanding events leading to tolerance and dependence in cells.\textsuperscript{146} However, equally important are the potential counter-adaptive mechanisms in cellular and synaptic physiology following chronic opioid exposure,\textsuperscript{145} as these opioid-induced biochemical changes may represent a pathological harm to neuronal cells, which can lead to neuropathophysiological consequences.\textsuperscript{87,94,147} Opioids induce alterations in the redox state of neurons by inducing ROS production as well as by decreasing the levels of GSH, the major antioxidant in the brain.\textsuperscript{94,98,148} However, these opioid-mediated effects on redox status of cells have only been marginally investigated. The results of my thesis indicate that opioids impinge upon EAAT3-mediated cysteine uptake, modify the redox state of the neurons and
exert a consequential influence on global epigenetic and transcriptional status, as opposed to targeting individual genes, indicated by contemporary studies. My studies also characterized the involvement of PI3-kinase, ERK, PKA and MAPK as downstream effector proteins for their involvement in the effects of opioids on the redox and methylation status of the neuron. Additionally, I also observed the effects of these opiate-induced redox-based epigenetic and transcriptional changes in the context of retrotransposon elements, which can have global impact on genomic instability, subsequently affecting transcriptional homeostasis. Lastly, my results also indicate that redox-based interventions like NAC and MeCbl, which support indirectly the replenishment of neuronal GSH concentrations and can also interfere with the effects of opioids on redox status and abrogate the consequential epigenetic and transcriptional effects of opiate drugs. This is the first study to characterize redox-based signaling pathway as a novel mechanism of opioids for mediating global epigenetic and transcriptional changes. My thesis recasts the effects of opioids in terms of biochemical and physiological consequences in the neuronal cells, which can be reversed by using redox-based interventions.
A. EAAT3 is cysteine transporter, controls redox-state in neurons.

The importance of EAAT3 as a cysteine transporter is highly underscored. It shares sequence and structural homology with other glutamate transporters, which has obscured the key critical difference in substrate specificity of EAAT3 for cysteine vs. glutamate transport.\textsuperscript{19,22} Glutamate is a primary endogenous ligand for the NMDA receptor, which is involved in the development of \(\mu\)-OR tolerance and associated with abnormal pain sensitivity and withdrawal syndrome. Glutamate transporters (GTs) play a critical role in this neural mechanism of opiate abuse by regulating the synaptic levels of glutamate under the influence of opioids.\textsuperscript{96,136,149–151} However, there is inconclusive evidence to implicate EAAT3 for mediating glutamate transport under the influence of morphine. In fact, studies find other GTs like GLT-1 and GLAST to be more involved than to EAAT3 for mediating the effects of opioids via glutamatergic regulation.\textsuperscript{96,149} Additionally, recent studies led by Drs. Koji Aoyama and Masahiko Watabe,\textsuperscript{10} and by Raymond Swanson,\textsuperscript{22} have shed light on the ability of EAAT3 to transport cysteine. Studies show ubiquitous expression and localization of EAAT3 in all regions of the brain in rats, even in areas where there is no glutamatergic signaling.\textsuperscript{152} In glutamatergic neurons, EAAT3 is not confined to the synapse, but expressed over the entire soma, strongly indicating a non-glutamate function of EAAT3.\textsuperscript{19,152} Additionally, in rats, EAAT3 knock-down in the brain can elicit oxidative stress, but not increased extracellular levels of glutamate.\textsuperscript{4} Finally, studies have shown that EAAT3 preferentially transports cysteine over glutamate.\textsuperscript{18,22} However, in spite of these findings, EAAT3 is still classified as a glutamate transporter, and involvement of aberrant EAAT3 activity or regulation in mediating oxidative stress in a neurological setting are largely unnoticed and unstudied.
My thesis shows how opioids impinge upon EAAT3 via opioid receptor, and regulate cysteine transport through EAAT3 in cultured SH-SY5Y neuroblastoma cells. As indicated earlier, studies have investigated effects of acute and chronic opioid exposure on EAAT3-mediated glutamate transport and signaling in a variety of conditions and models. Results indicated that under certain conditions, different signaling pathways can regulate the surface expression of EAAT3. Specifically, PI3K/Akt and PKA pathways are activated at earlier time points after treatment with opioid exposure, with MAPK and ERK activity playing a role in long-term regulation. My studies show that short-term (30 min and 4 hours) opioid-induced inhibition of EAAT3-mediated cysteine uptake is mediated by PKA (Fig. 25), whereas the stimulation of cysteine uptake is PI3 kinase-dependent (Fig. 24). Long-term inhibition of cysteine uptake by morphine is dependent on MAP kinase activity (Fig. 26). These results are consistent with studies indicating differential involvement of several protein-kinases to regulate EAAT3 activity based on duration of morphine exposure.

Importantly, activation of PI3K via Gi/o G-protein under the influence of morphine has been reported previously to be a key downstream element in promoting neuronal survival with unknown effector proteins. Consistent with this, my results show that the activation of PI3K by exposure of neuronal cells to morphine leads to an increase in cysteine uptake via EAAT3, which can further contribute to promote redox homeostasis, maintain cellular functionality and prevent apoptotic neuronal death, generally observed under the influence of morphine exposure. The alternate inhibition and stimulation of EAAT3-mediated cysteine uptake can also be explained based on the activation of PI3K as one of the compensatory changes which adapt to earlier inhibitory stimuli to prevent cell apoptosis. However, eventually these adaptive mechanisms might be outcompeted by other signaling mechanisms activated under prolonged
morphine exposure; e.g. PKA, MAPK, which can result in inhibition of EAAT3-mediated cysteine uptake. The PKA pathway activates the ubiquitin-proteasomal system under the influence of morphine, which results in down-regulation and degradation of EAAT3.\textsuperscript{132,133} Similarly, GTRAP3-18 is a glutamate transporter associated protein which regulates EAAT3 surface expression and is up-regulated under the influence of chronic morphine exposure.\textsuperscript{23,25}

The δ-OR has been shown to directly co-localize with EAAT3 and to inhibit EAAT3 surface expression. Further, activation of δ-OR with met-enkephalin increases EAAT3 surface expression.\textsuperscript{26} Met-enkephalin is an endogenous opioid and is a natural selective ligand for the δ-OR. I found that met-enkephalin treatment increased EAAT3 mediated cysteine uptake (Fig. 21). Hence, opioids can regulate the surface expression and activity of EAAT3 via different opioid receptors (δ-OR and μ-OR) and multiple secondary signaling pathways. Fig. 70 summarizes my results as well as some of the relevant literature on EAAT3 regulation under the influence of morphine and other opioids and illustrates a probable mechanism by which opioids might impinge upon EAAT3-mediated cysteine transport.
**Figure 70: Summary** Morphine action on EAAT3 via the μ-OR is mediated by (1) PI3K (2) MAPK (3) PKA (4) GTRAP3-18 (5) PTEN ubiquitin Nedd4 pathway, whereas the effects of met enkephalin on EAAT3 are mediate via the (6) δ-OR. Activation of (5) PTEN leads to activation of ubiquitin proteosomal system, which down regulates EAAT3 from the surface and degrades it (7). N-acetylcysteine (NAC, 8) and methylcobalamin (MeCbl, 9) can affect the GSH / GSSG and the SAM / SAH ratios respectively, which can further lead to epigenetic changes (10) and (11) consequently changes in mRNA expression and protein translation.
B. Influence of opioids on redox status

The human brain’s ability to comprehend, consolidate and recall memory is a unique ability achieved over evolution. This ability distinguishes us from any other organism on earth. There is constant structural reorganization in the brain, forming and pruning synapses to perform various cognitive and other activity-dependent functions, although the gross anatomy of the brain remains the same starting in adulthood, with roughly about 100 billion neurons making an estimated 100-500 trillion synapses. Neurons in the adult human brain are non-dividing and are generally non-replaceable, as the neuronal divisions would erase all of the synapses and axonal networks. Generally, it is believed that neuronal survival and stability is critical for storing information and maintaining stored memory. The uniqueness of the human brain is reflected in several mechanisms that have evolved to stabilize and maximize the longevity of this neuronal network.

The unique redox environment in human brain is one such mechanism employed for maintaining a lower redox capacity than the rest of the body, and oxidative stress is one of the many ways that the brain employs to keep cells out of the cell cycle. Brain is a unique redox compartment with lower cysteine and GSH levels in CSF as compared to blood, and even the transsulfuration pathway is partially blocked in adult human mammalian cells, including neurons, which can be inferred by the high levels of cystathionine in the brain compared to other tissues. This partial block of transsulfuration in neurons makes EAAT3 mediated cysteine uptake the primary and in some cases the only source of cysteine in mature human neurons. Hence, EAAT3 is a highly critical regulator of glutathione levels, the antioxidant capacity and redox status in brain. This limited antioxidant levels can allow any minor fluctuations in EAAT3 to induce large changes in the neuronal redox poise. The results from my studies show that the
modulation of cysteine uptake by opioids is able to change intracellular thiol levels and shift the redox potential of the cell. Additionally, several in vitro and in vivo studies have shown that morphine induces oxidative stress by either increasing the levels of ROS or by reducing the levels of GSH in brain and CSF. This is consistent with my results, wherein I observed a large reduction in intracellular levels of GSH (Fig. 30) and a reduced GSH / GSSG-based redox status in neuronal cells upon morphine exposure (Fig 32).

Furthermore, I also show that these changes in intracellular thiols and thioester metabolites subsequently can lead to alterations in the SAM / SAH ratio, which regulates over 1,000 methylation reactions, including DNA methylation. I observed a similar reduction in the levels of SAM / SAH (Fig. 30, 32) as well as global DNA methylation (Fig. 40) after morphine exposure. Thus the effects of opioids on EAAT3-mediated cysteine uptake are reflected in sequential changes in neuronal redox and methylation states.

The relationship between opioids and neuronal redox state is not coincidental. Opioids act on pathways, which stimulate attention, awareness and memory formation. These mechanisms stimulate neurons to grow axons and dendrites and form new synapses, as well as induce axon pruning and synapse retracting. However, these synapses, dendrites and axons are maintained at the cost of underlying metabolic changes. The neurons require ATP to maintain the ion gradient needed for each action potential sent along its axon, and that ATP production induces ROS generation in the electron transport chain. When the level of antioxidant is low, ROS accumulation can lead to downstream compensatory adaptive responses in the transsulfuration pathway as well as the methylation capacity of the cells, inducing global epigenetic changes and increasing transcription of several redox-pathway related genes including
EAAT3. The periodic increases observed in levels of EAAT3-mediated cysteine uptake might be reflective of these compensatory mechanisms, as GSH is decreased under the influence of morphine. However, during chronic opioid exposure, these compensatory pathways fail to restore physiological redox balance, leading to oxidative stress, which can result in neuropathological manifestations including apoptosis and neurodegeneration. This tightly linked control of antioxidant and neuronal activity might be a way of ensuring a narrow window of GSH-based redox potential in the brain. Other research has shown that EAAT3 transcription is linked to redox through the Keap1-Nrf2 pathway.\textsuperscript{33,161} When redox-sensitive cysteine residues on Keap1 become oxidized, Nrf2 disassociates and causes an increase in the transcription of EAAT3, but not the other EAATs. Additionally, the activation of Nrf2 is not the only mechanism increasing the surface expression of EAAT3.\textsuperscript{161} The role of EAAT3 in regulating redox in the human brain is undeniable, and the regulation by opioids seems to link both ROS production and mitigation. The mechanism of opioid-mediated epigenetic consequences via the redox signaling pathway is depicted below (Fig. 70).
Figure 71: **Redox-based epigenetic signaling.** Opioids alter EAAT3 mediated cysteine uptake, affecting GSH synthesis, which shifts redox status and the reducing potential. This shift affects methionine synthase activity further affecting the probability of DNA methylation i.e. epigenetic changes, subsequently inducing changes in gene transcription. Protein methylation and changed gene transcription cause changes in protein function and phenotype.

Performing an in vitro washout experiment after morphine exposure led to increased redox and methylation potential of the cells and indicated a period of recovery from the effects of morphine in the cells (Figs. 60, 63-66). This is reflective of compensatory adaptive changes in cellular responses, which serve to re-establish GSH levels of GSH during the oxidative stress period under the influence of morphine exposure. However, morphine washout might abnormally increase these adaptive changes, consequently leading to increased GSH levels as
well as inducing alterations in downstream regulated processes like methylation capacity, including DNA methylation, resulting in epigenetic consequences. Changes in glutamate transporter expression under the influence of morphine withdrawal are well characterized from in vivo studies. However, mechanistic studies investigating the consequences after changes in the EAAT3 levels have not as yet been carried out, which would be important for understanding the importance of EAAT3-mediated redox effects during the withdrawal period.

**Figure 72: Gene priming.** Epigenetic mechanisms mediate gene priming and desensitization under the influence of drugs of abuse. Many of these changes are latent, meaning that they are not reflected by stable changes in steady-state mRNA levels. Instead, these changes would induce subsequent changes in chromatin structure, such that a later drug administration/washout would induce a given gene to a greater (primed) or lesser (desensitized) extent based on the epigenetic modifications induced by previous chronic drug exposure. A, acetylation; M, methylation; P, phosphorylation; pol II, RNA polymerase II. (Adapted from Robison et al.)

1.02
Recent preliminary investigations have characterized gene transcription changes during the withdrawal period.\textsuperscript{114,162} However epigenetic changes underlying the transcriptional changes during the withdrawal period was not investigated. Additionally, the relationship between redox-mediated epigenetic changes and gene transcription changes during the withdrawal period also needs to be explored further. Some preliminary in vivo work suggests promising results, as these results support a link between the redox-based epigenetic changes.\textsuperscript{124} However, more studies need to be performed to firmly establish a link between redox changes in the cell and consequent epigenetic and gene expression changes. Fig. 72 depicts the hypothesis for the increased gene expression levels during the washout or relapse drug use. This might help in explaining the increased EAAT3-mediated cysteine uptake during the washout phase. Additionally, the genes involved in mediating the compensatory adaptive changes might also be regulated in this way. However, in the absence of the drug (i.e. during the washout phenomenon), these genes overhaul the signaling pathways as indicated in Fig. 73.
Figure 73: Effect of in vitro washout on cellular redox equilibrium. Inhibition of cysteine uptake can alter cellular redox potential, resulting in adaptive changes in gene expression via epigenetic effects, which restore the redox equilibrium. However, in the absence of opioids during the washout phenomenon, these adaptive changes can lead to increased cysteine uptake as well as intracellular redox potential.

The elevated levels of GSH observed during the washout period if confirmed in clinical studies, can have double-edged consequences. GSH is involved in protein S-glutathionylation, which is a specific posttranslational modification resulting from the disulfide adduction of GSH to a reactive cysteine on a target protein.\textsuperscript{163,164} This glutathionyl disulfide can serve to protect proteins from irreversible cysteine oxidation and/or serve as a functional modification analogous to phosphorylation.\textsuperscript{165} S-Glutathionylation plays an important role in cellular homeostasis and regulation of the balance between cell survival and cell death, as glutathionylation increases protein half-life.\textsuperscript{166} This appears to be an important signaling mechanism for maintaining GSH and GSSG homeostasis in the cells. However, on the other hand it might also prove to be dangerous, since glutathionylation of proteins like proteosomal proteins could lead to
aggregation of ubiquitin’s substrate protein. The proteosome is involved in protein degradation of unnecessary or damaged proteins, which could have neurodegenerative and neuropathological consequences if proteosomal activity is impaired.

**Figure 74: Glutathionylation of the proteosome can lead to protein aggregation and cell death.** Polyubiquitinated proteins are degraded properly in a fully functional proteosome in the appropriate thiol redox state (Proteosome-SH). Deactivation of the proteosome by glutathionylation (Proteosome-SSG), leads to impaired protein degradation, increased protein aggregation, and eventually cell death. (Adapted from Johnson, W. et al)

**C. MS links neuronal redox status and epigenetics.**

MS activity is highly sensitive to inhibition by oxidation and is reciprocally stimulated when redox status shifts to a reduced state, such as when cysteine uptake is increased, accounting for the neurotrophin-induced increase in the levels of MS observed previously. Our laboratory’s previous studies in postmortem human cortex revealed a progressive, age-dependent decrease in MS mRNA level, which amounted to 400-fold decrease across the lifespan. Additionally, it was found that age-dependent alternative splicing of MS mRNA results in the deletion of exons, which affect its sensitivity to oxidation. Thus, MS activity in human brain is highly responsive to the age-dependent increase in demand for antioxidant, which is consistent
with the low level of GSH in neurons. Hence, drugs that induce even minor fluctuations in redox status will exert significant effects on MS activity. In fact, previous results from our laboratory have showed that IGF-1 increases MS activity in SH-SY5Y cells, attributed to its activation of EAAT3-mediated cysteine uptake along with elevated GSH levels. Strikingly, the IGF-1-induced increase in MS activity was also associated with a similar increase of global DNA methylation.

Results from my thesis indicate that opioids also regulate MS activity via the redox state of the cell. My results show that morphine impaired cysteine uptake led to decreased GSH synthesis, triggering an increase in transsulfuration, as depicted by elevated levels in cystathionine. The subsequent negative shift in the GSH / GSSG ratio results in MS inhibition, shown by elevated levels of homocysteine and decreased levels of methionine. MS inhibition leads to an increase in SAH, a potent inhibitor of SAM-dependent methylation reactions, and may result in SAM accumulation. The decreased MS activity, reflected by an increase in homocysteine, can also at least in part be attributed to a GSH requirement for synthesis of methylcobalamin. Cobalamin is the cofactor of MS, which accepts the methyl-group from 5-methyltetrahydrofolate (5-MTHF) and transfers it to homocysteine subsequently to form methionine. However, in periods of oxidative stress, it is more likely that the oxidation state of cobalamin that forms methylcobalamin, Cbl(I), will be oxidized to Cbl(II), which cannot be methylated, limiting MS activity. Oxidation of cobalamin provides a critical redox-sensitive switch for controlling the methylation cycle and is also directly involved in influencing epigenetics in response to neuronal redox changes. Hence, drugs that alter redox status can therefore be expected to exert epigenetic effects via the intermediate involvement of MS. Indeed, our laboratory has also found that pretreatment with ethanol (0.1%) significantly
decreased MS activity and DNA methylation, which is pertinent to ethanol inhibition of cysteine uptake. Our laboratory has also shown previously that dopamine stimulates MS activity in SH-SY5Y cells through activation of D4 dopamine receptors and also stimulates cysteine uptake by more than 200%. Importantly, a number of studies have linked genetic variations in the D4 receptor (e.g. the 7-repeat VNTR) to drug abuse involving alcohol, cocaine, amphetamines and opiates. Hence, although my thesis studies have only investigated the consequential effects of opioids impinging on the redox-based modulation of the methylation state in neuronal cell cultures, this mechanism of exerting regulatory control on MS via the redox pathway and inducing global epigenetic changes seems to be linked to several drugs of abuse. Indeed, it might be a possible central mechanism involved in linking redox-based epigenetic changes further leading to drug-specific consequences. Notably, stimulant drugs, which increase dopamine, will increase cysteine uptake and DNA methylation, whereas ethanol and opiates decrease cysteine uptake and DNA methylation.

In consideration of the above hypothesis, redox-based interventions which replenish antioxidant homeostasis might be useful for abrogating the effects of these drugs of abuse on redox-state and methylation capacity, which might be a potential therapeutic strategy for disorders related to elicit drug use. The use of N-acetylcysteine (NAC) in restoring GSH levels is well established, as NAC passively diffuses through cell membrane and is a precursor for GSH synthesis. NAC is emerging as a useful agent in the treatment of psychiatric disorders including addiction, compulsive and grooming disorders, schizophrenia and bipolar disorder, however, the mechanism of its therapeutic activity is as yet not established. As indicated earlier, MeCbl is involved in regulating the activity of MS and can promote the formation of methionine from HCY, thereby promoting redox-methylation homeostasis by balancing the
transsulfuration pathway vs. the methionine cycle. Some of the disorders that may be preventable or treatable with methylcobalamin include autism, chronic fatigue syndrome, Parkinson's disease, peripheral neuropathies, Alzheimer's disease, muscular dystrophy and neurological aging.\textsuperscript{174–176}

My studies indicate that pretreatment with NAC and methylcobalamin can prevent the short-term and prolonged effects of morphine on intracellular redox and methylation status in neuronal cell cultures. Both of these interventions did not alter the effects of morphine on EAAT3; however, both replenish the levels of intracellular glutathione and reverse the effects of morphine. Additionally, the epigenetic effects of morphine were also reversed by these redox-based interventions. Hence, my thesis proposes a novel therapeutic strategy for treatment of opiate-based effects and pathophysiological effects, by using NAC and MeCbl. My results support further \textit{in vitro} as well as \textit{in vivo} testing for efficacy of these redox based interventions to treat opiate-addiction and other forms of drug addiction. Interestingly, NAC, along with naltrexone, is already used for treatment of symptoms associated with alcoholism as well as cocaine addiction. Hence, my studies might provide a mechanistic explanation for the use of these redox modulators in treating these psychiatric disorders.

D. Opioids, CNS inflammation and Endogenous stress system.

The interplay between CNS inflammation and oxidative stress is complex.\textsuperscript{177} However, opioid-induced effects on redox state are a prototypical example, which could be useful to explain the link between CNS inflammation and oxidative stress. My studies reported that opioids induced oxidative stress by decreasing GSH levels. However, other studies have reported generation of free radicals due to an imbalance between high cellular levels of reactive oxygen
and nitrogen species (ROS and RNS), both of which can induce mitochondrial dysfunction, damage neuronal precursors and impair neurogenesis. Excess production of ROS and NOS can result in the induction of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and NFκB, which initiates inflammatory processes under the influence of morphine.

My studies analyzing the functional pathways for all the differentially expressed transcripts also indicated altered mRNA levels for proteins involved in cytokine-cytokine interaction. Thus, regulators of ROS production may play a critical role in limiting tissue damage and preventing or inhibiting inflammation and prolonged immune reactions. NFκB is also an important transcription factor and is involved in regulation of transcription of several genes. Several genes which impinge upon or are mediators of redox homeostasis are under the influence of NFκB. Hence, redox regulates transcription factors, which further affect the process of chromatin condensation as well as mRNA levels. Additionally, studies have shown that neuroinflammation and expression of pro-inflammatory cytokines, such as TNF-α, are associated with development of morphine tolerance, pain transmission and the generation of inflammatory and neuropathic pain. Studies from our laboratory have previously found that TNFα induces a decrease in MS enzyme activity and a transient increase in GSH. 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. Hence, activation of the cytokine pathway under the influence of opioid exposure allows the cell to regulate oxidative stress and restabilize its redox status.

The effects of cytokines are mediated via hypothalamic-pituitary-adrenal axis (HPA axis) by the release of steroid hormones as well as opioid peptides like enkephalins and endorphins.
Generally, HPA axis is involved in regulating various body functions under normal physiological conditions, and via the breakdown of pro-opio-melanocortin (POMC) under the influence of stress,\textsuperscript{182} in this case under the influence of a physiological stressor namely oxidative stress. HPA-axis and POMC play an important role in what is known as the general adaptation syndrome (GAS).\textsuperscript{182} Opioids impinge upon this internal stress modulators and induce inflammatory changes by producing pro-inflammatory cytokines via regulation of redox-based epigenetic and transcription state of the neuronal cells.\textsuperscript{183} These pro-inflammatory processes are also mediators of aging via the same agents (e.g. TNFα and NFκB).\textsuperscript{184} Redox status imbalance is noted as one the strongest inducer of the process of aging, and cytokines might be one of the mediators of this process.

E. Opioids, epigenetics and neurodevelopmental disorders.

Epigenetics allows a single genome, present in all cells of an individual organism, to assume a unique pattern of stable gene expression for different cell types. Generally, an epigenetic mode of regulation 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 template for \textit{de novo} methylation.\textsuperscript{185} After implantation, the majority of the genome becomes hypermethylated, whereas extra-embryonic tissues remain hypomethylated (Fig. 75).
**Figure 75: Changes in DNA methylation during mammalian development.** Summary figure of changes in DNA methylation during development and differentiation. Figure adapted from *Human molecular genetics: Textbook*.185

*De novo* methylation orchestrates the structure and function of tissues by turning gene expression on and off. Epigenetic modifications cause chromatin remodeling by changing the way DNA interacts with itself and with accessory proteins. This remodeling can open up the DNA, making it more accessible and can recruit additional proteins such as transcription factors to increase transcription. Epigenetic remodeling can also condense DNA, decreasing chances of transcription.³ DNA methylation was once thought to be static and unchanging, however, it is now known that DNA methylation is a very dynamic process,³ closely associated with cell differentiation and neuronal plasticity. DNA methylation is carried out by a class of enzymes; called DNA methyltransferases (DNMTs). DNMT1 is responsible for maintaining DNA methylation. As the cell divides, DNMT1 methylates the daughter strand of DNA according to
the methylation of the mother strand. DNMT2 primarily methylates RNA, and DNMT3 is responsible for de novo methylation of DNA.\(^{185}\) Both, de novo and hemi methylation depend on the levels of SAM, which in turn are dependent on the action of the enzyme MS and the redox status of the cell.

Problems impinging on epigenetic pathways have been shown to cause developmental disorders including Rett, Angelman, Prader-Willi and Fragile X syndromes.\(^ {186-188}\) Additionally, dysfunctional redox regulation is associated with neurological disorders such as autism, schizophrenia and Alzheimer’s disease.\(^ {189-191}\)

Rett, Angelman, Prader-Willi and Fragile X syndromes are debilitating developmental disorders with distinct epigenetic causes. Rett syndrome is caused by mutations in the methyl-CpG binding protein-2 (MeCP2) transcription factor protein. MeCP2 protein binds to methylated DNA and acts as a transcriptional suppressor or activator, and in cases where MECP2 is mutated, the transcriptional regulation is diminished and information written to the genome in form of DNA methylation is lost. Angelman and Prader-Willi syndromes arise from improper methyl-imprinting of DNA as well as MeCP2 binding. Fragile X syndrome is caused by expansion of a CGG repeat in the 5′ UTR of the fragile X mental retardation 1 (FMR1) gene.\(^ {192}\) This mutation causes hypermethylation and silencing of FMR1. Numerous studies have linked autism to problems with redox, methylation and epigenetics.\(^ {190,193-195}\) Autistic children have low plasma levels of both GSH and SAM.\(^ {190,193-195}\) These findings, along with other studies from our laboratory, support a redox/methylation hypothesis of autism. Thus, a number of neurodevelopmental disorders are manifestations of disturbances in redox and methylation homeostasis affected due to epigenetic consequences. In absence of
proper clinical treatment available for most of these disorders, various non-clinical treatments are employed by parents, and support groups for these patients. Mostly used interventions are methylcobalamin, folic acid, glutathione supplements which are based on restoring redox/methylation homeostasis.

Recent studies have also indicated a critical role for MeCP2 in neural and behavioral responses to psychostimulants, and MeCP2 is also critical for the increase in BDNF transcription during cocaine self-administration. Additionally, most of the actions of psychostimulants such as amphetamine and cocaine are attributable to increased levels of dopamine. Data from our laboratory have indicated that dopamine stimulates cysteine transport and MS activity, resulting in increased DNA methylation, which would promote MeCP2 binding and epigenetic consequences, whereas morphine and ethanol inhibit EAAT3 activity, decrease GSH levels and inhibit MS, resulting in decreased DNA methylation and less MeCP2 binding. Dopamine stimulation of MS activity would induce DNA methylation, which, for example, is responsible for the cocaine-induced elevation in BDNF transcription, and morphine as well as ethanol would lead to contrasting effects. Taken together, these observations suggest that drugs of abuse might exert their effects by altering the methylation state of CpG sites via their action on redox status and MS activity, as well as alter MeCP2 binding and subsequent transcription status, which is highly implicative in the dynamics of neuronal and synaptic plasticity changes observed under the influence of drugs of abuse.

An increasing amount of evidence indicates that ancestral environmental perturbations can influence the physiology and behavior of descendants. Most studies of this sort focus on maternal effects, although there are examples of paternal phenotype transmission between
generations. Regarding drugs of abuse, the adult offspring of female rats exposed to morphine during adolescence show increased anxiety (female offspring), elevated morphine-induced analgesia (male offspring) and augmented behavioral sensitization to morphine (male and female offspring). Maternal exposure to cocaine leads to decreased levels of global DNA methylation in the hippocampus region of male offspring; whereas paternal cocaine administration leads to impaired working memory in female offspring and causes hyperactivity and increased perseveration in a T-maze among male progeny. Additionally, DNA hypomethylation in the parents of autistic children has also been found. Thus, drugs of abuse impinge upon mechanisms critically important for development and can lead to debilitating neuro-pathological manifestations via the redox-based signaling in the descendants of drug-dependent individuals which could be not only life-long, but also affect additional future generations in a profound manner.
**Figure 76: Transgenerational effects of drugs of abuse.** Multiple generations may be affected by exposure to drugs of abuse, either because several generations are exposed at the same time or because of a truly inherited, or transgenerational, effect. Reports support both phenomena. When a pregnant female is exposed to a chemical or experience, it may directly affect her (F0 generation), her child (F1) and even her grandchild (F2), exposed as an egg while its mother was a fetus. A male’s sperm may also be affected by an exposure, affecting his child. Transgenerational effects may emerge in later generations. (Adapted from Skinner, M.K. et al.)

Repetitive DNA elements, including transposable elements such as LINEs, long terminal repeats (LTRs) and Alu repeats, constitute approximately 50% of the human genome, and their transcription and their capacity for retrotransposition are suppressed by CpG methylation.\textsuperscript{209} In my studies, LINE-1 methylation showed time-dependent changes in promoter methylation in response to morphine. Methylation of non-promoter regions of LINE-1 was decreased at 4 hrs of treatment, but an increase in promoter methylation was observed at 4 hrs. Corresponding qPCR results indicated that morphine at 4 hrs inhibited
the expression of LINE-1, whereas the LINE-1 expression was increased after 24 hrs of morphine treatment. Hence, morphine can induce changes in the transcription of LINE-1 regions. These can have global impact on the genome. Studies suggest that altered LINE-1 transcription is involved in several diseases including neurodevelopmental disorders like Rett syndrome.\textsuperscript{43,210} Additionally, LINE-1 methylation is reportedly altered under the effect of drugs of abuse like cocaine and morphine.\textsuperscript{53}

The LINE-1 replication machinery is used by other retrotransposon like SINEs, Short-interspersed nuclear element, e.g., Alu repeats.\textsuperscript{41} Together, L1, Alu and other repetitive elements contribute to genomic plasticity in all cells including neurons, especially neurons in hippocampus and striatum, which have a much higher rate of de novo retrotransposition (almost 800 new insertions are estimated in hippocampal cell) as compared to other tissues, such as blood, heart and liver.\textsuperscript{54,211} Thus, epigenetic regulation is critically vital for the ability of a cell to suppress retrotransposon machinery. Epigenetic marks like methylated CpG help maintain a repressive chromatin model.\textsuperscript{212} However, under environmental or drug-induced perturbations, these repressive marks are exchanged with transcriptional-activation complexes which can induce active chromatin conformations, for example, when the Wnt3a-βcatenin complex binds to the L1 promoter and induces transcriptional activation.\textsuperscript{51,213} Notably, L1 insertions are reported to affect actively expressed genes which are important for synaptic transmission.\textsuperscript{211} Thus, these L1-based transposition events could have a huge impact on neuronal diversity and could contribute to drug-dependent plasticity in an individual. L1-events are reported to be at the highest during the earliest developmental stages, and, hence, any redox-based perturbations, which alter epigenetic marks on the chromatin would result in a greater impact of L1 insertions,
genomic instability and further consequences in transcriptional activation of other genes. This relationship is illustrated in the figure below.

**Figure 77:** Epigenetic-based regulation of L1 retrotransposon insertions in the mouse nervous system. 5MeC is an epigenetic mark that regulates excess L1 activity. The repressive chromatin remodeling is mediated by the actions of histone deacetylase HDAC1 and the methyl-CpG-binding protein MeCP2. Psychostimulants and drugs of abuse as well as loss of MeCP2 could substantially increase L1 RNA based transposition, which results in genome toxicity and insertions further inducing changes in neuronal gene expression. (Adapted from Muotri, A.R et al)  

Thus these observations: (a) trans-generational inheritance of epigenetic effects under the influence of drugs of abuse; (b) redox-based epigenetic implications in developmental disorders; (c) redox-based epigenetic and transcriptional changes in non-LTR like LINE-1; (d) redox-based intervention useful to reverse the effects of drugs of abuse as well as treat neurodevelopmental disorders, taken together show a link between
redox and methylation homeostasis. Disturbance of this link is associated with profound implications not only in phenotypic neurological disorders, but also trans-generational effects. Some studies, which might be helpful in clarifying this role, are proposed below. If successful, these studies could not only link the unexplained cause of rise in neurodevelopmental disorders, but could also lead to therapeutic strategy in forms of redox-based interventions.

Thus, my thesis demonstrates a link between the influence of opioids on neuronal-cellular redox and methylation status and the molecular observations previously associated with opioids. It advances our understanding of redox-based machinery responsible for regulating effects of opioids and potentially even other drugs of abuse, on methylation reactions, including DNA methylation, which modulates gene transcription. My findings support the primary importance of redox homeostasis in human brain. While they do not directly address neuropsychiatric and neurodegenerative disorders, they do suggest a possible that abnormalities affecting the fundamental process of redox-based epigenetic changes might have neurological manifestations. Further, these epigenetic changes can be inherited and might be a contributing factor in neurodevelopmental disorders like autism, Rett, Angelman, Prader-Willi and Fragile X, which are linked with impaired redox and methylation status. These conditions might then be potentially treated by using redox-based nutrients or interventions in a similar way as is currently used for treating the symptoms of drug addiction.
VI. Future Studies.

SH-SY5Y cells are an immortalized cell line that has been used for numerous studies of receptor mechanisms, including opioid dependence and tolerance. Hence, the use of SH-SY5Y cells to study the opioid-mediated receptor signaling via the redox-mediated epigenetic changes can be justified. However, these neuronal cells are not perfect models of neurons. SH-SY5Y cells are a neuroblastoma-derived cell line, and the transformed nature of this cell-line makes the cells genetically unstable. Additionally, as indicated earlier, the redox environment in mature neurons is different as compared to SH-SY5Y cells, as the latter has a more permissive transsulfuration pathway as compared to neurons. Minor perturbations in redox status may then be expected to have even larger and broader consequences in neurons. Hence, it would be really useful and interesting to investigate the effects of opioids on primary neurons, for example, in rat cortical neurons, or even human stem cell-derived primary neurons. These experimental conditions would better mimic normal neuronal cells, as compared to the SH-SY5Y cell line, allowing the results to be more strongly extrapolated for clinical studies.

Another important study to be performed would be to characterize redox-based changes in neurons derived from post-mortem brain samples / skin grafts of opiate addicts, using induced pluripotent stem cells (iPSCs) from opiate drug addict patients. The systematic generation of neurons from patients with opiate addiction can provide important insights into disease pathology, progression and mechanism. Since redox plays a critical role in differentiation and epigenetic factors are key mediators of neuronal differentiation, it would important to understand the interplay between these two processes and how each of them is regulated. Additionally, investigating the effects of
opioids on these two closely interdependent pathways in iPSCs would also allow us to characterize the molecular/cellular signaling mechanism, which could further aid in drug screening processes.

Next, as mentioned during the discussion, transgenerational effects of opioids and other drugs of abuse and the involvement of redox-based epigenetic signaling need to be investigated in animal models. Studies could be performed wherein the parent generation is administered drugs of abuse, especially during pregnancy. Effects of this administration on redox-based epigenetic signaling and retrotransposition can be investigated in parent, F0 and F1 generations. Brain-specific redox metabolites and epigenetic changes could be evaluated after characterizing routine behavioral, social and cognitive tests. Any changes could be compared against neurodevelopmental models of autism, Rett syndrome or other neurological disorders.

Furthermore, the effect of other drugs of abuse like cocaine and amphetamine on the redox-based epigenetic signaling mechanism needs to be investigated. As indicated earlier, dopamine and ethanol are already observed to affect redox status in the cells, and also alter MS activity and DNA methylation. Besides, psychostimulant drugs like cocaine and amphetamine act via modulating the levels of dopamine, and cocaine has been observed to induce epigenetic changes as well as alterations in LINE-1 methylation and retrotransposition.53 Hence, it would be interesting to characterize the implications of the redox-based signaling mechanism in mediating the effects of additional drugs of abuse, via measurements of cysteine uptake, intracellular thiols and MS activity. Redox-based epigenetic signaling might prove to be the central mechanism regulating the effects of
drugs of abuse. Since redox-based metabolic modulators like NAC and MeCbl abrogated the effects of opioids, these interventions might also be useful for intervening on effects of other drugs of abuse, which also needs to be investigated. This might prove to be an important therapeutic strategy for treating drug withdrawal symptoms as well as drug relapses.

Currently, Yiting Li, another Ph.D. student in our lab is treating SH-SY5Y cells with various cobalamin (Cbl) species like cyanocobalamin (CNCbl), glutathiocobalamin (GsCbl) etc., including MeCbl and measuring cysteine uptake levels. She is also investigating the levels of these Cbl species in rat and post-mortem human brain samples. As mentioned earlier, MeCbl regulates MS activity and my studies reported MeCbl to replenish the levels of redox-based epigenetic signaling by abrogating the effects of morphine. Hence, an effect of morphine and other drugs of abuse on the levels of various species of cobalamin would extend my thesis results. MeCbl delivery to the brain via nasal spray could also be tested in animal models of drug addiction. This could potentially provide further support to my results of replenishing redox status with MeCbl in brain compartment of drug addict patients. Further, other forms of Cbl (except MeCbl) could also be identified which, might be useful in replenishing the redox-based effects of opioids. These proposed and current studies will not only provide a much better understanding of how drugs of abuse, including opioids, could induce changes in the EAAT3-mediated cysteine uptake, redox-based epigenetic signaling and retrotransposon transposition, but could also provide a novel therapeutic strategies for treating drug addiction.
### VII. Appendix

#### Table 2: DETs after 4 hours of morphine exposure

<table>
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<tr>
<th>Gene Name</th>
<th>Symbol</th>
<th>(p)-value(Morphine_4hr vs. Control)</th>
<th>Ratio(Morphine_4hr vs. Control)</th>
<th>Fold-Change(Morphine_4hr vs. Control)</th>
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<td>C14orf99</td>
<td>4.67E-07</td>
<td>3.08317</td>
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<td>3.67E-07</td>
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<td>-1.26874</td>
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Effects of H-89, LY294002 and PD98059 on global DNA methylation

Fig: 1 Exposure to H-89 for 4 hours and PD98059 for 24 hours did not alter global DNA methylation levels
Effects of H-89, LY294002 and PD98059 on intracellular thiol levels

Fig: 2 Exposure to H-89 for 4 hours and PD98059 for 24 hours did not alter any thiol metabolites, however, exposure to LY294002 for 2 hrs resulted in decreased cysteine and GSH levels (p<0.05)
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<tr>
<td>GCLC</td>
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VIII. References:


Regulation of Gene Expression by Chronic Morphine and Morphine Withdrawal in the Locus Ceruleus and Ventral Tegmental Area.


Dopamine D4 receptor gene (DRD4) is associated with Novelty Seeking (NS) and substance abuse: the saga continues… *Publ. Online 08 August 2001 Doi101038sjmp4000918 6,* (2001).


