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# Oral S-adenosyl methionine (SAM) mediates disruptions in methyl group metabolism due to retinoic acid therapy and alters neurotransmitter metabolism: implications for major depressive disorder

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**Oral S-adenosyl methionine (SAM) mediates disruptions in methyl group metabolism due to retinoic acid therapy and alters neurotransmitter metabolism: Implications for major depressive disorder**

by

**Anne Lorraine Smazal**

A thesis submitted to the graduate faculty  
In partial fulfillment of the requirements for the degree of

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Program of Study Committee:

Kevin Schalinske, Major Professor  
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Iowa State University  
Ames, Iowa  
2013

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## LIST OF ABBREVIATIONS

13CRA: 13-*cis* retinoic acid, isotretinoin

5,10-CH<sub>3</sub>-THF: 5,10-methylenetetrahydrofolate

5-CH<sub>3</sub>-THF: 5-methyltetrahydrofolate

5-HIAA: 5-hydroxyindolacetic acid

5-HT<sub>1A</sub>: serotonin receptor 1<sub>A</sub>

ADHD: attention deficit hyperactivity disorder

APL: acute promyelocytic leukemia

ATRA: all-*trans* retinoic acid, tretinoin

BHMT: betaine-homocysteine S-methyltransferase

CBS: cystathionine β-synthase

COMT: catechol-*O*-methyl transferase

CRABP: cellular retinoic acid binding protein

CRBP II: cellular retinol binding protein II

DAT: dopamine transporter

DMG: dimethylglycine

GAMT: guanidinoacetate N-methyltransferase

GNMT: glycine N-methyltransferase

HAM-D: Hamilton Rating Scale for Depression

KO: genetic knockout

MAO: monoamine oxidase

MAOI: monoamine oxidase inhibitor

MAT: methionine adenosyl transferase

MS: methionine synthase

MTHFR: methylenetetrahydrofolate reductase

NDRI: norepinephrine-dopamine reuptake inhibitor

NET: norepinephrine transporter

NTT: neurotransmitter transporter

PEMT: phosphatidylethanolamine N-methyltransferase

PNMT: phenylethanolamine N-methyltransferase

RAR: retinoic acid receptor

RARE: retinoic acid response element

RBP: retinol binding protein

RXR: retinoid X receptor

SAH: S-adenosyl homocysteine

SAM: S-adenosyl methionine

SERT: serotonin transporter

SLC: solute carrier superfamily of membrane transport proteins

SNP: single nucleotide polymorphism

SNRI: selective norepinephrine reuptake inhibitor

SSRI: selective serotonin reuptake inhibitor

TCA: tricyclic antidepressant

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## ABSTRACT

Disruptions in methyl group metabolism have been associated with a number of disease states, including birth defects, cardiovascular disease, and neurological disorders. Therefore, characterization of the factors that regulate folate and methyl group supply is essential for the development of disease treatments and prevention. Glycine N-methyltransferase (GNMT), an enzyme essential to methyl group balance, is markedly increased by retinoic acid treatment. Furthermore, retinoid therapy has been shown to impact neurotransmitter metabolism, thereby contributing to the development of major depressive disorder. Recently, it has been shown that depressed patients have low levels of folate, other B-vitamins, and S-adenosyl methionine (SAM), all compounds essential for maintaining effective methyl group metabolism. The following studies were conducted to further characterize the impact of oral retinoic acid administration, and to investigate the potential prevention of any detrimental effects by administering oral SAM. In the preliminary study (Chapter 2), as expected, GNMT activity was significantly increased in rats receiving all-*trans* retinoic acid (ATRA). Additionally, whole brain serotonin levels were decreased by 46% in rats receiving ATRA, an effect that was partially attenuated by oral SAM.

The experiment was repeated with an improved dosing method, longer duration of treatment, and another form of retinoic acid, 13-*cis* retinoic acid (13CRA). Retinoic acid was shown to increase liver lipids and triglycerides, decrease hepatic SAM, and mildly elevate serotonin, in contrast to the first experiment. ATRA slightly decreased whole brain dopamine transporter (DAT) and 13CRA slightly decreased whole brain norepinephrine transporter (NET); these effects were not seen in rats that received supplemental SAM. Furthermore, SAM abrogated liver lipid content in rats receiving 13CRA, partially prevented increases in liver triglycerides, and restored hepatic SAM levels in both retinoic acid groups. Rats that received only SAM supplementation had extremely high dopamine, and increased

norepinephrine. SAM supplementation may be an effective therapy for both the mitigation of retinoic acid-related side effects and the prevention or treatment of major depressive disorder.

## CHAPTER 1: LITERATURE REVIEW

### Introduction to Methyl Group Metabolism

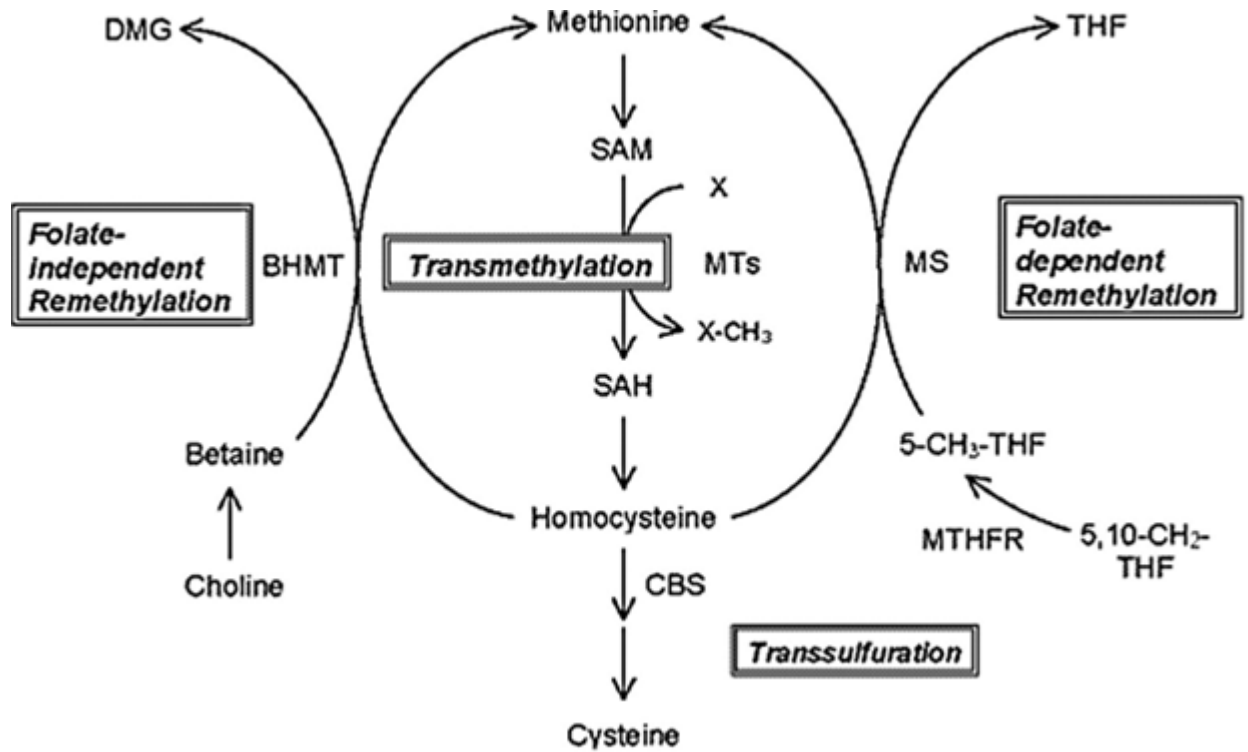
Methyl group, folate, and homocysteine metabolism involves a series of interdependent pathways which, when disturbed, can reflect or cause a pathological state. Methyl groups are one-carbon units derived from dietary nutrients, including folate, betaine, methionine, and choline, that must be successfully activated and transported for use in hundreds of metabolic functions. The process begins with the conversion of methionine, an essential amino acid, into *S*-adenosyl methionine (SAM), which can transfer a methyl group to a variety of substrates. Following this transmethylation and the removal of the adenosine group, SAM becomes homocysteine, which can be remethylated or catabolized into cysteine.

Aberrations in methyl group metabolism have been linked to a wide variety of pathologies, including atherosclerosis, osteoporosis, cardiovascular disease, and mental disorders<sup>1-3</sup>. Clinically, high serum homocysteine, or hyperhomocysteinemia, is used to detect impaired methyl group metabolism. However, correction of hyperhomocysteinemia using nutritional supplements or dietary modifications has been inconsistently successful. Methyl balance is controlled by a variety of genetic, hormonal, and nutritional factors, and understanding the significance of each contributing pathway remains an active goal in research.

### Transmethylation

The non-polar, sulfur-containing amino acid methionine represents a starting point for methyl group metabolism. Methionine reacts with ATP and methionine adenosyltransferase (MAT) to form *S*-adenosyl methionine (SAM), considered the universal methyl group donor (Figure 1.1). MAT has three

different isoforms, which differ in their regulation, kinetics, and primary site of activity<sup>4</sup>. Specifically,



**Figure 1.1: Methionine is converted to homocysteine via a transmethylation reaction. Homocysteine can be remethylated in either a folate-dependent or folate-independent manner. From Williams & Schalinske<sup>5</sup>.**

MAT I and MAT II are regulated via product inhibition by SAM and are nearly saturated under normal methionine levels. In contrast, MAT III is not regulated by SAM, and can thus function under higher methionine levels. MAT II is expressed in extrahepatic tissues, while MAT I and III are functional in the liver.

Numerous methyltransferases catalyze the transfer of a methyl group from SAM to a substrate, forming S-adenosyl homocysteine (SAH) and methylated substrate. The broad range of methyltransferase enzymes and potential substrates illustrates the necessity of transmethylation reactions and the body's reliance on available methyl groups. Methylation is a key post-translational modification essential for protein function, while methylation of lipids produces essential components of plasma membranes. Methylation of small molecules influences neurotransmitter synthesis, cell

signaling, and numerous other important functions. Epigenetic modifications, including methylation patterns of RNA and DNA, control gene expression and are increasingly recognized as contributors to health and disease. Given the essentiality of SAM, the primary methyl group donor, it is imperative that these methyltransferase reactions be regulated. To that end, the ratio of SAM to its post-reaction metabolite, SAH, can inhibit SAM-dependent reactions. Glycine N-methyltransferase is an enzyme primarily expressed in the liver, kidney, and pancreas that can help maintain the SAM:SAH ratio<sup>6,7</sup>. It functions by converting glycine to sarcosine, thus forming SAH from SAM. The SAM:SAH ratio therefore serves as a gauge of transmethylation potential.

Homocysteine is formed from SAH following the removal of the adenosyl group by SAH hydrolase. The two major contributors to the formation of homocysteine are phosphatidylethanolamine N-methyltransferase (PEMT), which synthesizes phosphatidylcholine, and guanidinoacetate N-methyltransferase (GAMT), which synthesizes creatine. Collectively, PEMT and GAMT account for roughly 85% of SAM-dependent transmethylation<sup>8</sup>.

Homocysteine can be converted to cysteine by vitamin B<sub>6</sub>-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase or recycled by two separate remethylation pathways (Figure 1.1). Conversion to cysteine represents a catabolic process by which homocysteine is converted first to cystathionine via condensation with serine, then to cysteine or other metabolites, such as glutathione, pyruvate, and taurine. CBS activity is positively and allosterically regulated by SAM; therefore, increased production of homocysteine should correspond with an increase in catabolism.

### **Folate-Dependent Remethylation**

Homocysteine can be remethylated in a folate-dependent manner to form methionine. This process requires 5,10-methylenetetrahydrofolate (5,10-CH<sub>2</sub>-THF), which is irreversibly converted by methylenetetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrofolate (5-CH<sub>3</sub>-THF). This

conversion is dependent on availability of folate groups, which cannot be formed de novo by mammals and must therefore be obtained in the diet. Methionine synthase (MS), which is dependent on vitamin B<sub>12</sub>, catalyzes the transfer of the methyl group from 5-CH<sub>3</sub>-THF to homocysteine, forming tetrahydrofolate (THF) and methionine.

The products and substrates for these remethylation reactions can control and are themselves controlled by transmethylation reactions. Specifically, SAM allosterically inhibits MTHFR, while 5-CH<sub>3</sub>-THF formed by MTHFR can inhibit GNMT<sup>6</sup>. If methyl group supply is low, low levels of SAM will not inhibit MTHFR, creating elevated levels of 5-CH<sub>3</sub>-THF. 5-CH<sub>3</sub>-THF can bind and inhibit GNMT, thereby lowering transmethylation and increasing intracellular SAM<sup>6</sup>. When methyl group supply is high, elevated SAM inhibits MTHFR, which lowers the production of 5-CH<sub>3</sub>-THF, thus increasing GNMT activity and increasing the consumption of SAM. The reciprocal regulation of transmethylation and folate-dependent remethylation allows for finely tuned control of methyl group availability.

### **Folate-Independent Remethylation**

Homocysteine can also be converted to methionine using betaine, a derivative of choline. In this pathway, betaine-homocysteine S-methyltransferase (BHMT) transfers a methyl group from betaine to homocysteine, forming methionine and dimethylglycine (DMG). As in folate-independent remethylation, this process is regulated by dietary intake of methyl groups, availability of B-vitamins, and the SAM:SAH ratio. Dietary choline is oxidized to betaine, which provides the methyl group for the BHMT reaction. If dietary protein supply is in excess, the resulting elevated SAM inhibits BHMT activity to normalize methyl group supply. Correspondingly, if dietary methionine is low, BHMT expression increases to preserve methyl groups<sup>9</sup>. In contrast to folate-dependent remethylation, which occurs in all tissues, betaine-dependent remethylation is mostly limited to the liver and kidney.

## Disruption of Methyl Group Metabolism and Pathology

As evidenced by the variety of regulatory factors discussed, maintenance of methyl group metabolism is essential for good health. The foundation of the pathway lies in sufficient dietary intake of several nutrients, including folate, B<sub>6</sub>, B<sub>12</sub>, choline, and protein.

Folate deficiency in pregnant women has been shown to cause a variety of birth defects, specifically improper closure of the neural tube, causing debilitating and lethal problems like spina bifida. In 1998, the United States mandated the enrichment of all cereal grains and grain products with folic acid, a public health initiative that decreased the rate of neural tube defects by 25 percent<sup>10</sup>. Other countries have similar mandates. Deficiencies in B<sub>6</sub> and B<sub>12</sub> contribute to depression, anemia, cognitive changes, and elevated homocysteine. Likewise, choline deficiency can result in impaired liver function and elevated homocysteine. Homocysteine is thought to be a biomarker, causal factor, or both in numerous pathologies. Specifically, homocysteine is an independent risk factor for cardiovascular disease, a fact supported by numerous human studies and animal studies utilizing genetic and diet-based alterations to methyl group metabolism<sup>1,11,12</sup>. Hyperhomocysteinemia is thought to mediate cardiovascular damage by promoting oxidative stress and endothelial cell dysfunction. Elevated homocysteine has also been implicated in osteoporosis, Parkinson's disease, Alzheimer's disease, depression, diabetes, and autoimmune disorders.

Methyl balance can also be dramatically changed by genetic factors. For example, the common MTHFR single nucleotide polymorphism C677T causes an accumulation of 5-CH<sub>3</sub>-THF, blocking its use in other biologically significant reactions. This "methyl trapping" can also result from a diet deficient in B<sub>12</sub>, which is required for MS functionality. Both the C677T polymorphism and low dietary B<sub>12</sub> have been shown to cause hyperhomocysteinemia<sup>13,14</sup>. Likewise, genetic defects in CBS and cystathionine  $\gamma$ -lyase,

termed homocysteinuria, result in elevated serum homocysteine and excretion of homocysteine in the urine<sup>15</sup>.

Previously, our lab has shown that retinoid therapy can substantially impact methyl group metabolism. Administration of retinoic acid has been shown to increase both GNMT abundance and activity, an effect specific to the liver<sup>16</sup>. This was accompanied in another study by DNA hypomethylation<sup>17</sup>. Retinoid therapy also appeared to support normal BHMT activity in diabetic rats and modulate serum homocysteine in adrenalectomized rats<sup>18</sup>. Given these effects, it is important to further characterize the effects of retinoic acid administration and potential methods to abrogate their impact on methyl balance.

## **Retinoid Therapy**

### **Introduction to Retinoid Compounds**

Since its discovery in 1915, Vitamin A and its related compounds and derivatives have been widely studied for nutritional and therapeutic purposes<sup>19</sup>. The term Vitamin A refers to several fat-soluble retinoid compounds, all containing a beta-ionone ring with an attached isoprenoid chain. These can be consumed from animal sources as retinyl esters and from plant sources as carotenoids, which are then converted to retinol on a limited basis<sup>20</sup>. Following enzymatic digestion in the stomach and intestine, retinyl esters and protein-bound carotenoids are converted to free retinol and free carotenoids, incorporated with bile into a micelle, and absorbed into an enterocyte. Once in the enterocyte, carotenoids can be converted to retinal. Retinal is either incorporated with cellular retinol binding protein II (CRBP II) and a fatty acid to form CRBP II-retinyl palmitate, or converted into retinoic acid, bound to albumin, and released to portal circulation. Similarly, free retinol is packaged with a fatty acid and CRBP II into CRBP II-retinyl palmitate. This complex is incorporated into a chylomicron to enter lymphatic circulation. Within the hepatocyte, retinoid compounds have several fates. Retinyl esters are



hydrolyzed, releasing free retinol and fatty acids. The free retinol can be re-esterified and stored as retinyl ester. Alternately, free retinol can be released into the bloodstream with retinol binding protein (RBP), converted to CRBP-retinal and then retinoic acid, or conjugated with glucuronic acid for excretion in the bile<sup>21</sup>. Once released into the blood, the RBP-retinol complexes with transthyretin for transport to the target tissue. If transported to the eye, retinol is converted to retinal, which is essential for the production of rhodopsin in the photoreceptor rod cells. In other cells, retinol can be converted to retinoic acid and attached to cellular retinoic acid binding protein (CRABP) or intracellular lipid-binding protein. Once transported into the nucleus and released from binding proteins, retinoic acid binds retinoic acid receptor (RAR) before forming a heterodimer with retinoid X receptor (RXR) and docking at a retinoic acid response element (RARE) on DNA to modulate gene expression<sup>22</sup>. In this way, retinoic acid can powerfully increase cell proliferation, apoptosis, differentiation, and turnover.

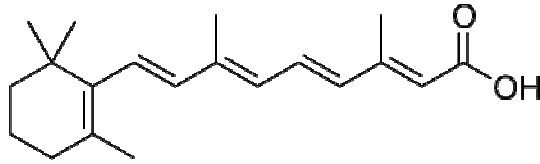
Importantly, Vitamin A is a fat soluble vitamin that can be stored in the body, primarily in the liver as retinyl ester. This is highly beneficial in cases of Vitamin A deficiency: a supplement containing 200,000 IU can meet the patient's daily requirement for four to six months<sup>23</sup>. Because Vitamin A deficiency is rare in the developed world, this technique is typically utilized by humanitarian organizations in southwestern Asia, equatorial Africa, and Central America, where vitamin A deficiency is a serious public health issue. However, excessive vitamin A stored in the liver can be highly toxic, causing extreme skin and eye dryness, changes in blood lipids, and liver damage, including steatosis and fibrosis<sup>24,25</sup>.

## **Therapeutic Retinoid Compounds**

### ***All-trans retinoic acid***

*All-trans* retinoic acid (ATRA), also called tretinoin, is available as a prescription drug and widely used as an acne treatment. As a form of Vitamin A, ATRA interacts with retinoic acid receptors to modify gene

expression. Inducing the rapid turnover of epithelial cells has proved an effective therapy, both topically



**Figure 1.2: All-trans retinoic acid (ATRA).**

and orally, for acne vulgaris and keratosis pilaris. After

ATRA was shown to successfully treat acute promyelocytic

leukemia (APL), retinoid therapy has been investigated for

the treatment of several forms of cancer. APL is thought

to be caused by chromosomal translocation, involving the fusion of the retinoic acid receptor gene and

the promyelocytic leukemia gene, which prevents proper differentiation of myeloid cells. ATRA

encourages the differentiation of promyelocytes, thus reducing the development of oncogenic cells<sup>26</sup>.

ATRA has also been shown to effectively promote apoptosis and inhibit growth of human breast cancer

cells by down-regulation of Bcl-2, an anti-apoptotic protein<sup>27</sup>. Other tumoral diseases that respond well

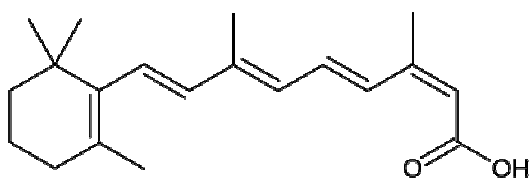
to ATRA therapy include ovarian cancer, bladder cancer, neuroblastoma, squamous cell carcinoma, and

several others<sup>28,29</sup>.

### ***13-cis retinoic acid***

In the early 1960s, pharmaceutical company Hoffman-La Roche (now known as Roche in North America)

developed a new, synthetic retinoid known as 13-cis retinoic acid or simply isotretinoin (figure 1.3).



**Figure 1.3: 13-cis retinoic acid (13CRA) also called isotretinoin**

Information on the early development and testing of the

drug is varied and contradictory. Dr. Werner Bollag, a

Swiss scientist, performed the initial exploration of the

drug and discovered its incredible potency in treating

acne in 1971, but dismissed the drug as a reasonable acne treatment because of the high rate of birth

defects in his test subjects. Later, Drs. Gary Peck and Frank Yoder began evaluation of isotretinoin as a

therapy for keratinization disorders, particularly lamellar ichthyosis<sup>30</sup>. Lamellar ichthyosis is a rare

autosomal disorder in which an infant is born with a collodian membrane, which sheds within weeks to

reveal scaly, hyperkeratinized skin. Isotretinoin was highly effective at clearing the hyperkeratinized skin

lesions. During trials for the treatment of lamellar ichthyosis, Peck and Yoder noticed that acne lesions were also cleared. Undeterred by the teratogenic effects, they published the initial paper on the use of isotretinoin to treat cystic acne in 1978<sup>31</sup>. The Food and Drug Administration approved the use of isotretinoin for severe, recalcitrant acne in 1982. Marketed by Hoffman-La Roche as Accutane, the drug was initially well received and became known as the gold standard of last-resort care for nodular skin lesions.

However, after widespread reports of severe side effects, the safety of isotretinoin was publicly questioned. Because the effects of any form of retinoic acid are nonspecific and directly impact gene expression, usage of therapeutic retinoid compounds carries a risk of extreme systemic side effects. Retinoic acid is a well-established teratogen, causing severe alterations to the central nervous system, craniofacial structures, and growth retardation. Isotretinoin patients were initially strongly encouraged to avoid pregnancy during treatment, a guideline that proved ineffective due to underreporting of negative events and the decentralization of isotretinoin distribution following the expiration of Hoffman-La Roche's patent in February 2002<sup>32</sup>. In the United States, a program restricting the prescription of isotretinoin was instated in 2000 and updated in 2006. Female patients were required to take pregnancy tests monthly prior to filling their prescription and complete an online questionnaire confirming their knowledge of teratogenic effects and birth control. However, other side effects were less predictable and poorly managed, including dramatic changes in blood lipids<sup>33,34</sup>, chronic dry eyes<sup>35</sup>, bowel inflammation<sup>36</sup>, loss of night vision<sup>35</sup>, impairment of bone turnover, and depression<sup>37</sup>. The link between isotretinoin and depression came into the national spotlight after B.J. Stupak, the 17-year-old son of U.S. Representative Bart Stupak, died from a self-inflicted gunshot wound in 2000 after several weeks of isotretinoin usage. Adamant that his son was a happy, popular, and well-liked student, Stupak claimed that isotretinoin was responsible for B.J.'s death. Although the U.S. FDA Adverse Events

Reporting System confirmed nearly 450 reports of depression in patients taking isotretinoin between 1982 and 2001, including 40 suicides, it has been speculated that these events are underreported<sup>38</sup>.

The link between isotretinoin and depression is disputable and poorly understood. Hoffman-La Roche and many others argue that severe acne, not Accutane, causes severe depression, low self-esteem, and poor self-image, all of which are related to suicidal ideation<sup>39,40</sup>. Additionally, the majority of Accutane users are adolescents or young adults, a population widely understood to be at a higher risk for depression and suicidal ideation.

This debate spurred a variety of investigations into the effects of retinoid compounds on mood stability, both in cell line and animal models. Four behavioral tests are thought to best evaluate depression in animal models<sup>41</sup>. First, the forced swimming test measures behavioral despondency, with shorter swimming durations indicating hopelessness<sup>42</sup>. Second, voluntary intake of a saccharin solution measures anhedonia, or a loss of pleasure from activities. Third, an open field test, in which the animal is placed in an enclosed space and monitored for movement and willingness to explore, demonstrates anxiety<sup>43</sup>. Fourth, the tail suspension test, which measures immobility in rodents when suspended only by their tails, can be used as a more general indicator of mood in development of antidepressants<sup>44</sup>. In 2005, forced swimming, open-field, and saccharine consumption tests were used to evaluate behavioral effects of oral retinoic acid treatment in a rat model. Rats administered either ATRA or isotretinoin at extremely high doses (10-15 mg/kg bw and 7.5-22 mg/kg bw) did not demonstrate any increases in depressive behavior after 6 to 10 weeks of daily treatment<sup>45</sup>. A follow-up study using similar doses and 15 to 19 weeks of treatment also indicated no changes in depressive related behavior<sup>46</sup>.

However, in 2006, O'Reilly and others showed that six weeks of isotretinoin treatment at 1 mg/kg bw, comparable to human prescribed doses of 0.5 to 2 mg/kg bw, increased depressive behavior

in mice. Mice receiving isotretinoin displayed markedly different responses in both tail suspension and forced swimming tests<sup>37</sup>.

Furthermore, in vitro measurements of brain tissue have shown that isotretinoin interferes with the trafficking and metabolism of serotonin, a neurotransmitter considered essential for maintenance of a “good mood.” Among the changes were increased serotonin receptor 1A (5-HT<sub>1A</sub>) and increased serotonin transporter (SERT), which indicates lower synaptic serotonin concentration, thought to be a major contributor to major depressive disorder<sup>47</sup>. Retinoic acid compounds have also been shown to impede neurogenesis and regulate both dopaminergic and noradrenergic signaling in development brain tissue, all of which can affect mood stability<sup>48-51</sup>.

Although the U.S. national registration program for isotretinoin patients was updated to include a standard screening for depressive thoughts, Hoffman-La Roche has never publicly acknowledged the risk of depression or suicide associated with their product. However, following a large class-action lawsuit won by isotretinoin patients who developed inflammatory bowel disease, the company ceased distribution in the United States<sup>52,53</sup>. Generic isotretinoin is still readily available by prescription, though patients with a personal or family history of mood disorders are generally advised to pursue other treatments.

## **Major Depressive Disorder**

Major depressive disorder, more commonly referred to as depression, is a mental illness affecting an estimated 7-14% of all American adults every year<sup>54,55</sup>. Because this figure only reflects reported cases of depression, a poorly recognized and highly stigmatic disease, the true number of cases may be much higher. The American Psychiatric Association states the symptoms as a pervasively low mood that adversely affects everyday life for an extended period of time. Major depressive disorder is the leading worldwide cause for work hours lost due to disability, and women are nearly 50% more

susceptible than men<sup>56</sup>. Persons affected by major depressive disorder experience difficulty focusing on tasks, eating regularly, and maintaining a normal sleep schedule. If left unmanaged, depression leads to suicide, which consistently ranks as a top cause of death worldwide.

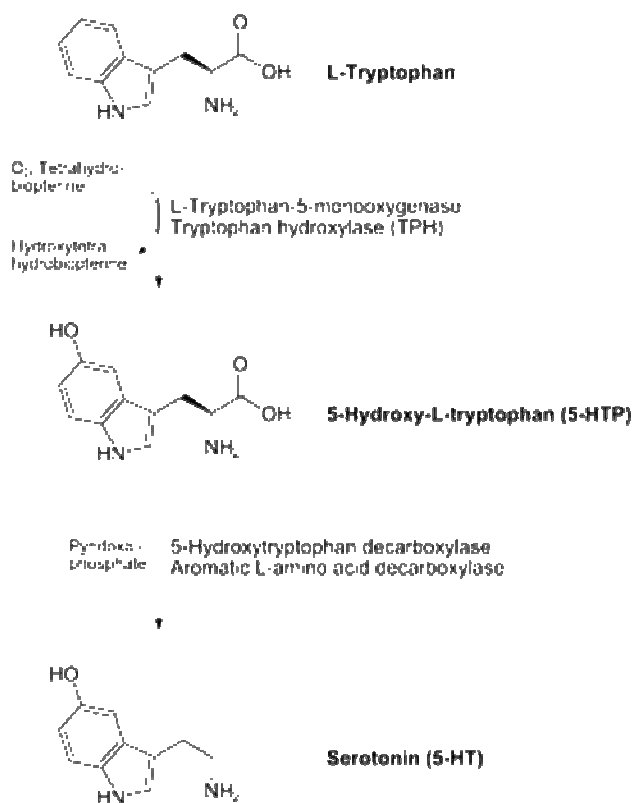
Researchers have hypothesized two main physiological bases for the development of depression. The first is decreased hippocampal neurogenesis as a result of stress<sup>57</sup>. Increased glucocorticoid levels can impact cell proliferation, differentiation, and survival in two specific regenerative regions of the brain, the olfactory bulbs and the dentate gyrus of the hippocampus. In turn, hippocampal atrophy can impact mood. Decreases in hippocampal volume have been shown to correlate with total lifetime length of depression (rather than age) in several MRI studies comparing depressed patients, recovered depressed patients, and healthy controls<sup>58,59</sup>. The second proposed theory is a “chemical imbalance” in the neurotransmitters that control mood. This “Monoamine Hypothesis” proposes that malfunctions in the synthesis, release, uptake, and degradation of neurotransmitters may result in a prolonged debilitating mental state. Since its proposal in 1965 by Joseph Schildkraut, the Monoamine Hypothesis has guided development of drug therapies<sup>60</sup>. Early compounds included monoamine oxidase inhibitors (MAOIs), which block the degradation of mood-related neurotransmitters, and tricyclic antidepressants (TCAs), which impede transport of serotonin and norepinephrine. Given the risk of severe side effects, MAOIs and TCAs have largely been replaced by selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), and norepinephrine-dopamine reuptake inhibitors (NDRIs)<sup>61</sup>. These selective reuptake inhibitors are generally considered safer. Selective reuptake inhibitors block binding sites on neurotransmitter transporters or receptors, leading to an effective increase in synaptic serotonin, dopamine, or norepinephrine.

Currently, depression is diagnosed according to the results of the Hamilton Rating Scale for Depression (HAM-D), a structured interview originally developed in 1960<sup>62</sup>. Although widely considered the “gold standard” for diagnosis, the efficacy and consistency of the scale is often debated<sup>63,64</sup>. The HAM-D involves both patient responses and a clinician’s observations and includes assessment of agitation, changes in appetite, and changes in ability to focus on tasks<sup>65</sup>. Interview responses are scored by severity, and the sum score is used to indicate depression. Though 20 is the accepted threshold score required for inclusion in a clinical trial, many clinicians disagree, further undermining the validity of the scale. The interview is periodically repeated throughout treatment to measure improvement or regression.

Because no objective biomarker currently exists for the diagnosis of depression, much less the identification of specific defects in brain chemistry, many patients try several different types of antidepressants before experiencing relief. In fact, it is estimated that only 30-40% of patients achieve complete remission after starting an initial antidepressant regimen<sup>66</sup>. The prevalence of treatment-resistant depression has spurred the development of augmentative therapy, or the combination of an antidepressant with talk-based therapy, nutritional supplements, another type of antidepressant, or other alternative therapies<sup>67</sup>.

## **Serotonin**

Perhaps the most widely researched mood-related neurotransmitter is serotonin, a tryptophan-based monoamine synthesized both by the serotonergic neurons of the central nervous system and by the enterochromaffin cells of the gut in a reaction requiring B<sub>6</sub> (pyridoxal phosphate; Figure 1.4)<sup>68,69</sup>. Gut serotonin is absorbed into the bloodstream and stored in platelets, and its activity appears to promote wound healing via vasoconstriction<sup>69</sup>. Brain serotonin is associated with mood, appetite, sleep, learning, and memory<sup>70</sup>.



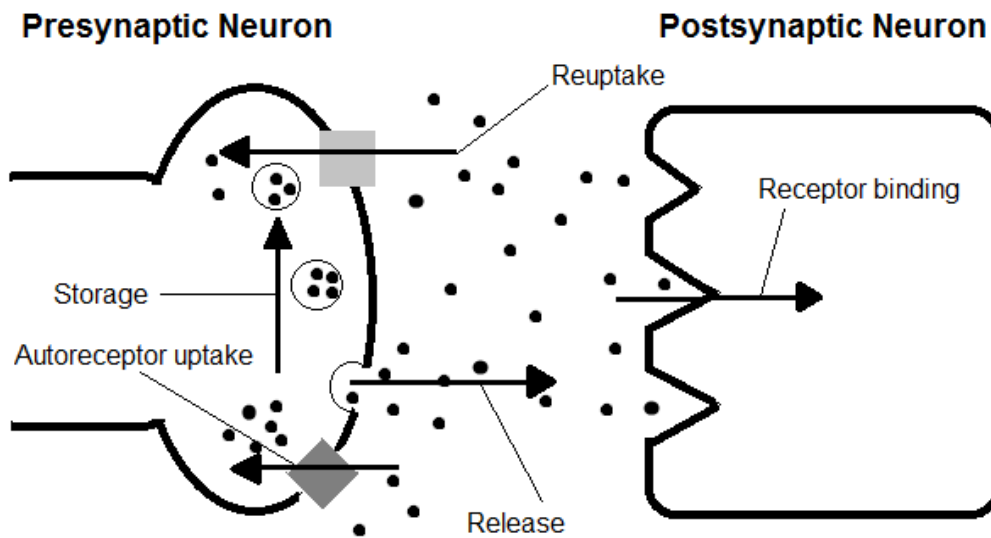
**Figure 1.4: Synthesis of serotonin (5-HT)**

Serotonin is released into the synaptic cleft by the presynaptic cell using vesicular transport. Following release, synaptic serotonin can be recycled back into the presynaptic neuron by the serotonin reuptake transporter (SERT), taken up via serotonin receptors into the postsynaptic neuron for response propagation, taken up by presynaptic autoreceptors to indicate synaptic concentrations, or degraded, chiefly via catechol-*O*-methyl transferase (COMT) and monoamine oxidase (MAO; Figure 1.5)<sup>68</sup>. Low levels of synaptic serotonin have been shown to cause depressive symptoms<sup>71</sup>.

In 1975, research into modulation of neurotransmitter transporter (NTT) activity exploded when scientists at Eli Lilly developed and screened derivatives of diphenhydramine, an antihistamine with some antidepressant properties, for enhanced selectivity for neurotransmitter transporters. Lilly 110140 displayed high selectivity for serotonin transporter inhibition and was revealed to have a number of antidepressant effects, including increased hippocampal neurogenesis<sup>72,73</sup>. The compound became known as fluoxetine and traded as Prozac. Although fluoxetine was not the first SSRI approved in the United States, it was the most aggressively marketed and widely tested, and Prozac has since become a collective synonym for antidepressant medications. Modulation of serotonin reuptake remains a



preeminent target for drug development.



**Figure 1.5: Neurotransmitter signaling**

## Dopamine

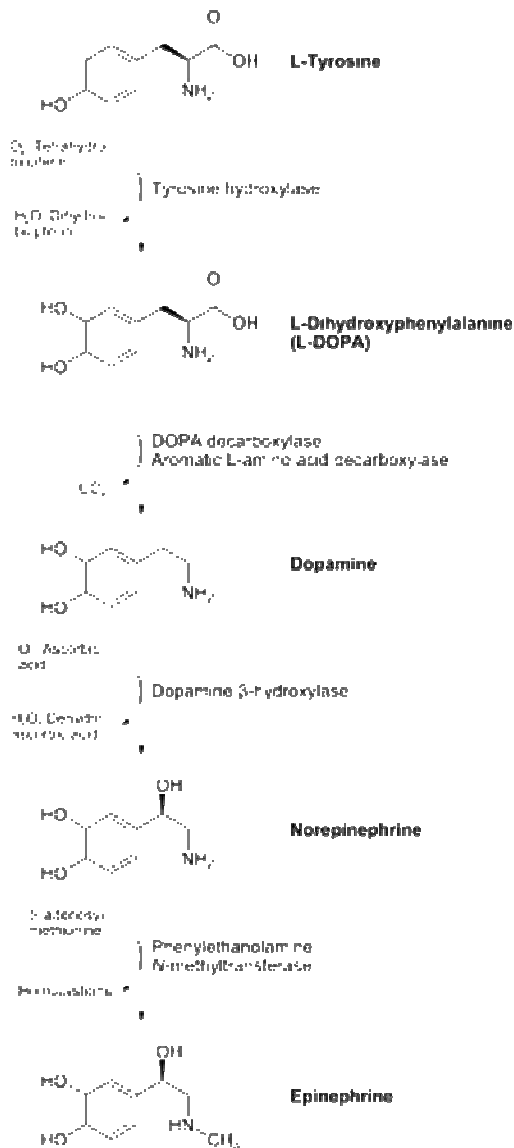
Dopamine is a monoamine neurotransmitter strongly linked to reward-based satisfaction. It is synthesized in neurons and the adrenal medulla from tyrosine or phenylalanine (Figure 1.6). Pleasurable activities cause a rapid release of dopamine in the brain, leading to feelings of well-being and happiness. Highly addictive drugs, including cocaine and methamphetamines, cause a similar but intensified response, and dopamine metabolism is thought to be a chief contributor to physical addiction<sup>74</sup>. Low dopamine levels are linked to a wide variety of diseases, chiefly depression, schizophrenia, and Parkinson's disease<sup>75,76</sup>. Of these, Parkinson's disease is considered the most degenerative, as the loss of motor control and tremors characteristic to the disease are very difficult to control with medication without severe side effects.

Modulation of dopamine synthesis, trafficking, and degradation is essential for the effective treatment of these diseases. Dopamine metabolism closely mirrors serotonin metabolism, utilizing a specific dopamine transporter (DAT) and postsynaptic receptors. Impaired dopamine trafficking results

in severe disruption of both motor control and cognitive function, as observed in patients suffering from Parkinson’s disease. Parkinson’s disease is usually characterized by a loss of motor control, often manifested as “tremors,” an involuntary trembling in the hands. Levodopa, or L-DOPA, is a dopamine precursor that can cross the blood-brain barrier and is used to manage Parkinson’s disease symptoms.

However, L-DOPA also causes a large variety of side effects, including impaired methyl group metabolism, hypertension, and arrhythmia<sup>77</sup>.

Recently, regulation of dopaminergic signaling via pharmaceutical intervention has been shown to treat depression and hyperactivity disorders, aid in smoking cessation, and reduce addictive cravings<sup>78</sup>. These drugs, known as norepinephrine-dopamine reuptake inhibitors (NDRIs), block the action of NET and of DAT, inhibiting reuptake<sup>79</sup>. As a relatively new class, NDRIs are considered atypical depressants and are often combined with more traditional therapies. The most popular NDRI, bupropion (marketed as Wellbutrin) is approved for treatment of



**Figure 1.6: Synthesis of dopamine and norepinephrine.**

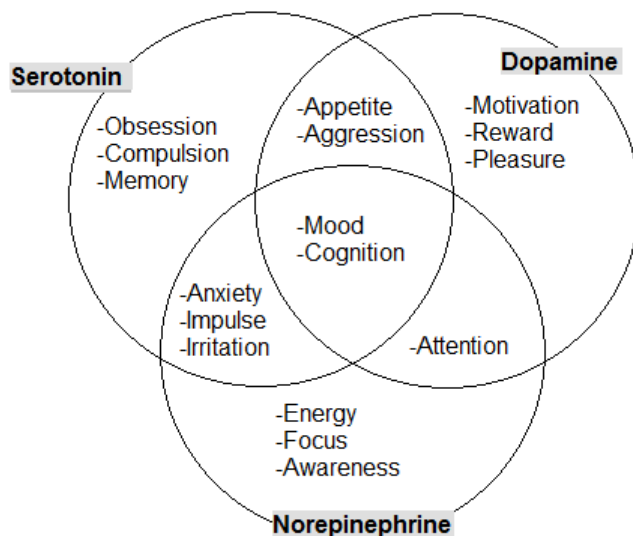
depression and for smoking cessation, but is used off-label to treat addiction, sexual dysfunction, and obesity<sup>80</sup>.

## Norepinephrine

Norepinephrine is a catecholamine synthesized from dopamine in medullary chromaffin cells<sup>81</sup>. The synthesis reaction requires methyl group donation from SAM. As part of the sympathetic response, norepinephrine stimulates increased heart rate, increased blood pressure, and increased blood supply to the brain. Given these effects, norepinephrine is thought to contribute to mood balance by regulating motivation, pleasure, and attention (Figure 1.7)<sup>71,82</sup>. Norepinephrine is released and metabolized similar to serotonin and dopamine, but utilizes a specific norepinephrine transporter (NET) and adrenergic receptors. Norepinephrine is degraded by COMT and MAO, but can also be converted to epinephrine by phenylethanolamine N-methyltransferase (PNMT)<sup>81</sup>. Given its structural similarity to dopamine, drugs designed for either often have crossover effects.

Selective norepinephrine reuptake inhibitors (SNRIs), including Cymbalta, Effexor, and Lexapro,

have gained popularity for treatment of depression with concurrent general anxiety disorder and are often combined with SSRIs for nonresponsive patients<sup>61</sup>.



**Figure 1.7: Emotional regulation via serotonin, dopamine, and norepinephrine**

## Neurotransmitter Transporters and Depression

Because most pharmaceutical depression treatments revolve around modulation of neurotransmitter transporters

(NTTs), it is important to discuss the activity and regulation of monoamine NTTs. As previously mentioned, serotonin, norepinephrine, and dopamine each utilize a specific transporter (SERT, NET, and DAT, respectively). SERT, NET, and DAT are all sodium/chloride dependent members of the solute carrier superfamily (SLC), which are anchored to the plasma membrane of monoaminergic neurons<sup>83</sup>. Following vesicular release of neurotransmitters into the synaptic cleft, NTTs transport a large portion of the released neurotransmitter back into the presynaptic cell, effectively lowering the synaptic concentration of that neurotransmitter and contributing to the presynaptic pool. NTT-facilitated reuptake is the primary regulatory mechanism by which cellular stores of serotonin, dopamine, or norepinephrine are maintained.

Knockout (KO) models in mice have consistently demonstrated the essentiality of NTT function. Although it may seem counterintuitive, given that selective inhibition of NTTs by antidepressant drugs increases synaptic neurotransmitter concentration and generally relieves depression, NTT-KO mice have marked decreases in whole brain neurotransmitter concentration. For example, one study showed that DAT-KO mice had 95% less whole brain dopamine than wild-type controls, even when dopamine synthesis was more than doubled, indicating the importance of dopamine reuptake to maintain presynaptic stores of dopamine<sup>84</sup>. These mice also displayed severe hyperactivity and could be calmed by amphetamine administration, a response mirrored in attention-deficit/hyperactivity disorder (ADHD) and opposite of the expected response in a healthy subject. Similarly, NET SNPs contribute to depression<sup>82</sup>. NET-KO mice display hyperactivity, similar to DAT-KO mice; however, this is accompanied by excessive sympathetic responses to stimuli, including tachycardia.

Similar responses are seen in SERT-KO mice, with anxiety-associated behaviors replacing hyperactivity, and SERT alterations have been implicated in several mental disorders. The promoter of the SERT gene contains a polymorphic region, which produces either short or long repeats. The short

repeats are associated with susceptibility to anxiety and depression in humans<sup>85</sup>. Single nucleotide polymorphisms (SNPs) in the SERT gene are associated with bipolar disorder, autism, obsessive-compulsive disorder, eating disorders, and depression<sup>86,87</sup>. Additionally, certain SERT polymorphisms have been linked to both depression and non-response to SSRIs.

Recreational drugs powerfully inhibit NTTs<sup>84</sup>. In particular, cocaine nonselectively inhibits SERT, NET, and DAT. Cocaine addiction is primarily attributed to DAT inhibition, which results in a strong “reward” feeling. Methamphetamines and 3,4-methylenedioxymethamphetamine (MDMA, also called ecstasy) impact both NET and SERT.

Other factors that impact NTT function include glycosylation, a post-translational modification thought to contribute to transporter stability, and stress-related cytokines. Specifically, TNF- $\alpha$ , an inflammatory cytokine, potently promotes SERT activity<sup>88</sup>. As previously discussed, SSRIs, SNRIs, and NDRI selectively block some NTT function, causing an increase in synaptic neurotransmitter concentration<sup>61</sup>. The mild depletion of cellular neurotransmitter concentration is tempered by receptor desensitization, a response that contributes to the overall antidepressant activity of the drug.

## **Depression and Methyl Group Metabolism**

Impaired methyl group metabolism has long been associated with psychiatric symptoms. In 1975, analysis of 272 patients at a mental health facility showed that depressed patients had low serum levels of folate, while psychotic patients had low serum B<sub>12</sub><sup>89</sup>. Further studies showed that high plasma homocysteine and low serum folate both increased the risk of depression and increased the duration of depressive episodes, an effect that is especially pronounced in pregnant and postpartum women<sup>90-92</sup>.

Supplementation with folate and B-vitamins has been moderately successful in relieving depressive symptoms.

Because patients with low serum folate and hyperhomocysteinemia also have low SAM levels, it has been suggested that oral supplementation of SAM, the ubiquitous methyl donor, may alleviate or prevent depression. Several clinical trials have shown that SAM is an effective antidepressant, and superior to some selective serotonin reuptake inhibitors<sup>93-95</sup>. The use of SAM as an antidepressant is especially attractive for its low cost, wide availability, and high tolerability<sup>96</sup>. Marketed as AdoMet, SAM-e, and adementionine, SAM has been used for the treatment of depression for over twenty years in Europe but has only recently become available in the United States. Interestingly, SAM supplementation does not increase serum homocysteine in humans, contrary to expectation<sup>97,98</sup>.

The specific mechanism of antidepressant activity caused by SAM is under debate. Because SAM and other compounds essential to methyl group balance are required for neurotransmitter synthesis, it may be a simple question of methyl group supply<sup>99</sup>. Acute intraperitoneal SAM administration has been shown to transiently increase serotonin in the rat forebrain at supraphysiological doses (>200mg/kg bw), an effect that was matched by increased degradation into 5-hydroxyindolacetic acid (5-HIAA)<sup>100</sup>. Other groups have shown that acute intraperitoneal SAM administration at lower doses (10 mg/kg bw) modifies dopamine and norepinephrine metabolism inconsistently across brain regions. Using mice genetically modified (via ApoE knockout) to be prone to seizures, supplemental SAM was shown to reduce aggressive behaviors and seizure frequency<sup>101</sup>. Most studies have focused on clinical trials of SAM in depressed patients rather than the molecular mechanisms of the supplement's activity.

## CHAPTER 2: ORAL ADMINISTRATION OF RETINOIC ACID LOWERS BRAIN SEROTONIN CONCENTRATION IN RATS

### Abstract

Major depressive disorder has been associated with lowered functionality of serotonin, dopamine, and norepinephrine in the brain. Oral retinoid therapy has been reported to induce sudden and severe depressive symptoms in adolescents and adults. Retinoid compounds have been shown to negatively impact neurotransmitter synthesis and transport in vitro, while animal models of retinoid therapy and brain health have traditionally utilized intraperitoneal administration of retinoic compounds and focused on depressive behaviors. To our knowledge, no previous in vivo studies have used oral administration to demonstrate the impact of retinoid compounds on neurotransmitters in the rat brain. In this study, male Sprague-Dawley rats received oral administration of either all-*trans* retinoic acid, all-*trans* retinoic acid plus S-adenosyl methionine (SAM), or vehicle daily. After 9 days, rats were sacrificed and brain tissue was collected for analysis. Rats receiving all-*trans* retinoic acid had a significant (48%;  $p < 0.05$ ) decrease in brain serotonin concentrations compared to control, an effect that was partially prevented by SAM supplementation. Neither norepinephrine nor dopamine levels were impacted by oral dosage of all-*trans* retinoic acid. To our knowledge, this is the first demonstration of lowered brain serotonin concentrations resulting from oral administration of retinoic acid. SAM represents a potential therapeutic strategy to prevent major depressive disorder owing to retinoid usage.

### Introduction

Recent developments in pharmacology have shown retinoid compounds to be highly effective for the treatment of nodular skin lesions and several cancers<sup>102</sup>. Of particular interest is retinoic acid, the most potent form, which has powerful effects on gene expression and has been successfully utilized as

an oral medication. However, the effects of retinoic acid are both strong and nonspecific, causing a variety of undesired side effects. Specifically, patients taking retinoic acid must be closely monitored for increases in blood lipids resulting from impaired liver function and sudden changes in mood or behavior<sup>24</sup>. A small but significant portion of retinoid patients experience sudden onset of depressive symptoms, including suicidal ideation. Although retinoic acid has been shown to alter serotonergic signaling and neurogenesis in vitro, the mechanism of this effect remains unclear<sup>47,48</sup>.

Impaired methyl group metabolism is well established as a hallmark of several pathologies. Retinoic acid has been shown to disturb methyl group metabolism by activating and inducing glycine N-methyltransferase (GNMT)<sup>16,18</sup>. GNMT acts to optimize the intracellular ratio of *S*-adenosyl methionine (SAM) to its metabolite, *S*-adenosyl homocysteine (SAH), a primary indicator of transmethylation potential. Recently, impaired methyl group metabolism has been implicated as a contributor to depressive symptoms. This is attributed to a deficiency or depletion of folate, other B-vitamins, choline, and SAM<sup>3</sup>. Supplementation with these nutrients has been moderately successful in treating depression<sup>92</sup>. Owing to the relationship between retinoids, impaired methyl group metabolism, and depression, we sought to characterize the neurological effects of retinoic acid therapy. In addition, we hypothesized that supplemental SAM would mitigate these consequences by correcting the depletion of intracellular methyl groups.

## Materials and Methods

All animal studies were approved by the Institutional Animal Care and Use Committee and were performed according to Iowa State University Laboratory Animal Resources Guidelines. Male Sprague Dawley rats (N=19; Harlan Teklad, Madison, WI) weighing 100-130 g were housed singly in plastic cages on a 12 hr light:dark cycle. Rats were watered and fed ad libitum using a diet containing vitamin-free casein (20%), cornstarch (55%), glucose (15%), mineral mix (AIN93, 3.5%), vitamin mix (AIN93, 1%), corn



oil (5%), choline bitartate (0.2%; Sigma-Aldrich, St. Louis, MO) and methionine (0.3%; Sigma-Aldrich, St. Louis, MO). Following a 10 d acclimation period, rats were randomly divided into three groups. The first group (n=6) received a daily oral dose of corn oil, administered via positive displacement pipet at 2.5  $\mu$ l per gram body weight. The second group (n=6) received all-trans retinoic acid (ATRA) (Sigma-Aldrich, St. Louis, MO) at 30  $\mu$ mol/kg bw suspended in corn oil at 2.5  $\mu$ l/g bw. The third group (n=7) received the same retinoid dose plus 4mg/kg bw S-adenosyl methionine (SAM). The SAM (Nature Made, Northridge, CA) was a commercially available supplement sold as a 400 mg dose in a 1000 mg pill that was crushed and powdered prior to suspension in the retinoid and corn oil mixture. After 9 d, rats were fasted overnight and anesthetized by intraperitoneal injection of ketamine and xylazine (90:10 mg/kg bw). Whole blood was obtained via cardiac puncture and immediately centrifuged for the isolation of plasma. Whole liver, brain, and kidney tissue was snap frozen in liquid nitrogen for later analysis.

Whole brain concentration of serotonin, dopamine, and norepinephrine was quantified using commercially available ELISA kits (Rocky Mountain Diagnostics, Colorado Springs, CO). For the assessment of serotonin, whole brain tissue was homogenized in 5 volumes (g/mL) of 0.5 M hydrochloric acid containing 1 g/L ascorbic acid. For the assessment of dopamine and norepinephrine, a separate homogenate was prepared in 5 volumes (g/mL) 0.01 M hydrochloric acid containing 1 mM EDTA and 4 mM sodium metabisulfite. Both homogenates were centrifuged at 10,000  $\times$  g for 10 min at 4°C. The supernatant was collected and stored at -80°C prior to use.

Hepatic and renal GNMT activity was assessed using a modified method of Cook and Wagner<sup>18</sup>. In summary, triplicate aliquots of 250  $\mu$ g protein, plus an equivalent heat-killed control for each sample, were combined with a reaction mix containing 0.2 M Tris (pH 9.0), 5 mM dithiothreitol, 0.02 mM S-adenosyl-L-[methyl-<sup>3</sup>H]methionine (PerkinElmer, Waltham, MA), and 2 mM glycine. After a 30 min incubation at 25°C, 10% trichloroacetic acid was added to stop the reaction. Activated charcoal was

added, vortexed, and centrifuged to remove the unreacted SAM<sup>3</sup>H. The supernatant was isolated and used for liquid scintillation counting.

Plasma samples were derivatized for the quantification of circulating homocysteine using a previously described method<sup>103</sup>. During derivatization, N-acetylcysteine (1mM) was added for use as an internal standard. Homocysteine was assessed by HPLC, using a 100 µL injection of sample onto a µBondapak C<sub>18</sub> Radial-Pak column (Waters, Milford, MA) in a mobile phase of 4% acetonitrile and 96% potassium phosphate buffer (0.1 M, pH 2.1).

Liver and brain SAM concentrations were analyzed by the method of Bottiglieri with minor alterations<sup>104</sup>. Briefly, liver tissue was homogenized in two volumes of 0.4 M perchloric acid, while brain tissue was homogenized in 5 volumes (g/mL) of 0.01 M hydrochloric acid containing 1 mM EDTA and 4 mM sodium metabisulfite. Liver and brain samples were injected at volumes of 80 µL and 40 µL, respectively, on a µBondapak C<sub>18</sub> Radial-Pak column (Waters, Milford, MA) and a mobile phase consisting of 4% acetonitrile and 96% 0.1 M sodium acetate containing 5 mM heptanesulfonic acid and adjusted to pH 4.5. Absorbance was detected using a Waters 2487 Dual Absorbance Detector at 254 nm.

Statistics were calculated using SigmaPlot 9.0 software (Systat, Chicago, IL). Means were compared using a one-way ANOVA, followed by the Fisher least significant difference post-test. When applicable, the means of treatment groups were compared to control using the student's t-test.

## Results

**SAM supplementation lowered total weight gain, but did not affect relative liver and kidney size.** Rats receiving SAM supplementation with ATRA gained 20% less weight than control and ATRA rats (P<0.05). However, relative liver and kidney sizes remained consistent across treatment groups (Table 2.1).

**Neither ATRA nor SAM significantly changed concentrations of SAM in the brain and liver.** Although the data suggest that ATRA may lower liver and brain SAM, HPLC analysis revealed no significant differences between mean group levels of SAM in the brain and liver (Table 2.2). Although SAM supplementation appeared to increase tissue SAM, SAM levels were greatly varied within groups. It was noted that liver SAM had no correlation to brain SAM in individual rats. Furthermore, the SAM:SAH ratio was not impacted in either tissue.

**Neither ATRA nor SAM significantly changed serum homocysteine levels.** While ATRA appeared to raise serum homocysteine, this effect was not statistically significant. As with the tissue concentrations of SAM, the levels of serum homocysteine were varied within groups. SAM did not alter homocysteine levels (Table 2.2).

**Retinoic acid caused elevated hepatic GNMT activity.** In accordance with similar studies in our lab, ATRA increased hepatic GNMT activity by 129% ( $p < 0.05$ ) regardless of SAM supplementation (Figure 2.1). As expected, this result was tissue specific; renal GNMT was not significantly impacted by ATRA or by SAM.

**Retinoic acid lowered whole brain serotonin, but did not affect norepinephrine and dopamine.** Oral supplementation of ATRA lowered whole brain serotonin by 46% ( $p < 0.05$ ), an effect that was partially attenuated by SAM (Figure 2.2 A). In contrast, ATRA did not significantly impact whole brain concentrations of norepinephrine and dopamine (Figure 2.2 B,C).

## Discussion

Previous studies have shown that chronic administration of retinoic acid induces depressive behaviors in mice<sup>37</sup>. Changes in neurotransmitter synthesis and transport are regarded as the physiological basis for mood disorders. Of particular interest are serotonin, norepinephrine, and

dopamine, the three neurotransmitters that have been successfully targeted by pharmaceutical intervention for the treatment of depression. Serotonin modulates numerous behavioral and emotional processes, including mood, anger, attention, and memory. In the present study, oral administration of ATRA was shown to lower whole brain serotonin, providing a partial biochemical basis for the correlation of mood changes with retinoid therapy. However, this finding is in direct contrast to the literature<sup>47</sup>. In vitro studies involving retinoic acid and serotonin metabolism suggest that retinoic acid may affect the stability of serotonin mRNA or the stability of its transporter and receptor mRNA transcripts, showing a possible mechanistic basis for the reduced whole brain serotonin<sup>47</sup>.

ATRA did not appear to affect whole brain concentration of norepinephrine and dopamine. Dopamine is primarily produced in two regions of the brain, the substantia nigra and the ventral tegmental area, while norepinephrine is produced from dopamine by specialized chromaffin cells. Because our homogenate was prepared from the whole brain rather than of one region, changes in norepinephrine and dopamine concentrations could have been masked.

SAM administration did not affect levels of serotonin, norepinephrine or dopamine, an effect that is somewhat surprising given the role of SAM in the synthesis of norepinephrine from dopamine, tyrosine, and phenylalanine and its overall use as a successful antidepressant. Furthermore, we would expect successful SAM administration to increase whole brain and liver SAM concentration. However, problems with the administration of SAM could have prevented proper absorption of the compound and prevention of its activities. This is supported by the inconsistencies in SAM concentrations in the liver and brain within the group receiving supplemental SAM. While the marked increase in hepatic GNMT activity confirms the successful administration of retinoic acid, the oil based vehicle poorly suspended the powdered SAM and was not well tolerated. Rats receiving ATRA developed skin lesions at the point of contact with the positive displacement pipet, an effect that seemed exacerbated by the addition of

powdered SAM to the mixture. Furthermore, as a sulfur-containing amino acid, the SAM likely had a disagreeable taste, which could partially explain the impaired growth of the ATRA+SAM group. The impaired growth was not accompanied by alterations to relative liver or kidney size. In future studies, these challenges should be addressed to ensure consistent and tolerable administration of ATRA and SAM.

To our knowledge, this is the first demonstration that oral administration of ATRA lowers whole brain serotonin in rats. This further supports the importance of close monitoring of mood changes in patients receiving retinoid therapies. Given the inconclusive nature of our findings related to the concurrent administration of SAM, further research and development of an alternate administration vehicle are pertinent.

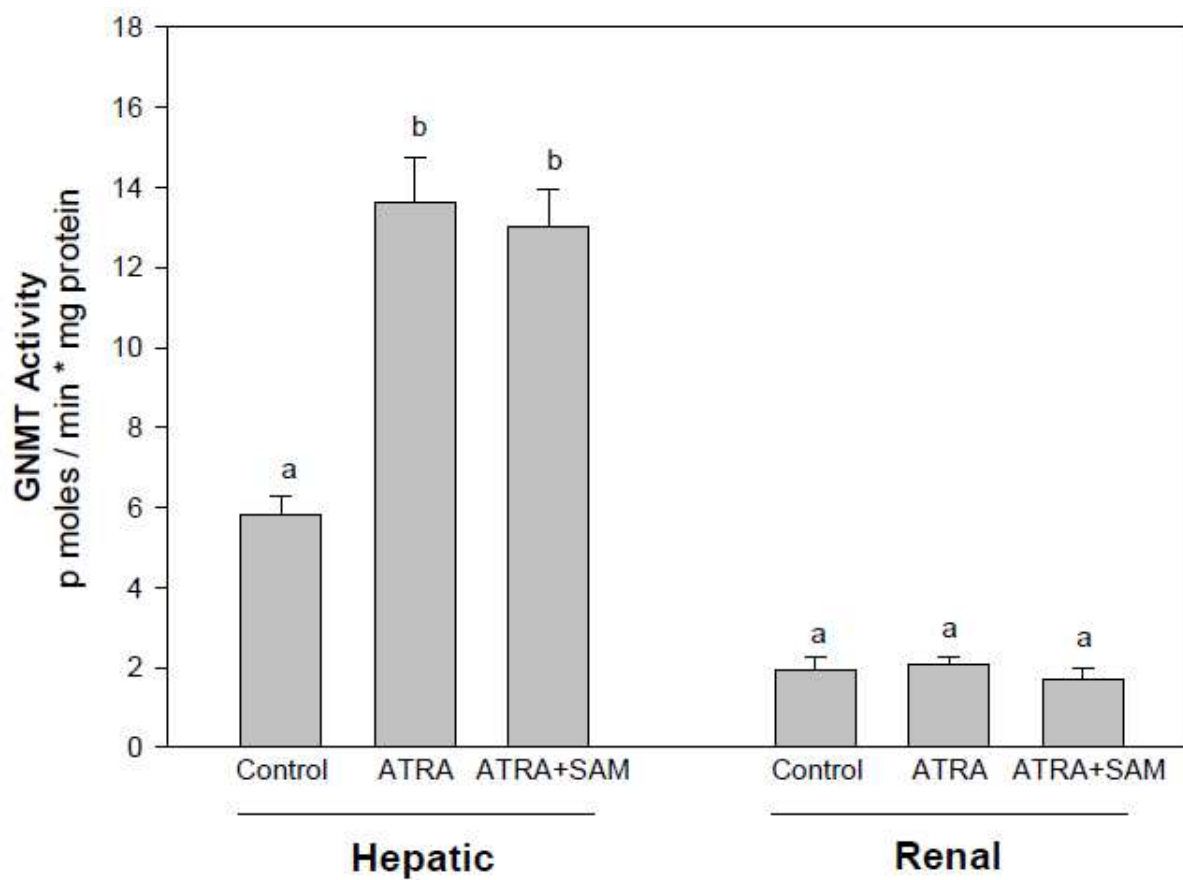
**Table 2.1:** SAM supplementation significantly reduces total weight gain, but does not impact relative kidney or liver sizes.

	Total weight gain, g	Relative liver size, %	Relative kidney size, %
Control	128.0±3.5 <sup>a</sup>	3.7±0.1	0.7±0.1
ATRA	124.3±5.3 <sup>a</sup>	3.5±0.1	0.7±0.1
ATRA+SAM	96.6±7.1 <sup>b</sup>	3.5±0.1	0.7±0.1

Data shown as group mean ±SE; superscript letters indicate significant differences between groups (p<0.05)

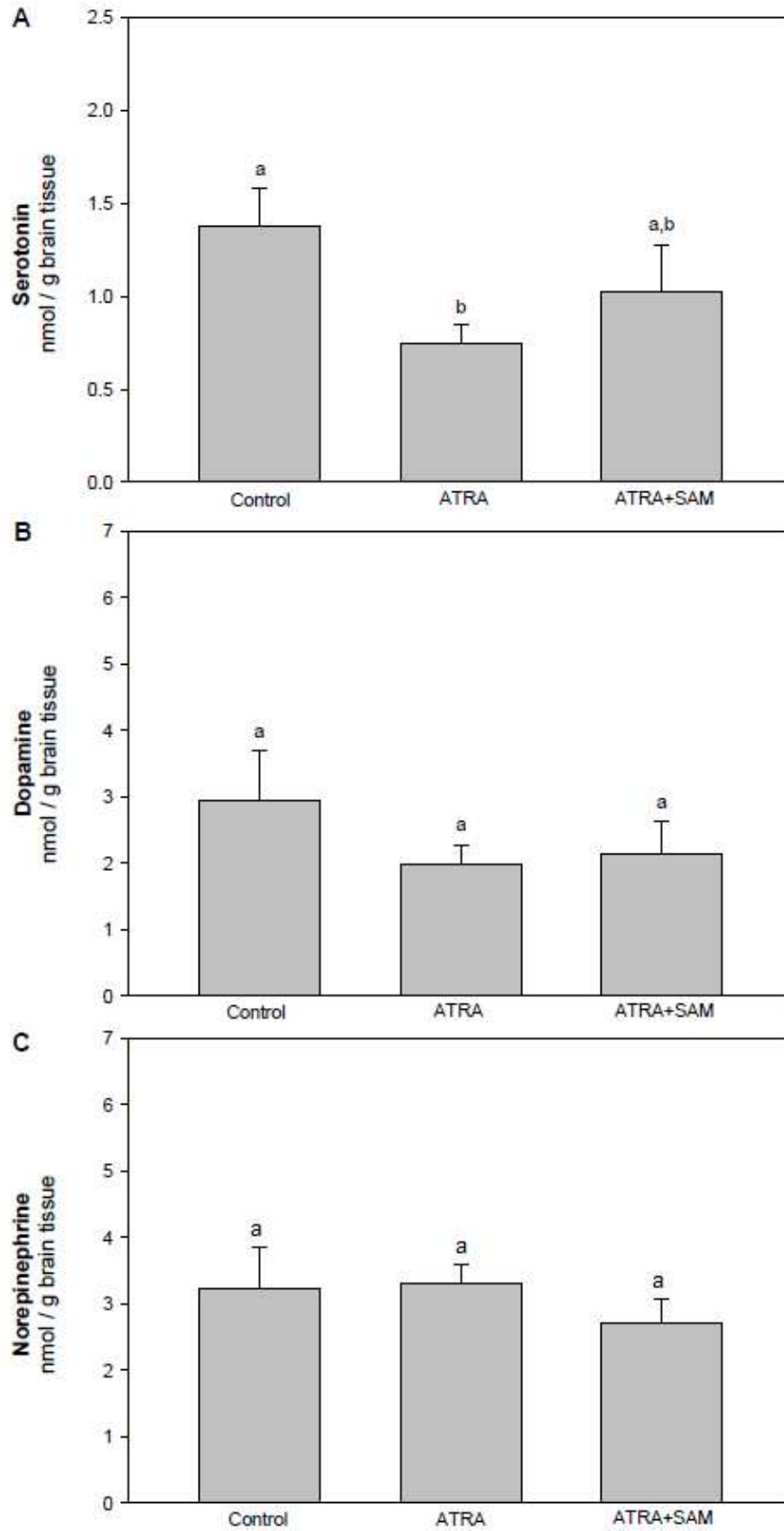
**Table 2.2:** Indices of methyl group metabolism were unchanged by retinoic acid and SAM supplementation.

	Liver			Brain			Plasma
	SAM nmol/g	SAH nmol/g	SAM:SAH	SAM nmol/g	SAH nmol/g	SAM:SAH	Hcy μmol/L
Control	25.2±1.4	9.6±1.3	3.0±0.5	10±2.4	1.5±0.3	5.5±1.7	6.3±1.6
ATRA	23.3±3.2	9.4±1.9	3.7±0.3	6.0±1.2	0.7±0.3	6.0±3.6	9.3±1.9
ATRA+SAM	30.5±2.9	13.0±2.9	3.1±0.7	12.2±4.6	2.0±0.8	7.5±4.0	9.9±1.6



**Figure 2.1:** Hepatic and renal GNMT activity. Data shown as mean  $\pm$ SE. Letters indicate significant differences between groups in a particular tissue ( $p < 0.05$ ).





**Figure 2.2:** Whole brain concentration of serotonin (A), dopamine (B), and norepinephrine (C).

## CHAPTER 3: ORAL ADMINISTRATION OF S-ADENOSYL METHIONINE INCREASES MOOD-RELATED NEUROLOGIC COMPOUNDS AND MITIGATES LIVER DYSFUNCTION SECONDARY TO ORAL ALL-TRANS RETINOIC ACID AND 13-CIS RETINOIC ACID THERAPY

### Abstract

As retinoid therapy gains popularity for the treatment of skin lesions and various cancers, it is increasingly important to understand the side effects and consequences of retinoid treatment, which include development of hepatic steatosis, alterations in blood lipids, impaired methyl group metabolism, and depressive symptoms. The focus of this study was the impact of all-*trans*- and 13-*cis* retinoic acid on one-carbon metabolism and the potential for mitigation of these effects by oral S-adenosyl methionine (SAM), the universal methyl group donor. Rats receiving all-*trans* retinoic acid had 42% increased liver lipids, 71% increased liver triglycerides, and 65% decreased hepatic SAM. Rats receiving 13-*cis* retinoic acid had 42% increased liver lipids, 57% increased liver triglycerides, and 39% decreased hepatic SAM. Both forms of retinoic acid caused moderate, but significant increases in whole brain serotonin and decreases in whole brain neurotransmitter transporters. These effects were partially or completely prevented by concurrent oral administration of SAM. In addition, SAM supplementation in healthy rats was shown to increase whole brain dopamine by 1,650% and whole brain norepinephrine by 70%. In contrast to other studies, SAM was not shown to impact serotonin levels. Our data indicates that the depressive effect of retinoic acid therapy may be due to a functional deficit in methyl groups as a result of impaired methyl group metabolism. Furthermore, oral SAM seems to be a powerful agent in the prevention or treatment of major depressive disorder caused by retinoic acid administration or by other factors.

## Introduction

As retinoid therapies gain popularity for the treatment of numerous pathologies, from skin lesions to cancer, it is important to characterize and understand the mechanisms of their side effects. Researchers have noted increases in blood lipids, hepatic steatosis, and alterations in methyl group metabolism after treatment with retinoid compounds<sup>18,33</sup>. Specifically, increases in hepatic glycine N-methyl transferase (GNMT) and corresponding depletion of methyl groups occur following administration of all-*trans* retinoic acid. In addition, 13-*cis* retinoic acid (13CRA) has been linked to acute onset of major depressive disorder and suicidal ideation. 13CRA has been shown to modulate levels of serotonin and its transporter and receptors. Disruptions in methyl group metabolism have long been noted as causes of cognitive and mood disorders, including depression, Alzheimer's disease, and Parkinson's disease<sup>76,105</sup>. Supplementation with B-vitamins and folate has enjoyed moderate clinical success in alleviating depressive symptoms<sup>91,92,106</sup>. Recently, S-adenosyl methionine has been touted as a potential treatment for depression, both alone and in conjunction with prescription serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs)<sup>94,107</sup>.

Given the relationships between retinoid therapy, dysfunctional methyl group metabolism, and brain health, it is important to explore the potential of SAM to mitigate the side effects of retinoic acid. In this study, two types of retinoic acid, ATRA and 13CRA, were administered with supplemental SAM to characterize the impact of SAM supplementation on overall health, with a particular focus on the support of mood-related compounds.

## Materials and Methods

**Animals.** All protocols were approved by the Institutional Animal Care and Use Committee and performed according to Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-

Dawley rats (N=37; Harlan Teklad, Indianapolis, IN) were obtained at 100-130 g, singly housed in plastic cages in a room with a 12 hr light:dark cycle, and given ad libitum access to food and water. The diet was a lower-protein formulation containing vitamin-free casein (10%), cornstarch (65%), glucose (15%), mineral mix (AIN93, 3.5%), vitamin mix (AIN93, 1%), corn oil (5%), choline bitartate (0.2%; Sigma-Aldrich, St. Louis, MO) and methionine (0.3%; Sigma-Aldrich, St. Louis, MO). After an acclimation period, the rats were randomly divided into six groups. The first group (n=6) received daily oral administration of honey thinned with distilled water (3:1) at 1.5  $\mu\text{L/g}$  bw and served as a control. The second group (n=6) received 10 mg/kg bw crushed SAM (Nature Made, Northridge, CA) suspended in the same diluted honey vehicle. The third and fourth groups (n=6) received all-*trans*-retinoic acid (ATRA) or 13-*cis*-retinoic acid (13CRA) at 30  $\mu\text{mol/kg}$ , respectively. The fifth and sixth groups received all-*trans* retinoic acid (ATRA) or 13-*cis* retinoic acid (13CRA), respectively, at 30  $\mu\text{mol/kg}$ , plus 10 mg/kg bw crushed SAM. Treatment doses were based on previous retinoid studies and recommended human dosages. All doses were administered orally in the 3:1 honey and water mixture at 1.5  $\mu\text{L/g}$  bw using a positive displacement pipet. After 20 daily doses, all rats were fasted overnight, anesthetized by intraperitoneal injection of ketamine and xylazine (90 and 10 mg/kg bw, respectively), and sacrificed. Whole blood was obtained via cardiac puncture and immediately centrifuged for the isolation of plasma. Liver, kidney, and brain tissue was collected and snap frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**Enzyme analysis.** Assessment of hepatic and renal GNMT assessment was performed as previously described in Chapter 2.

**HPLC analysis.** Assessment of hepatic and neural SAM and SAH was performed as described in Chapter 2; however, both liver and brain extractions were injected at 80  $\mu\text{L}$ .

**DNA methylation assessment.** Global DNA methylation was assessed using a colorimetric ELISA (Epigentek, Farmingdale, NY). The kit utilized 100 ng of DNA, which was isolated from liver tissue using a

QuickGene 810 with the appropriate kit (Autogen, Holliston, MA). Results were compared to positive and negative controls provided by the kit and calculated according to the known methylation of the positive control.

**Neurotransmitter and transporter assessment.** Quantification of whole brain serotonin, dopamine, and norepinephrine were performed as described in Chapter 2, using ELISA kits (Rocky Mountain Diagnostics, Colorado Springs, CO). For the detection of serotonin transporter (SERT), dopamine transporter (DAT), and norepinephrine transporter (NET), whole brain homogenate was prepared using 5 volumes (g/mL) of buffer containing 0.25 M sucrose, 10 mM sodium phosphate, 1mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. Following homogenization, samples were centrifuged at  $14,500 \times g$  for 30 min and stored at  $-80^{\circ}\text{C}$  until use. Each transporter was assessed using a separate commercially available ELISA kit (SERT and NET: USCN Life Science Inc., Houston, TX; DAT, Bio Medical Assay, Beijing, China).

**Liver lipid quantification.** Total liver lipid content was assessed using the Folch extract procedure<sup>108</sup>. One gram of liver tissue was added to 10 mL chloroform and methanol (2:1 v/v) and homogenized using a Potter-Elvehjem homogenizer, then filtered, diluted to 20 mL with chloroform:methanol, and added to 4 mL of calcium chloride solution (0.05%). After overnight separation, the upper phase was discarded and the lower phase was washed. Triplicate 5 mL aliquots of the lower phase were added to weighing pans with a known mass. After drying overnight, the pans were weighed to calculate the extracted lipid. An additional 5 mL aliquot was preserved at  $-20^{\circ}\text{C}$  for the analysis of liver triglycerides. Liver triglycerides were detected using the prepared aliquots of liver lipid and a colorimetric lipase kit (BioAssay Systems, Hayward, CA). Briefly, 10  $\mu\text{l}$  samples were added to an enzyme mix containing lipase, ATP, and a dyeing reagent, allowed to incubate at room temperature for 30 minutes, and read on a Bio-Tek EL-340

microplate reader at 562 nm. Results were compared to standards provided by the kit and to total lipid concentration.

**Statistics.** Statistics were calculated using SigmaPlot 9.0 software (Systat, Chicago, IL). Means were compared using a two-way ANOVA, followed by the Fisher least significant difference post-test. When variance was great, a two-way ANOVA followed by the Holm-Sidak method post-test was used to establish comparisons versus control. When applicable, the means of treatment groups were compared to control using the student's t-test.

## Results

**Oral SAM supplementation is well tolerated in rats.** No changes in weight gain, relative liver size, or relative kidney size were observed (Table 3.1).

### **Retinoic acid treatment and SAM supplementation impact markers of methyl group metabolism.**

ATRA causes a 64% reduction in hepatic SAM ( $p < 0.05$ ), an effect that was reversed by supplemental SAM. A similar, but not significant decrease was observed in rats receiving 13CRA. Neither treatment affected the SAM:SAH ratio. SAM supplementation significantly increased brain and liver SAM in control rats (370% and 154%, respectively;  $p < 0.05$ ) but no change to the SAM:SAH ratio was observed in either tissue. Plasma homocysteine was not impacted by either treatment (Table 3.2).

**ATRA and 13CRA exert similar effects on hepatic and renal GNMT.** Both ATRA and 13CRA caused similar increases in hepatic GNMT activity (60% and 80%, respectively;  $p < 0.05$ ). Supplemental SAM was able to partially alleviate the elevated hepatic GNMT activity, but this response was highly varied within each treatment group. Neither treatment significantly impacted renal GNMT activity (Figure 3.1).

**Neither retinoic acid nor supplemental SAM impacted global DNA methylation.** No changes in the percentage of 5-methyl cytosine in total DNA were observed, regardless of treatment (Figure 3.2).

**SAM supplementation increased whole brain concentration of dopamine and norepinephrine, but not serotonin.** SAM supplementation without concurrent retinoic acid administration caused whole brain dopamine concentration to increase 15-fold ( $p < 0.05$ ) (Figure 3.3 B). While a one-way ANOVA revealed no significant changes in norepinephrine concentration, a student's t-test showed that rats receiving SAM supplementation exhibited a 70% increase in mean norepinephrine concentration when compared to control rats, regardless of retinoic acid treatment (Figure 3.3 C). While serotonin concentrations appeared to be doubled by SAM, the effect was not significant (Figure 3.3 A). Neither retinoid treatment appeared to significantly alter whole brain concentration of norepinephrine and dopamine. However, when compared to control using a student's t-test, both retinoid treatments increased whole brain serotonin ( $p < 0.05$ ).

**Retinoic acid affects neurotransmitter transporter abundance.** Analysis of the three corresponding transporters for serotonin, dopamine, and norepinephrine (SERT, DAT, and NET, respectively) revealed significant alterations in DAT and NET, but not SERT (Figure 3.4). ATRA lowered DAT by 30% relative to control, and 13CRA lowered NET by 23% relative to control ( $p < 0.05$ ). Both of these effects were completely prevented by SAM supplementation.

**Retinoid therapy increases liver lipids.** Both ATRA and 13CRA induced 42% increased mean total liver lipid concentration ( $p < 0.05$ ) (Figure 3.5). Supplemental SAM blocked this effect in rats treated with 13CRA, but not in rats receiving ATRA. Further, ATRA and 13CRA increased liver triglycerides by 71% and 57%, respectively ( $p < 0.05$ ). This effect was partially attenuated by supplemental SAM.

## Discussion

Retinoid therapy has been associated with an increase in depressive episodes in otherwise healthy patients; however, the mechanism of this effect remains under debate. Here, we hypothesized

that oral administration of two forms of retinoic acid, ATRA and 13CRA, may modify neurotransmitter metabolism and liver health by disturbing methyl group balance. As previously demonstrated, both ATRA and 13CRA significantly increased hepatic GNMT activity. Accordingly, hepatic SAM was depleted in both retinoic acid groups.

Several aberrations in methyl group metabolism have been shown to contribute to hepatic steatosis, or an excess accumulation of fat that impedes liver function. Ethanol, low dietary methionine, low dietary protein, low folate intake, and low betaine intake have all been implicated in the pathogenesis of the disease<sup>109,110</sup>. The relationship between depleted hepatic SAM supply and increased hepatic lipids is thought to be due to changes in the activity of two SAM-dependent enzymes, DNA methyltransferase and phosphatidylethanolamine N-methyltransferase. These enzymes are responsible for DNA methylation and the synthesis of phosphatidylcholine from phosphatidylethanolamine, respectively. DNA methylation is an essential component of epigenetic regulation of gene expression. Phosphatidylcholine is required for effective transport and synthesis of very low density lipoproteins. In certain conditions, including high ethanol intake and methyl group depletion, changes in these processes result in DNA hypomethylation and an accumulation of liver lipids. Furthermore, liver toxicity and elevated serum triglycerides are well-known side effects of retinoid therapy, particularly 13CRA<sup>33,111</sup>. This is thought to be due to upregulation of hepatic palmitoyl-CoA synthetase<sup>112</sup>. As expected, the group receiving retinoic acid had elevated liver lipids and triglycerides, and depleted hepatic SAM. Interestingly, supplemental SAM was able to completely attenuate liver lipids and partially attenuate hepatic triglycerides in rats treated with 13CRA, but only partially lessened elevation of liver triglycerides in rats treated with ATRA. However, no appreciable changes in DNA methylation were observed. This suggests that the accumulation of liver lipids could be mostly attributed to retinoic acid's influence on palmitoyl-CoA synthetase and only moderately exacerbated by depletion of SAM. Furthermore, SAM



supplementation seems to be a viable preventative therapy for hepatitis steatosis, especially due to <sup>13</sup>CRA usage.

In contrast to our previous study, SAM administration had no discernible effect on weight gain, relative liver size, or relative kidney size. Taken with the significant increase in both liver and brain SAM, this data indicates that oral SAM is well tolerated in rats when administered in the appropriate vehicle.

The results of the neurotransmitter quantification experiments can be interpreted in several ways. It is important to consider not only the absolute measurement of whole brain serotonin, norepinephrine, and dopamine, but also the measurements in the context of their transporters. As previously discussed, each transporter is the chief regulator of its respective neurotransmitter concentration in the intracellular pool and in the synaptic space. Depressed patients have been shown to have low SERT, DAT, and NET. Because these NTTs are responsible for replenishing the intracellular neurotransmitter pool by recycling a portion of the neurotransmitter released into the synapse, low NTT is thought to contribute to a depressed mood by depleting total neurotransmitter levels, thereby lowering synaptic neurotransmitter concentration. In the context of select reuptake inhibitors, a class of antidepressants that bind and inhibit NTTs, lower functional NTT actually increases synaptic NT by failing to remove the portion that would normally be recycled into the presynaptic neuron. However, antidepressant treatment has been shown to blunt the activity of postsynaptic receptors, so any decrease in intracellular NT is matched by decreased removal by the postsynaptic cell. Therefore, the net effect of these antidepressants is increased synaptic serotonin, dopamine, or norepinephrine, contributing to a stable mood.

In this study, both the SAM and the retinoic acid treatments impacted neurotransmitter concentration, NTT concentration, or both. First, we will consider the impact on serotonergic signalling. In contrast to our previous study, whole brain serotonin was significantly increased in rats receiving

either form of retinoic acid. This matches the findings of O'Reilly, Trent, and others, who have shown that 13-cis retinoic acid increases intracellular serotonin *in vitro*<sup>47</sup>. However, our analysis did not reveal a significant change in SERT, while the O'Reilly group observed significant decreases in SERT and the serotonin autoreceptor 5-HT<sub>1A</sub>, which inhibits serotonergic firing. They speculated that the depressive symptoms associated with retinoic acid treatment are due to low synaptic serotonin, caused by declines in reuptake and inhibition of serotonin release. Therefore, although serotonin may be increased, it is largely sequestered in the presynaptic neuron. Because our study evaluated whole brain serotonin, we cannot definitively say whether retinoic acid treatment increased serotonin sequestration. However, high serotonin has been shown to increase 5-HT<sub>1A</sub>, which inhibits neuronal firing<sup>113</sup>. It is possible that the higher serotonin levels seen in rats receiving retinoic acid could have, in turn, caused lower synaptic release of serotonin through the inhibitory activity of 5-HT<sub>1A</sub>.

Although rats treated with SAM seemed to have increased whole brain serotonin, the variance within each group was great, and no significant difference to the control group was observed. Rats treated with both retinoic acid and SAM did not have increased whole brain serotonin, indicating that SAM attenuated changes to serotonergic signalling secondary to retinoic acid treatment. SAM treatment also appeared to increase SERT, but this effect was not significant, suggesting that mild increases in whole brain serotonin were matched by increases in SERT to stabilize synaptic serotonin.

Oral SAM administration was shown to significantly increase whole brain dopamine. In rats receiving only SAM treatment, whole brain dopamine was increased more than 15-fold, while rats receiving concurrent retinoic acid experienced a comparatively mild increase, roughly 2- or 3-fold. ATRA-treated rats had normal whole brain dopamine, but lower DAT, an effect that was corrected by SAM therapy. In dopaminergic signalling, increased synaptic dopamine causes a compensatory increase in the autoregulatory D<sub>2</sub> receptor, which inhibits neuronal firing, thereby stabilizing synaptic dopamine. This is

very similar to the function of 5-HT<sub>1A</sub> in serotonergic signalling. However, D<sub>2</sub> receptors also control DAT functionality; increased D<sub>2</sub> activity upregulates DAT activity and possibly expression. Using D<sub>2</sub> autoinhibition of firing and increasing DAT-mediated reuptake, sudden increases in synaptic dopamine can be attenuated. However, no change in whole brain DAT was seen in SAM-treated rats, indicating two potential outcomes. First, the regulatory capacity of D<sub>2</sub> autoreceptors could have been overloaded by the extreme increase in dopamine. Second, the synthesis of dopamine could have been so high that reuptake was unnecessary to maintain a sufficient intracellular pool, and the excess dopamine was allowed to degrade. High dopamine is the chief physiological root of psychosis and mania. However, mildly increased dopamine is associated with feelings of reward and well-being, and the dopamine concentration at which positive feelings escalate to mania or psychosis is not well defined. In rats, chronic ketamine administration induces a psychotic state, and evaluation of dopamine levels in ketamine-treated rats is useful to determine the level at which increased dopamine induces psychosis. Some studies have reported the extracellular dopamine threshold to be 90-200% of control, and increases of 60% or less are not associated with psychotic behaviour in rats<sup>114,115</sup>. In humans, the threshold is not well defined. Psychotic patients have been shown to have 30% greater dopamine synthesis capacity than healthy patients<sup>115</sup>. In the same study, schizophrenic patients were found to have similar dopamine levels, yet no development of psychosis, a difference thought to be due to varied receptor sensitivity. Additionally, bupropion, a potent DAT inhibitor and widely successful antidepressant, has only been tenuously linked to psychosis<sup>116</sup>. Given the variance in reported dopamine levels in psychosis, both in rats and in human patients, it is difficult to define the overall effect of oral SAM.

Taken together, our data indicates that SAM supplementation in healthy adults may be inappropriate at this dose (10 mg/kg bw). In 2012, Green and others evaluated the safety of SAM supplementation in patients with 22q11 deletion syndrome, a genetic mutation that causes

varied abnormalities, including increased risk of depression and schizophrenia, congenital heart disease, and cleft palate. In this population, patients were carefully monitored for psychotic and manic symptoms, but none were observed, even at 1600 mg/day, an incredibly high dose<sup>117</sup>. Additionally, many debilitating symptoms of Parkinson's disease are thought to be due to low dopamine; SAM supplementation could be a viable treatment for Parkinson's patients. It seems that SAM supplementation could be an effective therapy for those vulnerable to methylation-related neurologic disorders.

Noradrenergic signalling was impacted by 13CRA and SAM. Rats receiving 13CRA had significantly lower whole brain NET, but no change in whole brain norepinephrine. In contrast, SAM supplementation significantly increased norepinephrine relative to control, but did not impact NET. Low NET is associated with tachycardia and mania caused by lowered clearance of norepinephrine, which stimulates sympathetic effects, from the synaptic space. This effect, while rare, has been reported in humans taking 13CRA<sup>118,119</sup>. Rats receiving both SAM and 13CRA had normal whole brain NET, indicating that SAM supplementation may be a feasible therapy in conjunction with 13CRA in patients suspected to be at risk for atrial tachycardia. The significant increase in norepinephrine seen in all rats receiving SAM, without an increase in NET, indicates moderately higher synaptic norepinephrine. Clinically, this may manifest as increased focus and energy. Norepinephrine levels also provide more information regarding dopaminergic signalling, because norepinephrine is synthesized from dopamine in a SAM-dependent manner. Given the extremely elevated dopamine levels and only moderately elevated norepinephrine, it seems that the majority of excess dopamine is not being converted to norepinephrine.

The overall impact of retinoic acid and supplemental SAM on neural health is debatable. ATRA seemed to increase whole brain serotonin, lower DAT, and normal whole brain norepinephrine. As discussed, lower DAT is implicated in depression. However, increases in whole brain serotonin without a

concurrent increase in SERT parallel the physiologic outcome of antidepressant therapy. The effects of 13CRA are slightly more definitive. 13CRA similarly increased whole brain serotonin, but did not impact dopaminergic signalling. However, 13CRA seemed to lower whole brain NET, indicating that while the levels of whole brain norepinephrine were normal, the synaptic norepinephrine may have been abnormally high. Tachycardia in 13CRA patients could be caused in part by low NET. SAM supplementation appeared to attenuate whole brain serotonin and mildly increase both dopamine and norepinephrine in rats receiving either retinoic acid treatment. Changes in neurotransmitter transporters were also attenuated by SAM. This suggests that supplemental SAM may prevent or treat depressive symptoms associated with retinoic acid therapy.

However, SAM supplementation in healthy adults may not be advisable. Although SAM supplementation alone did not appear to appreciably affect serotonin and only caused a mild increase in whole brain epinephrine, the marked increase in dopamine is concerning. Extremely high dopamine is a chief biochemical cause of psychosis, and the unchanged level of DAT indicates that the autoregulatory mechanisms were completely overwhelmed<sup>84</sup>. While most SAM distributors advise against coupling supplemental SAM with a prescription antidepressant without the approval or monitoring of the prescribing physician, this is mostly due to the risk of serotonin syndrome, or a manic episode due to extreme elevation of serotonin. Little information on supplemental SAM and psychosis is available in the literature. Our results indicate that supplemental SAM may cause psychosis due to dopaminergic signalling. Taken together with the positive effects of SAM in retinoic acid therapy, our data suggests that supplemental SAM is inappropriate for healthy adults at the dosage level (10 mg/kg) in this study.

Overall, our study showed numerous undesirable effects of retinoic acid therapy, including depleted hepatic SAM, development of fatty liver, and some modulation of serotonin, norepinephrine, and dopamine. Supplemental SAM appeared to be an excellent conjunctive therapy, preventing or

partially preventing nearly all negative effects of retinoic acid. However, supplemental SAM at 10 mg/kg did not have a convincingly positive impact on hepatic or neural health when administered to healthy rats. In fact, the extreme increase in dopamine may be very harmful. The use of SAM in patients at risk for liver disease or mental disorders may be advantageous, given the low cost and relative safety of the supplement, and further research in this avenue should be conducted.

**Table 3.1:** Neither retinoic acid treatment nor SAM supplementation impacts markers of metabolic health.

	Total weight gain g	Relative liver size %	Relative kidney size %
Control	100.84±4.63	2.90±0.12	0.70±0.03
+SAM	119.55±5.54	3.09±0.15	0.67±0.01
ATRA	109.52±5.01	3.20±0.15	0.63±0.01
+SAM	110.11±6.58	3.10±0.08	0.74±0.03
13CRA	117.95±6.03	3.11±0.06	0.70±0.04
+SAM	111.82±6.25	3.19±0.08	0.66±0.02

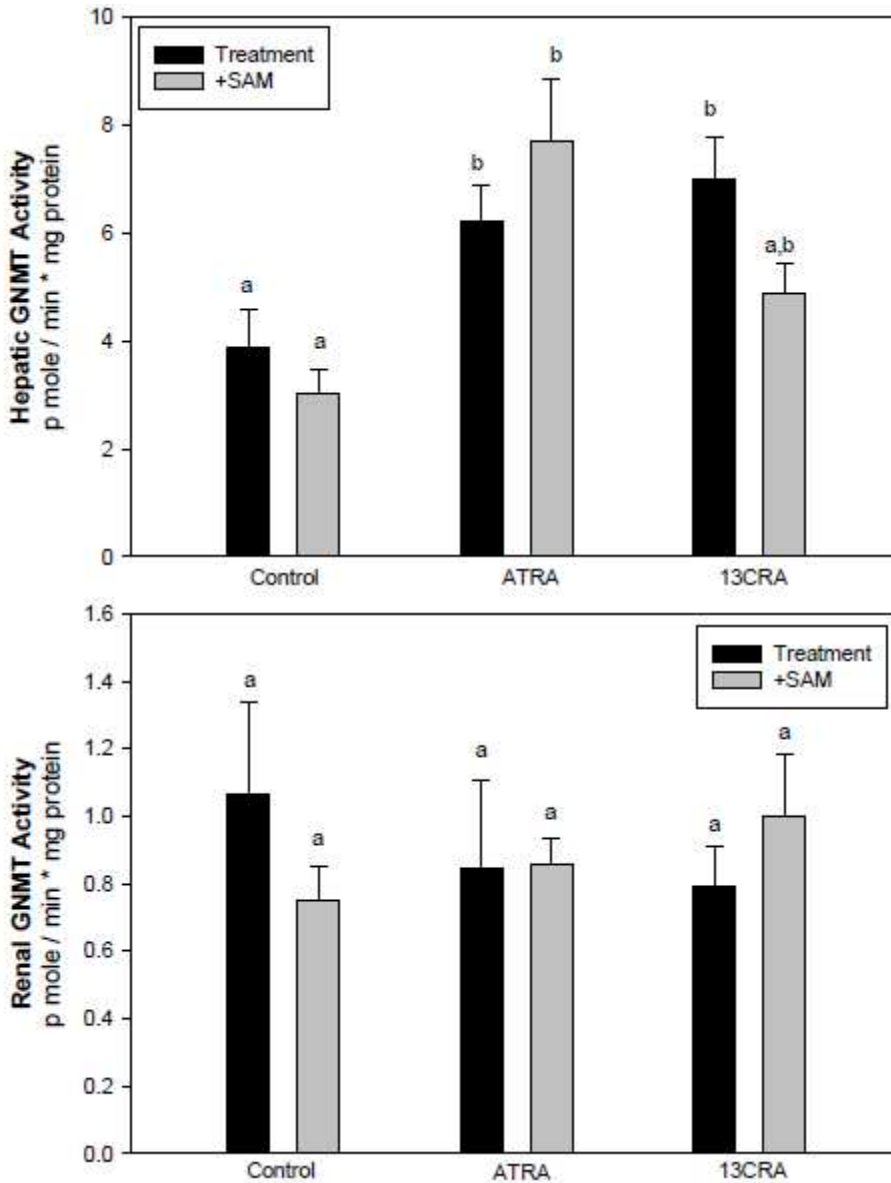
Values shown are group mean ±SE.

**Table 3.2:** Retinoic acid and SAM exert opposite effects on hepatic and neural SAM concentrations.

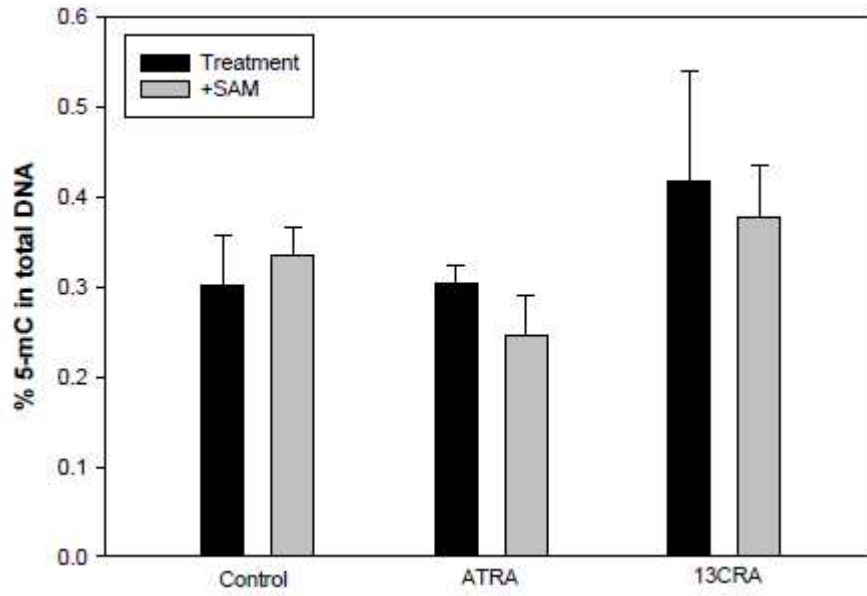
	Liver			Brain			Plasma
	SAM nmol/g	SAH nmol/g	SAM:SAH	SAM nmol/g	SAH nmol/g	SAM:SAH	Hcy μmol/L
Control	56.7±6.6 <sup>a</sup>	26.2±7.1 <sup>a</sup>	2.7±0.9	5.0±1.6	2.0±1.1 <sup>a</sup>	4.6±1.4	7.0±0.5
+SAM	100.6±12.1 <sup>b</sup>	34.5±9.6 <sup>a</sup>	3.9±0.8	18.5±3.6 <sup>*</sup>	6.8±1.5 <sup>b</sup>	2.8±0.3	6.0±0.6
ATRA	14.9±4.1 <sup>a,*</sup>	6.9±1.4 <sup>b</sup>	2.2±0.5	5.5±1.4	1.2±0.5 <sup>a</sup>	9.1±4.8	5.4±1.2
+SAM	83.2±17.5 <sup>a,b</sup>	22.8±10.9 <sup>a</sup>	5.4±1.6	15.3±7.8	6.4±3.1 <sup>b</sup>	3.1±1.3	6.9±0.5
13CRA	32.0±11.6 <sup>a</sup>	11.7±4.2 <sup>a</sup>	3.8±1.4	7.3±1.6	3.1±1.0 <sup>a</sup>	2.9±0.5	6.4±0.8
+SAM	80.8±15.0 <sup>a,b</sup>	21.2±6.3 <sup>a</sup>	5.6±1.4	9.9±3.2	5.7±3.0 <sup>b</sup>	8.2±4.1	6.2±0.8

Data expressed as group mean ±SE. Superscript letters indicate significant differences between group means as tested by two-way ANOVA followed by Fisher's LSD ( $p < 0.05$ ) while asterisks indicate significant differences to control using student's t-test.

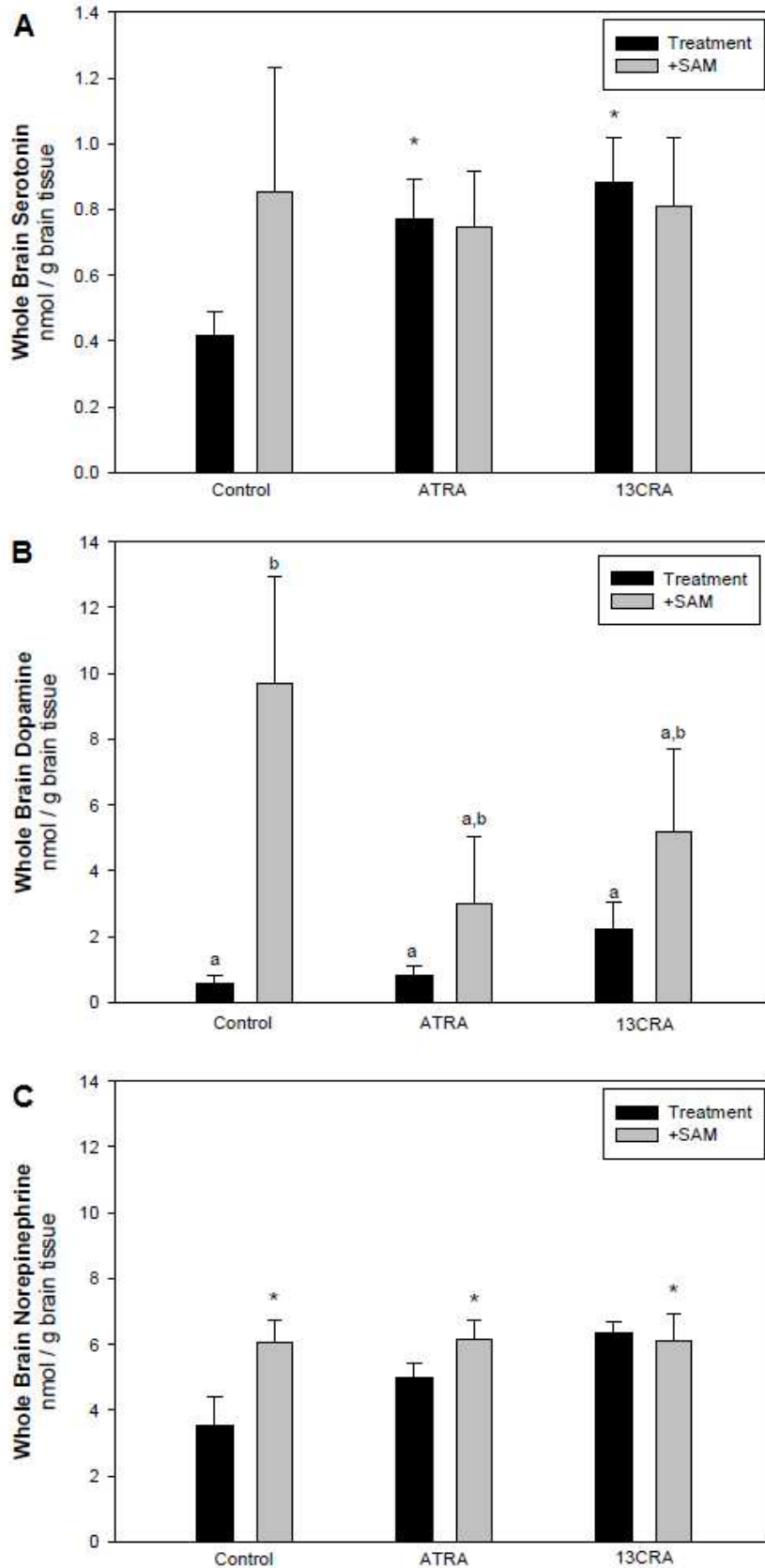




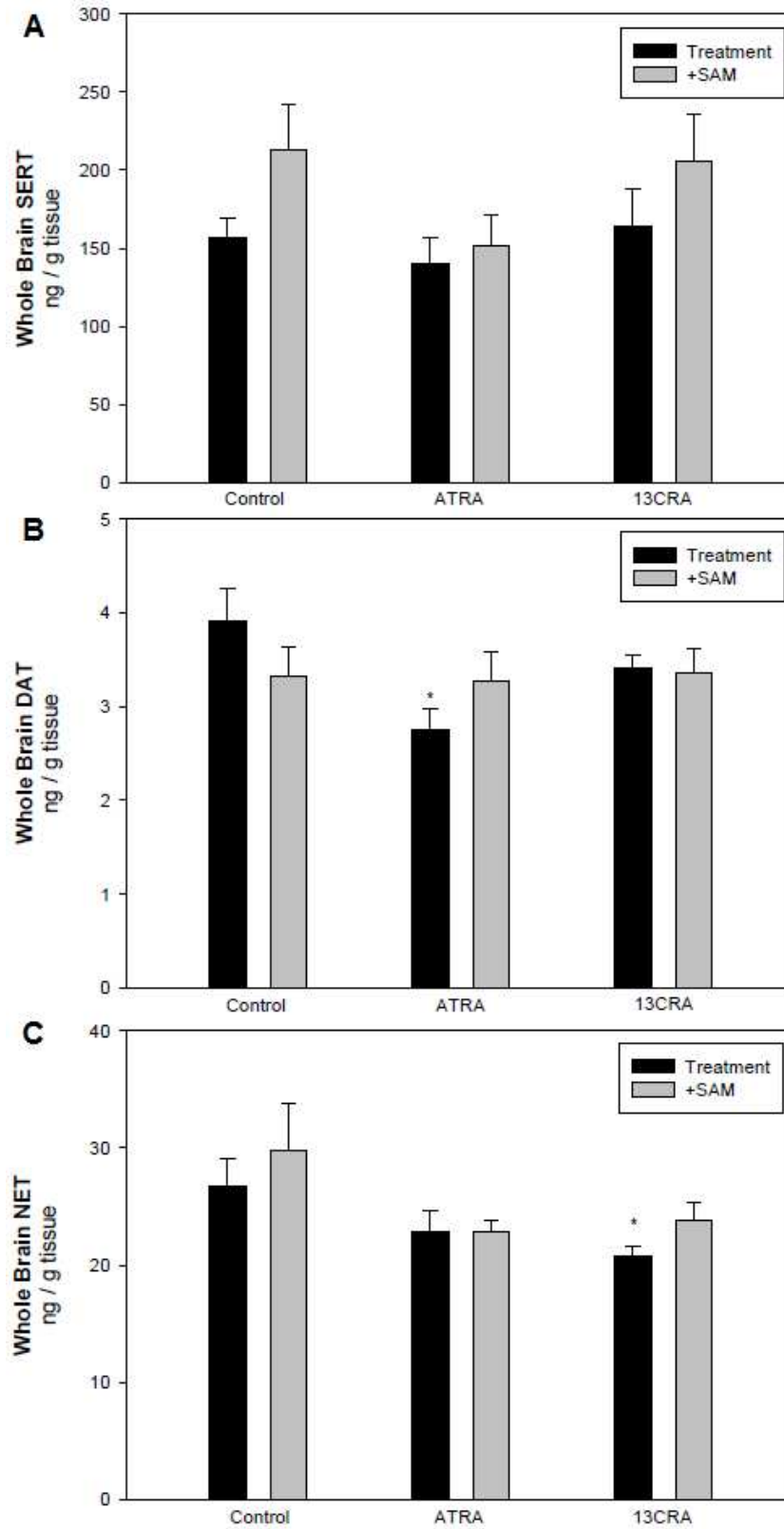
**Figure 3.1:** Retinoic acid treatment causes elevated hepatic, but not renal, GNMT activity. Supplemental SAM partially prevents increased GNMT activity due to 13CRA treatment. Values shown are group means  $\pm$ SE. Letters indicate significant differences between groups.



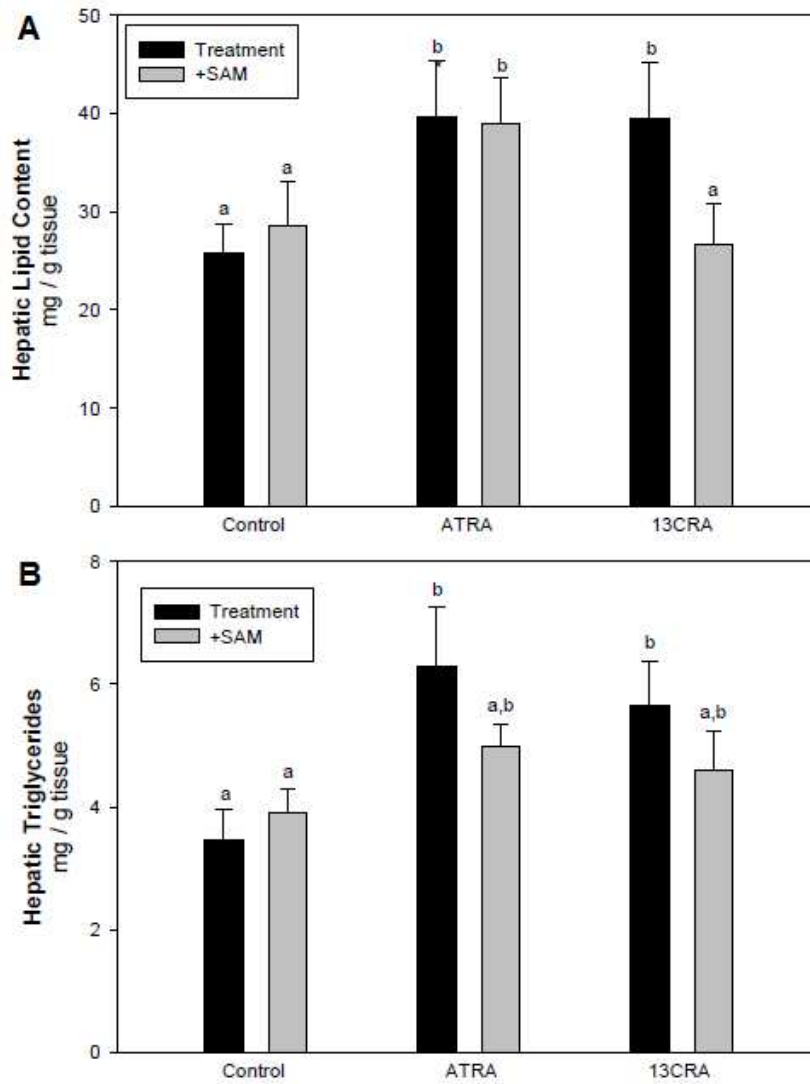
**Figure 3.2:** Neither retinoic acid treatment nor SAM supplementation significantly impacts global DNA methylation. Values shown are group means  $\pm$ SE.



**Figure 3.3:** Whole brain neurotransmitter concentrations for rats treated with retinoic acid and supplemental SAM. Values shown are group means  $\pm$ SE. Letters represent significant differences between group means ( $p < 0.05$ ). Asterisk indicates significant difference in group mean when compared to control using a student's t-test ( $p < 0.05$ ).



**Figure 3.4:** Whole brain neurotransmitter transporter concentrations for rats treated with retinoic acid and supplemental SAM. Values shown are group means  $\pm$ SE. Letters represent significant differences between group means ( $p < 0.05$ ). Asterisks indicate significant difference in group means when compared to control using a student's t-test ( $p < 0.05$ ).



**Figure 3.5:** Total liver lipids and triglycerides in rats treated with retinoic acid and supplemental SAM. Values shown are group means  $\pm$ SE. Letters represent significant differences between group means ( $p < 0.05$ ).

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