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Biosynthesis of fatty acid amides

Emma K. Farrell

University of South Florida

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Biosynthesis of Fatty Acid Amides

By

Emma K. Farrell

A dissertation submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

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N-acylamide, peptidylglycine α-amidating monooxygenase, lipidomics

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Dedication

This work is dedicated to my family
and the many who have contributed to it along the way.
Acknowledgements

No project of this size could be expected to be completed without the help of many others along the way. In roughly chronological order, I would like to thank these hard-working, careful scholars who have donated their time and expertise in the name of science. I would like to thank Ryan Aaron Matthews, who showed me how to run Western Blots. Terry Campbell, who showed me how to do RT-PCR. Jacob Shafer, who was an excellent labmate and whose ear I often had as well as his organic and computer expertise. Edward William Lowe, for helping with computer difficulties and being an excellent labmate. Dr. James R. Garey for many invaluable suggestions and endless patience. Sumit Handa, who took over ordering, provided SDS-PAGE gels, and was an excellent labmate. Zhenming An, who was an excellent labmate and fixed various things around the lab as needed. Tamanna Sultana, though we never spoke. Her extraction work was the foundation of much of mine. The tech support at Shimadzu for their hours of assistance. Yudan Chen, who was a wonderful technician and helped with cell and media extractions, solid phase extraction, cell culture, making fatty acid emulsions with BSA, organizing the lab, and various other things. Lamar Galloway, who synthesized N-acylglycines and did chromatography. Jian-Kang Chen whom I never met but sent us N_{18}TG_{2} cells when ours were depleted. Milena Ivkovic, who was an excellent labmate, did some of the RT-PCR work, and whose ear I also often had. Felipe Cameroamortegui, who helped with cell and media extractions and chromatography. Muna Barazanji, who helped with cell and media extractions, chromatography, cell culture, prep TLC development, and various other jobs, and was a pleasure to talk with. Kristen Amidei, who helped with cell and media extractions, solid phase extractions, cell culture, and whom I know has a bright future ahead of her in the laboratory. Mitchell Johnson for his correspondence about the N-acylglycines. My committee for inspiring me to do my best. And finally, my family for their continued support.
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List of Abbreviations:

2-AG 2-Arachidonoyl glycerol
5-HT 5-Hydroxytryptamine (Serotonin)
Abdh4 α,β-Hydrolase 4
ACBP Acyl-CoA binding protein
ACGNAT Acyl CoA:glycine N-acyltransferase
ACS Acyl-CoA synthetase
ACSL Long chain acyl-CoA synthetase
ASC Ascorbic acid
AT Amino transferase
BAAT Bile acid:amino acid transferase
BSA Bovine serum albumin
BSTFA N,O-Bis(trimethylsilyl)trifluoroacetamide
CB Cannabinoid receptor
CI Chemical ionization
CNS Central nervous system
COMT Catechol-O-methyltransferase
COX-2 Cyclooxygenase-2
CP Choroid plexus
CSF Cerebrospinal fluid
CYP45F Cytochrome P450
cyt c Cytochrome c
DBM Dopamine β-monoxygenase
DCM Dichloromethane
DMEM Dulbecco's Modified Eagle's Medium
DMSO Dimethylsulfoxide
DNA Deoxyribonucleic acid
dsRNA double stranded RNA
EA Elaidic acid
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene-bis(oxyethylenenitrito)tetraacetic acid
EI Electron impact
EMEM Eagle's minimum essential medium
erg Ether-à-go-go related gene
ESI Electrospray ionization
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerol-3-phosphate</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectroscopy</td>
</tr>
<tr>
<td>GP-NAE</td>
<td>Glycerophospho-N-acyl ethanolamine</td>
</tr>
<tr>
<td>GPR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HAEC</td>
<td>Human aortic endothelial cells</td>
</tr>
<tr>
<td>HdA</td>
<td>Heptadecanoic acid, D33 deuterated</td>
</tr>
<tr>
<td>HdG</td>
<td>N-Heptadecanoylglycine, deuterated with 33 deuteriums</td>
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<tr>
<td>HEK-293</td>
<td>Human embryonic epithelial kidney cells (ATCC# CRL-1573)</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPTLC</td>
<td>High performance thin layer chromatography</td>
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<td>Ion exchange</td>
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<td>Multiple ion chromatogram</td>
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<td>N-fatty acyldopamine</td>
</tr>
<tr>
<td>NAE</td>
<td>N-Acylethanolamine</td>
</tr>
<tr>
<td>NAG</td>
<td>N-Acylglycine</td>
</tr>
<tr>
<td>NAPE</td>
<td>N-acylphosphatidyl ethanolamine</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acylphosphatidyl ethanolamine-specific phospholipase D</td>
</tr>
<tr>
<td>NEG</td>
<td>N-Elaidoylglycine</td>
</tr>
<tr>
<td>NFDM</td>
<td>Nonfat dry milk</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NLG</td>
<td>N-linoleoylglycine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NMT</td>
<td>N-myristoyltransferase</td>
</tr>
<tr>
<td>NOE</td>
<td>N-oleoylethanolamine</td>
</tr>
<tr>
<td>NOG</td>
<td>N-oleoylglycine</td>
</tr>
<tr>
<td>NP</td>
<td>Normal phase</td>
</tr>
<tr>
<td>NPG</td>
<td>N-palmitoylglycine</td>
</tr>
<tr>
<td>NPOG</td>
<td>N-Palmitoleoylglycine</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl glycine alpha-amidating monooxygenase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDEase</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PFAM</td>
<td>Primary fatty acid amide</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>Pi</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>pNAE</td>
<td>phospho-N-acylethanolamine</td>
</tr>
<tr>
<td>POA</td>
<td>Palmitoleic acid</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>Ptase</td>
<td>phosphatase</td>
</tr>
<tr>
<td>rbf</td>
<td>Round-bottom flask</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SCP</td>
<td>Sheep choroid plexus cells</td>
</tr>
<tr>
<td>SDA</td>
<td>Semidehydroascorbic acid</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIM</td>
<td>Single ion monitoring</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>t-BDMS</td>
<td>tert-butyl dimethylsilyl ether</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline (25mM Tris, 140mM NaCl, 0.5% Tween-20)</td>
</tr>
<tr>
<td>TDA</td>
<td>Tridecanoic acid</td>
</tr>
</tbody>
</table>

xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDEA</td>
<td>N-Tridecanoylethanolamine</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential ion channel</td>
</tr>
<tr>
<td>TRPM</td>
<td>Transient receptor potential channels of melastatin</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid type 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Biosynthesis of Fatty Acid Amides

Emma K. Farrell

ABSTRACT

Primary fatty acid amides (PFAMs) and N-acylglycines (NAGs) are important signaling molecules in the mammalian nervous system, binding to many drug receptors and demonstrating control over sleep, locomotor activity, angiogenesis, vasodilatation, gap junction communication, and many other processes. Oleamide is the best-studied of the PFAMs, while the in vivo activity of the others is largely unstudied. Even less is known about the NAGs, as their discovery as novel compounds is much more recent due to low endogenous levels. Herein is described extraction and quantification techniques for PFAMs and NAGs in cultured cells and media using solvent extraction combined with solid phase extraction (PFAM) or thin layer chromatography (NAG), followed by gas chromatography-mass spectroscopy to isolate and quantify these lipid metabolites. The assays were used to examine the endogenous amounts of a panel of PFAMs as well as the conversion of corresponding free fatty acids (FFAs) to PFAMs over time in several cell lines. The cell lines demonstrated the ability to convert all FFAs, including a non-natural FFA, and an ethanolamine to the corresponding PFAM. Different patterns of relative amounts of endogenous and FFA-derived PFAMs were observed in the cell lines tested.

Essential to identifying therapeutic targets for the many disorders associated with PFAM signaling is understanding the mechanism(s) of PFAM and NAG biosynthesis. Enzyme expression studies were conducted to determine potential metabolic enzymes in the model cell lines in an attempt to understand the mechanism(s) of PFAM biosynthesis. It was found that two of the cell lines which show distinct metabolisms of PFAMs also demonstrate unique enzyme expression patterns, and candidate enzymes proposed to
perform PFAM and NAG metabolism are described. RNAi knockdown studies revealed further information about the metabolism of PFAMs and calls into question the recently proposed involvement of cytochrome c. Isotopic labeling studies showed there are two pathways for PFAM formation. A novel enzyme is likely to be involved in formation of NAGs from acyl-CoA intermediates.
1 Literature Review: Fatty Acid Amides – Biosynthesis, Degradation, and Pharmacological Significance

The identification of two biologically active fatty acid amides, \( N \)-arachidonoylethanolamine (anandamide) and oleamide, has generated a great deal of excitement and stimulated considerable research. However, anandamide and oleamide are merely the best-known and best-understood members of a much larger family of biologically-occurring fatty acid amides. In this review, the fatty acid amides that have been isolated from mammalian sources will be outlined, what is known about how these molecules are made and degraded \textit{in vivo} will be discussed, and their potential for the development of novel therapeutics will be highlighted. Relative to NAEs, much less is currently known about the NAAs, the NADAs and the PFAMs, except that they are found in biological systems (see refs. \textsuperscript{6,61,62} for earlier reviews).

The fatty acid amide bond has long been recognized in nature, being important in the structure of the ceramides\textsuperscript{65} and the sphingolipids\textsuperscript{71}. The first non-sphingosine based fatty acid amide isolated from a natural source was \( N \)-palmitoylethanolamine from egg yolk in 1957.\textsuperscript{90} Interest in the \( N \)-acylethanolamines (NAEs) dramatically increased upon

<table>
<thead>
<tr>
<th>Table 1-1: The Structures of the Fatty Acid Amides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acid Amide</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>NAE</td>
</tr>
<tr>
<td>NADA</td>
</tr>
<tr>
<td>NAA\textsuperscript{a}</td>
</tr>
<tr>
<td>PFAM</td>
</tr>
</tbody>
</table>

\textsuperscript{a}\text{R}_2 \text{ represents the functional groups that define the different amino acids. } \text{R}_1 \text{ represents various acyl chains (see Table 2).}
the identification of \(N\)-arachidonoylethanolamine (anandamide) as an endogenous ligand for the cannabinoid receptors in the mammalian brain.\(^4\) It is now known that a family of NAEs is found in the brain and in other tissues.\(^8,9^5\) In addition to the NAEs, other classes of fatty acid amides have been characterized, including the \(N\)-acylamino acids (NAAs),\(^9^6\) the \(N\)-acyldopamines (NADAs)\(^1^0^3\) and the primary fatty acid amides (PFAMs).\(^1^0^4,1^0^5\) Table 1-1 shows the structure of the amides, and Table 1-2 shows some of the commonly found acyl groups.

<table>
<thead>
<tr>
<th>Name</th>
<th>Carbon skeleton</th>
<th>Structure of (R_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic</td>
<td>20:4((\Delta^{5,8,11,14}))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Erucic</td>
<td>22:1((\Delta^{13}))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>16:1((\Delta^0))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Linoleic</td>
<td>18:2((\Delta^{9,12}))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Linolenic</td>
<td>18:3((\Delta^{9,12,15}))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Oleic</td>
<td>18:1((\Delta^0))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Elaidic(^a)</td>
<td>18:1(trans-(\Delta^0))</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Palmitic</td>
<td>16:0</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Stearic</td>
<td>18:0</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

The arrow points to carbon-2 in the fatty acid chain. \(R_1\) is the acyl group from Table 1-1.
1.1 N-Acylethanolamines

A series of long-chain NAEs have been identified in the mammalian brain, the most abundant being N-palmitoyl-, N-stearoyl- and N-oleoylthanolamine, each compromising ≥25% of total brain NAEs. Other less abundant NAEs found in the brain are anandamide, N-linoleoyl-, N-linolenoyl-, N-dihomo-γ-linolenoyl- and N-docosatetraenoylthanolamine. In addition to the brain, the NAEs are widespread in the peripheral tissues.

Anandamide is the best-understood NAE. The function of anandamide in mammals is mediated largely by its binding to the CB1 receptors (Kd = ~80 nM). Anandamide is also known to bind to CB2 receptors (Kd = ~500 nM), peroxisome proliferator-activated receptors (PPARα, Kd=20 µM and PPARγ, Kd=10 µM), to the transient receptor potential (TRP) vanilloid type 1 (TRPV1) channels (Kd ~ 2 µM), and the transient receptor potential channels of melastatin type 8 (TRPM8) (Kd ~ 1 µM). It is unclear how much the binding of anandamide to the non-CB1 receptors contributes to its total activity in vivo.

Anandamide is involved in the regulation of body temperature, locomotion, feeding and the perception of pain, anxiety and fear. The functions of the other known mammalian NAEs are not as well-established as anandamide, even though anandamide represents only 1-10% of brain NAEs. With the exception of N-dihomo-γ-linolenoyl- and N-docosatetraenoylthanolamine, the other NAEs do not bind to the CB1 and CB2 receptors. N-Oleoylthanolamine binds to PPARα and PPARβ, functioning to inhibit feeding behavior, as well as the TRPV1 receptor, and the G-protein-coupled receptor, GPR119. Stearoylthanolamine binds to specific, non-CB1 and CB2 receptors and yet exhibits activities similar to anandamide. N-Palmitoylthanolamine is neuroprotective and also modulates pain and inflammation. The anti-inflammatory effect of N-palmitoylethanolamine is mediated by its binding to PPARα. Ryberg et al. recently found that N-palmitoylethanolamine is a ligand for the orphan GPR55 receptor. It has been suggested that at least some of the activities of N-palmitoylethanolamine, N-oleoylthanolamine and N-stearoylthanolamine result from
the “entourage effect”: cellular levels of anandamide are stabilized or increased because the other NAEs compete with anandamide for enzymatic degradation.125

The most widely-accepted biosynthetic pathway for NAEs involves the cleavage of $N$-acylphosphatidylethanolamine (NAPE) to the corresponding NAE and phosphatidic acid (PA) by NAPE-specific phospholipase D (NAPE-PLD) (reaction 1 in Figure 1-1).130,131 NAPE is produced by the $N$-acylation of phosphatidylethanolamine in a reaction catalyzed by a calcium-activated transacylase (Figure 1-2).130 Recent evidence suggests that there are other PLD-independent pathways for NAE biosynthesis.10,67 One alternative pathway involves the phospholipase C-mediated cleavage of NAPE to yield a phospho-NAE (pNAE) which is then cleaved by a phosphatase to yield the NAE and inorganic phosphate (reactions 6 and 7 in Figure 1-1). Another alternative pathway involves sequential hydrolysis of the $O$-acyl chains of NAPE to produce free fatty acids and glycerophosphono-NAE (GP-NAE) (reactions 2 and 4 in Figure 1-1). Simon and Cravatt10 have found that a serine hydrolase, $\alpha/\beta$-hydrolase 4 (Abh4), can catalyze both $O$-deacylation steps required to convert NAPE to GP-NAE. Phosphodiesterase cleavage of GP-NAE will yield the NAE and glycerol 3-phosphate (reaction 5 in Figure 1-1). Other possible routes to the NAEs are direct hydrolysis of lysoNAPE (reaction 3 in Figure 1-1) or the 2-step conversion of GP-NAE to the NAE via phospho-NAE (reactions 8 and 7 in Figure 1-1). The PLD-independent pathways for NAE biosynthesis are exciting discoveries, suggesting that the body has redundant “back-up” methods to produce these important bioactive lipid amides that are made “on demand.”62,67 Future work will determine how these three pathways function to supply the required NAE levels.

For completeness, one last NAE synthetic strategy must be mentioned. There is data going back more than 40 years, showing that the NAEs can be produced in vitro from ethanolamine and free fatty acids (FFAs) in brain microsomes to form NAPEs, in a reaction that did not require ATP or CoA-SH.132 The authors hypothesize that the FFAs are taken into proteins and then exchanged to acceptor molecules (ethanolamines or phosphatidylethanolamines, in this case). However, in vivo significance of this chemistry is unclear.
Figure 1-1: Biosynthetic Pathways for N-Acylethanolamines

The enzymes catalyzing the individual reactions are in the shaded boxes and the numbers that refer to reactions in the text are in bold blue. The reader is referred to Simon and Cravatt\(^\text{10}\) and Liu et al.\(^\text{67}\) for greater details on NAE biosynthesis. Abdh4, \(\alpha,\beta\)-hydrolase 4; G3P, glycerol 3-phosphate; GP-NAE, glycerophospho-NAE; LPA, lysophosphatic acid; LysoNAPE, lyso N-acylphosphatidylethanolamine; LysoPLC/D, lysophospholipase C/D; NAE, N-acylethanolamine; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, NAPE-specific phospholipase D; PA, phosphatidic acid; PDEase, phosphodiesterase; P\(_i\), phosphate; PLA\(_2\), phospholipase A2; PLC, phospholipase C; pNAE, phospho-NAE; PTase, phosphatase (most probably tyrosine phosphatase, PTPN22 or inositol-5\(^\prime\)-phosphatase, SHIP1, in vivo).
NAE degradation is by hydrolysis to fatty acid and ethanolamine (see Equation 1-1). Three enzymes are known to catalyze this reaction: two fatty acid amide hydrolases (FAAH-1 and FAAH-2) and N-acylthanolamine-hydrolyzing acid amidase (NAAA). FAAH-1 and FAAH-2 both hydrolyze NAEs, but have different acyl group specificities (see Chapter 4 for more information). Note that FAAH inhibitors have been targeted as potential analgesics.

Figure 1-2: Biosynthesis of N-Acylphosphatidylethanolamine
Biosynthesis of N-acylphosphatidylethanolamine (NAPE). See text for more details.
1.2 N-Acylldopamines

A relatively small number of long-chain N-fatty acyldopamines (NADAs) have been isolated and characterized from mammalian systems, including N-palmitoyl-, N-stearoyl-, N-oleoyl- and N-arachidonoyldopamine.\(^1,63\) All of these NADAs are found in the mammalian brain, with the highest concentrations in the striatum, hippocampus and cerebellum.\(^1\)

N-Arachidonoyldopamine and N-oleoyldopamine were first identified as capsaicin-like endovanilloids that bound tightly to the TRPV1 receptor.\(^1,6,63\) As a consequence of their binding to the TRPV1 receptors, both of these N-fatty acyldopamines stimulated calcium influx in HEK-293 cells over-expressing either rat or human TRPV1 and produced hyperalgesia in rats.\(^1,63\) N-Arachidonoyldopamine also binds tightly to the CB\(_1\) receptor (K\(_d\) = 250-500 nM)\(^63,138\) and a non-CB\(_1\)/CB\(_2\) G-protein receptor (GPR) in the aorta.\(^64\) Other endogenous N-fatty acyldopamines include N-palmitoyldopamine and N-stearoyldopamine, both of which bind with to the TRPV1 or CB\(_1\) receptors with relatively low affinity (K\(_d\) values >5 \(\mu\)M).\(^63\) The biological role(s) fulfilled by N-palmitoyldopamine and N-stearoyldopamine are unclear, but there is evidence that both enhance the activity of N-arachidonoyldopamine via the entourage effect.\(^139\)

In addition to the long-chain N-fatty acyldopamines, N-acetyldopamine is a known metabolite in mammals. The function of N-acetyldopamine is unclear, but it has been shown to inhibit mammalian sepiapterin reductase (an enzyme in the tetrahydrobiopterin biosynthetic pathway) with a K\(_i\) = 400 nM.\(^140\)

There has been little work on the pathways for the biosynthesis and degradation of the N-acyldopamines. N-Acetyltdopamine is produced by the acetyl-CoA-dependent N-acetylation of dopamine\(^141\) and has been found in the urine, kidney and liver.\(^141,142\) It has
been proposed that the long-chain $N$-acyldopamines are made in vivo in a similar fashion, with the acyl donors being the corresponding acyl-CoA thioesters.\textsuperscript{1} Alternatively, the $N$-acyldopamines could be produced by the tyrosine hydroxylase-mediated oxidation of $N$-acyltyrosines, metabolites that were identified in mammals just this year (see Table 1-4). Huang et al.\textsuperscript{1} provide data in support of both biosynthetic pathways, as inhibition of tyrosine $\beta$-hydroxylase reduced formation of a NADA-like compound from tyrosine. Skimob et al. also show the increased formation of $N$-arachidonoyldopamine during the incubation of arachidonic acid and tyrosine as compared to the incubation of arachidonic acid and dopamine.\textsuperscript{143} Evidence suggests a decarboxylation preceding oxidation (Figure 1-3).

Degradation of the NADAs is thought to occur by FAAH-catalyzed hydrolysis to the fatty acid and dopamine\textsuperscript{1} or $O$-methylation by catechol-$O$-methyltransferase (COMT).\textsuperscript{1} $N$-Acetyldopamine can serve as a substrate for tyrosinase; thus, the long-chain $N$-acyldopamines could also be oxidized to a quinone by this enzyme.\textsuperscript{32} $N$-Acetylnoradrenaline is a known human metabolite\textsuperscript{66} suggesting that $N$-acetyldopamine and the longer-chain $N$-acyldopamines could serve as substrates for dopamine $\beta$-monooxygenase (DBM) (Table 1-3).
1.3 N-Acylamino acids

Mammalian N-acylamino acids have a long history, tracing their discovery to the conjugation of glycine to benzoate to form N-benzyglycine (hippurate) in the 1840s (see Caldwell et al. and reference cited therein). N\(^{\alpha}\)-Acetyl conjugates for all 20 of the common amino acids have been identified in mammals. In addition, the N\(^{\alpha}\)-acetyl conjugates of other amino acids, including β-alanine, allo-isoleucine, α-aminobutyric acid, GABA, 2-aminooctanoic acid, citrulline and N\(^{\varepsilon}\)-acetyllysine have also been characterized from mammalian sources. With the exception of N-acetylglutamate, which serves as an allosteric activator of carbamoyl phosphate synthetase I, the N-acylamino acid conjugates are trace metabolites that function in the excretion/detoxification of abnormally high levels of a particular amino acid. Similarly, a set of N-isovaleroylamino acids have been identified from patients suffering from isovaleric academia, with N-isovaleroylglycine being the most abundant metabolite. The function of these N-isovaleroylamino acids is also in excretion; one patient suffering from isovaleric academia was excreting 1.7 grams of N-isovaleroylglycine per day.

Glycination is essential in the synthesis of bile acids and metabolism of short chain fatty acids and small molecule xenobiotics. N-Conjugation of fatty acids to amino acids forming the long-chain N-fatty acylglycines is known, but is relatively uncommon in mammals, and a comprehensive listing of the known long-chain NAGs isolated from mammalian sources can be found in Chapter 4. The most common

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAAH</td>
<td>NADA + H(_2)O → FFA + dopamine</td>
<td>1</td>
</tr>
<tr>
<td>COMT</td>
<td>NADA + S-adenosyl-L-methionine → S-adenosyl-L-homocysteine + N-acyl-3-methoxytyramine</td>
<td>1</td>
</tr>
<tr>
<td>tyrosinase</td>
<td>NADA + (\frac{1}{2} O_2 \rightarrow H_2O + N)-acyl dopamine quinone</td>
<td>32</td>
</tr>
<tr>
<td>DBM</td>
<td>NADA + ascorbate + O(_2) → dehydroascorbate + H(_2)O + N-acylnoradrenaline</td>
<td>66</td>
</tr>
</tbody>
</table>

Abbreviations: COMT, catechol-\(O\)-methyltransferase; DBM, dopamine β-monooxygenase; FAAH, fatty acid amide hydrolase; FFA, free fatty acid; NADA, \(N\)-acyldopamine
mammalian \( N \)-fatty acylamino acids are conjugates of glycine, glutamine and taurine (Table 1-4). Many of the \( N \)-acylamino acids were discovered in the last 1-3 years, thanks to improvements in separation techniques and more sensitive mass spectrometry equipment. (See Chapter 2 for a discussion of these innovations and more details on metabolite analysis.)

Like the shorter chain \( N \)-acetyl and \( N \)-isovaleroyl amino acids, the major function of these longer chain amino acid conjugates appears to be in the detoxification and excretion of xenobiotic carboxylates.\(^9^6\) Glycine conjugation is particularly important in detoxification and elimination, as a careful analysis of the metabolism of most xenobiotic carboxylates reveals at least a trace of the corresponding \( N \)-acylglycine conjugate.\(^1^6^1\) In fact, the list of \( N \)-acylglycines shown in Table 1-4 is incomplete as glycine conjugates of many other carboxylates also have been reported.\(^1^5^9^,1^6^1\)
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>N-Acyl Group</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Arachidonoyl, Oleoyl, Palmitoyl, Stearoyl</td>
<td>2,3</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>Arachidonoyl, Docosahexaenoyl, Oleoyl, Palmitoyl, Stearoyl</td>
<td>2,3</td>
</tr>
<tr>
<td>Arginine</td>
<td>Stearoyl</td>
<td>3</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Oleoyl</td>
<td>3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>Arachidonoyl, Docosahexaenoyl, Oleoyl, Palmitoyl, Stearoyl, β-Citryl, Phenylacetyl</td>
<td>5,91,93</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Arachidonoyl, Docosahexaenoyl, Oleoyl, Palmitoyl, Phenylacetyl and other arylacetlys, 4-Phenylbutyryl, Stearoyl</td>
<td>3,91,93</td>
</tr>
<tr>
<td>Glycine</td>
<td>Arachidonoyl, Benzoyl, Butyryl, Bile acids, Decanoyl, Hexanoyl, Isobutyryl, 2-Methylbutyryl, 3-Methylcrotonyl, Octanoyl, Phenylacetyl and other arylacetlys, Palmitoyl, Propionyl, Suberyl, Tiglyl</td>
<td>2,7,93,96,101</td>
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<td>Histidine</td>
<td>Arachidonoyl, Docosahexaenoyl, Oleoyl, Palmitoyl</td>
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<td>(Iso)leucine</td>
<td>Arachidonoyl, Lactyl, Oleoyl, Palmitoyl</td>
<td>3,106</td>
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<td>Leucine</td>
<td>Lactyl</td>
<td>106</td>
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<tr>
<td>Methionine</td>
<td>Oleoyl, Palmitoyl, Stearoyl</td>
<td>3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Docosahexaenoyl, Oleoyl, Palmitoyl, Stearoyl, Succinoyl</td>
<td>3,112</td>
</tr>
<tr>
<td>Proline</td>
<td>Oleoyl, Palmitoyl, Stearoyl</td>
<td>3</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>Phenylacetyl</td>
<td>98</td>
</tr>
<tr>
<td>Serine</td>
<td>Arachidonoyl, Palmitoyl, Stearoyl</td>
<td>3,120</td>
</tr>
<tr>
<td>Taurine</td>
<td>Bile acids, Phenylacetyl and other arylacetlys, long chain, saturated acyl groups from C16:0-C26:0, long-chain, monounsaturated acyl groups from C18:1-C24:1, Linoleoyl</td>
<td>3,96,99,121</td>
</tr>
<tr>
<td>Threonine</td>
<td>Oleoyl, Palmitoyl</td>
<td>3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Oleoyl, Palmitoyl, Stearoyl</td>
<td>3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Oleoyl, Palmitoyl, Stearoyl</td>
<td>3</td>
</tr>
<tr>
<td>Valine</td>
<td>Lactyl, Linoleoyl, Palmitoyl, Stearoyl</td>
<td>106</td>
</tr>
</tbody>
</table>

^aN-Acetyl and N-isovaleroylamino acids were not included in this table.  
^bAmino acids not commonly found in proteins are italicized.  
^cIncluded here most of the more common N-acylglycine conjugates known. Many others have been identified as metabolites in various organic acid acidemias or in the detoxification of a xenobiotic carboxylate.  
^dIncluded in the family of long-chain fatty acyl groups found N-conjugated to taurine were odd-numbered acyl chains including C21:0, C21:1, C23:0, C23:1, C25:0, and C25:1. N-Tricosanoyltaurine was found to be one of the more abundant N-acyltaurines in mouse brain.  

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**Table 1-4: Mammalian N-Fatty Acylamino Acids**

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11
Amino acid N-fatty acyl conjugation may function primarily in excretion/detoxification; however, this chemistry does serve other roles in mammals. Bile acid conjugation to glycine or taurine increases bile acid solubility, renders the bile acids impermeable to cell membranes and is essential to proper liver function. In addition, β-citrylglutamate may have a role in spermatogenesis and in the differentiation of lens epithelial cells into fiber cells.

Most intriguing are the emerging roles of the long-chain N-fatty acylamino acids. Milman et al. isolated and characterized N-arachidonoyl-L-serine from bovine brain and showed that this novel N-fatty acylserine had vasodilatory properties. The Merkler lab has proposed that the N-fatty acylglycines are biosynthetic precursors to the PFAMs, being oxidatively cleaved to the corresponding PFAM and glyoxylate in a reaction catalyzed by peptidylglycine α-amidating monooxygenase (PAM). Recent evidence suggests that the N-fatty acylglycines may serve as more than simple PFAM pathway intermediates and may have independent functions as lipid messengers: N-oleoylglycine regulates body temperature and locomotion. N-arachidonoyltaurine activates TRPV1 and TRPV4 calcium channels of the kidney, N-arachidonoylglycine is an endogenous ligand for the orphan GPR18 receptor, and N-palmitoylglycine inhibits nociception and induces transient calcium influx. Similar to prostaglandins and endocannabinoids, the actions of fatty acyl glycines are likely to be mediated through G-protein-coupled receptors, as has been recently reported for N-arachidonoylglycine. For a more thorough list of endogenous long-chain mammalian signaling NAGs and their known function, see Chapter 4.

N-Arachidonoyl-γ-aminobutyric acid is analgesic, and N-arachidonoylglycine is analgesic, and inhibits FAAH and the GLYT2a glycine transporter. The function(s) served by N-arachidonoyllalanine is currently not understood. Another set of N-acyl amino acid conjugates that warrant some discussion are related to the conjugation of fatty acids to either the α-amino group of an N-terminal glycine residue or to the ε-amino group of internal lysine residue. The most common N-terminal acyl group found in eukaryotes is myristic acid, but other fatty acids, including lauric, (cis-Δ⁵)-tetradecaenoic (physeteric), (cis,cis-Δ⁵,Δ⁸)-tetradecadienic, and palmitic acids, have been identified as N-terminal fatty acids. Mammalian proteins decorated via an amide linkage
between the ε-amino group of an internal lysine and myristic acid\textsuperscript{170} or palmitic acid\textsuperscript{171} have been identified. Proteolytic degradation of N-terminal or ε-acyllysyl lipidated proteins could release the corresponding N-acylglycine or N\textsuperscript{ε}-acyllysine, but there are no reports showing that such metabolites have been detected in mammals.

One biosynthetic route to the N-acylamino acids utilizes an acyl-CoA thioester as the acyl group donor (Table 1-5). Enzymes known to catalyze this reaction include N-acetylglutamate synthase (a.k.a. amino acid N-acetyltransferase),\textsuperscript{68} bile acid coenzyme A:amino acid N-acyltransferase (BAAT),\textsuperscript{99} acyl-CoA:glycine N-acyltransferase (ACGNAT),\textsuperscript{107} a peroxisomal acyl-CoA:amino acid N-acyltransferase (ACNAT1),\textsuperscript{108} and acyl-CoA:L-glutamine N-acyltransferase.\textsuperscript{111} These enzymatic reactions are summarized in Table 1-5. N-Terminal acylation is catalyzed by N-myristoyl transferase (NMT), an enzyme which strongly prefers myristoyl-CoA as a substrate, and only transfers the acyl group to the α-amino moiety of an N-terminal glycine. Glycine and the α-amino moiety of other N-terminal amino acids are not NMT substrates.\textsuperscript{113} Evidence suggests that myristoyl-CoA or palmitoyl-CoA are also the acyl donors for the acylation of ε-amino group of internal lysine residues.\textsuperscript{169}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Product</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textbf{Acyl-CoA + amino acid} \rightarrow N-acylamino acid + CoA-SH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylglutamate synthase</td>
<td>N-Acetylglutamate</td>
<td>68</td>
</tr>
<tr>
<td>Bile acid coenzyme A:amino acid N-acyltransferase</td>
<td>Bile acyl-glycine and -taurine</td>
<td>99</td>
</tr>
<tr>
<td>Acyl-CoA:glycine N-acyltransferase</td>
<td>Short- and branched-chain N-acylglycines</td>
<td>107</td>
</tr>
<tr>
<td>Peroxisomal acyl-CoA:amino acid N-acyltransferase</td>
<td>N-Acyltaurines</td>
<td>108</td>
</tr>
<tr>
<td>Acyl-CoA:L-glutamine N-acyltransferase</td>
<td>N-Acylglutamines</td>
<td>111</td>
</tr>
<tr>
<td>N-Myristoyltransferase</td>
<td>N-Myristoylated proteins</td>
<td>113</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>Long chain N-acylamino acids</td>
<td>114-116</td>
</tr>
<tr>
<td>\textbf{NAE + 2NAD\textsuperscript{+} \rightarrow N-acylamino acid + 2NADH + 2H\textsuperscript{+}}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol/aldehyde dehydrogenase</td>
<td>Short- and medium-chain N-acylglycines</td>
<td>110,117-119</td>
</tr>
</tbody>
</table>
The data regarding the biosynthesis of the long-chain N-fatty acylglycines is not clear. *N*-conjugation of fatty acids to glycine via a fatty acyl-CoA thioester is an attractive possibility. The available evidence strongly suggests that ACGNAT does not catalyze this reaction *in vivo*: long-chain acyl-CoA thioesters are not ACGNAT substrates, and ACGNAT is found primarily in the liver and kidney while the PFAMs have been isolated from the brain. ACGNAT is not likely involved in the biosynthesis of other N-fatty acylamino acids, as amino acids other than glycine are very poor ACGNAT substrates. Other possible candidates that might catalyze this reaction *in vivo* include BAAT, which will produce *N*-fatty acylglycines at a low rate relative to the bile acid conjugates, or cytochrome c. The recent report that cytochrome c can catalyze the formation of *N*-oleoylglycine and *N*-arachidonoyl -glycine, -serine, -alanine and –γ-aminobutyric acid from the corresponding CoA thioester in a reaction stimulated by H\textsubscript{2}O\textsubscript{2} is very intriguing, but the *in vitro* significance is unclear. There is also evidence for lcNAG formation via the NAD\textsuperscript{+}-dependent oxidation of the NAEs by the sequential actions of a fatty alcohol dehydrogenase (ADH) or an ADH followed by a fatty aldehyde dehydrogenase (AIDH) (Table 1-5, bottom). Deuterium-labeled *N*-arachidonoylethanolamine (D\textsubscript{4} on the ethanolamine) incubated in RAW264.7 and C6 glioma cells is converted to D\textsubscript{2}-labeled *N*-arachidonoylglycine (see Chapter 4 for more in-depth discussion).

The catabolic fates of the *N*-acylamino acids are not well-defined. FAAH will hydrolyze the *N*-acyltaurines and *N*-arachidonoylglycine to the corresponding fatty acid and amino acid, but the other *N*-acylamino acids are not degraded by FAAH. *N*-palmitoylglycine is up-regulated in FAAH knockout mice, and increased levels of *N*-oleoylglycine and *N*-stearoylglycine were observed after injection with a FAAH inhibitor, but *N*-docosahexaenoyle glycine and *N*-linoleoylglycine were not increased under these conditions. The Merkler lab has shown that *N*-acylglycines are biosynthetic precursors to the PFAMs using purified PAM and in PAM-expressing neuroblastoma N\textsubscript{18}TG\textsubscript{2} cells. Marnett and co-workers have found that the *N*-arachidonoylamino acids are substrates for lipoxygenase (LO) and cyclooxygenase-2 (COX-2) *in vitro*, pointing either to a mechanism for the inactivation of the *N*-arachidonoylamino acids or for the formation of other bioactive, oxidized amino acid conjugates. Some NAAs,
including long chain NAGs, have also been shown to be substrates for cytochrome P450BM-3, a bacterial monooxygenase similar to eukaryotic P450s, resulting in production of ω-1, ω-2, and ω-3 hydroxylated NAAs.\textsuperscript{180,181} Much work is needed to better define the pathways of biosynthesis and degradation for the N-acylamino acids.

### 1.4 Primary Fatty Acid Amides

Arafat \textit{et al.}\textsuperscript{104} first isolated and characterized five PFAMs (palmitamide, palmitoleamide, oleamide, elaidamide and linoleamide) from luteal phase plasma in 1989. Because the function of the PFAMs was initially unknown, interest in these molecules was modest until Cravatt \textit{et al.}\textsuperscript{105} isolated oleamide and erucamide from the cerebrospinal fluid (CSF) of cat, rat and human and further demonstrated that the intraperitoneal injection of nanomole quantities of oleamide induced physiological sleep in rats. Research concerning oleamide has progressed rapidly since this first report and, in addition to its role in regulating the sleep/wake cycle, this PFAM has been shown to block gap junction communication in glial cells and between dendritic and T-cells, to regulate memory processes, to decrease body temperature and locomotor activity, to stimulate Ca\textsuperscript{2+} release, to modulate depressant drug receptors in the CNS, and to allosterically activate the GABA\textsubscript{A} receptors and specific serotonin receptor subtypes (see refs.\textsuperscript{87,182,183} and Chapter 3 for reviews). The mode of action of the hypothermic response may be by modulation of the 5-HT receptors since they modulate thermoregulation in the hypothalamus and GABA\textsubscript{A} receptors, which also regulate thermal control in the hypothalamus. GABA\textsubscript{A} knockout mice are no longer induced to sleep but retain hypothermia after oleamide injection.\textsuperscript{85} Thus, oleamide produces these activities through separate systems.

Like oleamide, other members of the PFAM are bioactive: linoleamide increases Ca\textsuperscript{2+} flux\textsuperscript{184} and inhibits the \textit{erg} (ether-à-go-go related gene) current in pituitary cells,\textsuperscript{185} erucamide stimulates angiogenesis\textsuperscript{186} and regulates fluid imbalance,\textsuperscript{187} and elaidamide may function as an endogenous inhibitor of epoxide hydrolase.\textsuperscript{188} For a more thorough and detailed description of the functions of endogenous PFAMs, see Chapter 3.
The PFAMs are degraded by fatty acid amide hydrolase, being hydrolyzed to the fatty acid and ammonia (Equation 1-2).\textsuperscript{8,167} One of the key unanswered questions regarding the PFAMs is how these novel brain lipid amides are produced in the body. A number of reactions have been proposed to account for PFAM production. Sugiura \textit{et al.}\textsuperscript{189} found that FAAH catalyzed the \textit{in vitro} production of oleamide from oleic acid and NH\textsubscript{3} (Equation 1-2 backwards). This reaction is unlikely to occur \textit{in vivo} because the K\textsubscript{M} for ammonia was high (65 mM), and the pH optimum for oleamide synthesis was > 9. Mouse neuroblastoma N\textsubscript{18}TG\textsubscript{2} cells secrete [\textsuperscript{1-14}C]-oleamide when cultured in the presence of [\textsuperscript{1-14}C]-oleic acid.\textsuperscript{177,190} Thus, these cells must contain the enzymatic machinery required for oleamide biosynthesis. Oleamide production in the N\textsubscript{18}TG\textsubscript{2} cells increases upon the inhibition of FAAH, providing further evidence against a role for this enzyme in PFAM production \textit{in vivo}. Bisogno \textit{et al.}\textsuperscript{190} proposed that PFAMs were produced by phospholipid aminolysis. However, incubation of [\textsuperscript{14}C]-oleic acid-containing phospholipids with NH\textsubscript{4}OH in the presence of N\textsubscript{18}TG\textsubscript{2} cell homogenates did not result in the formation of [\textsuperscript{14}C]-oleamide.
Currently, there are two proposed pathways for the biosynthesis of the long chain PFAMs that have some experimental support (Figure 1-4). One is the direct amidation of fatty acyl-CoA thioesters by ammonia as catalyzed by cytochrome c.\textsuperscript{191,192} The PFAM-synthesizing activity of cyt c yields a number of PFAMs, exhibits Michealis-Menten kinetics with a $K_M$ value for oleoyl-CoA of 21 $\mu$M and a pH optimum of 7.5, and is stimulated by H$_2$O$_2$. The optimal concentrations for H$_2$O$_2$ (2 mM) and NH$_3$ (125 mM) have led to the proposal of an “oleamide synthesome” in which several proteins are aggregated to provide the necessary ingredients for oleamide synthesis\textsuperscript{192} (see Chapter 4 for more details).

A second proposed pathway for PFAM biosynthesis involves the PAM-mediated cleavage of N-fatty acylglycines.\textsuperscript{165,178} The Merkler laboratory has shown that PAM is expressed in the oleamide-synthesizing N$_{18}$TG$_2$ cells and further demonstrated that pharmacological inhibition of PAM in N$_{18}$TG$_2$ cells results in the accumulation of N-oleoylglycine.\textsuperscript{177,193} A melding of the two proposed pathways could also lead to PFAMs:
first the cytochrome c-mediated production of the \(N\)-fatty acylglycine (Figure 1-4 cyt c reaction with glycine instead of ammonia) followed by PAM oxidation to the corresponding PFAM. As discussed by Mueller and Driscoll,\textsuperscript{174} there may be more than one pathway for the \textit{in vivo} production of the PFAMs, consistent with the fact that there are a number of pathways known for the \textit{in vivo} production of the NAEs (Figure 1-1). These are the most likely routes for PFAM biosynthesis, but a more detailed analysis can be found in Chapter 4. Outlined below in Figure 1-5 are potential pathways for the biosynthesis of the PFAMs that metabolically link together the PFAMs to the \(N\)-fatty acylglycines and the NAEs. The potential conversion of one class of fatty acid amide to another only adds another fascinating dimension to this family of bioactive compounds.
Pharmacological Importance of the Fatty Acid Amides

Because of the broad functions exhibited by the various members of the fatty acid amide family, a wide range of indications could benefit from a fatty acid amide-targeted approach.
drug, including cancer, cardiovascular disease, inflammation, pain, drug addiction, eating disorders, anxiety, ganglial motor disorders, and depression (see refs. 6,24,62,194,195 for recent reviews). Potential drug targets include the enzymes involved in fatty acid amide biosynthesis and degradation,196,197 transporters responsible for moving the fatty acid amides across the cell membranes,195 and analogs of the fatty acid amides themselves as agonists or antagonists for their respective receptors (Table 1-6).6,24,198

Although the role of oleamide as an endocannabinoid is a topic of debate,88 the vasodilatory actions of oleamide were attenuated in the presence of certain cannabinoid and TRPV1 receptor antagonists,199 indicating that oleamide-induced vasorelaxation is mediated, in part, by CB1 and non-CB1 cannabinoid receptors, as well as TRPV1 receptors. Endocannabinoids such as anandamide and 2-arachidonoyl glycerol (2-AG) are released in response to pathogenic events and activate cannabinoid receptors that in turn activate signaling pathways linked to neuronal repair and maintenance as well as neuroprotective responses.24 Indeed, the endocannabinoid system is currently being investigated as potential targets in treating inflammatory neurodegenerative diseases, including Parkinson’s,14,18,20 Huntington’s,14 Alzheimer’s,17,20 multiple sclerosis20 and amyotrophic lateral sclerosis.20
### A. N-Acylethanolamines

<table>
<thead>
<tr>
<th>NAE</th>
<th>Receptors(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anandamide</td>
<td>CB1, CB2, PPARα, PPARγ, TRPV1, and TRPM8</td>
<td>4-8</td>
</tr>
<tr>
<td>N-Dihomo-γ-linolenoylethanolamine</td>
<td>CB1 and CB2</td>
<td>9</td>
</tr>
<tr>
<td>N-Palmitoylethanolamine</td>
<td>CB1 and CB2</td>
<td>9</td>
</tr>
<tr>
<td>N-Oleoylethanolamine</td>
<td>PPARα, PPARγ, TRPV1, and GPR119</td>
<td>5,8,46</td>
</tr>
<tr>
<td>N-Linolenoylethanolamine</td>
<td>PPARα, GPR55</td>
<td>5,54</td>
</tr>
<tr>
<td>N-Linoleoylethanolamine</td>
<td>TRPV1</td>
<td>{Movahed, 2005 #29}8</td>
</tr>
</tbody>
</table>

### B. N-Acyldopamines

<table>
<thead>
<tr>
<th>NDA</th>
<th>Receptors(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Arachidonoyldopamine</td>
<td>CB1, TRPV1, and non-CB1/CB2 GPCR</td>
<td>1,63,64</td>
</tr>
<tr>
<td>N-Oleoyldopamine</td>
<td>PPARα, PPARγ, and TRPV1</td>
<td>1,63</td>
</tr>
</tbody>
</table>

### C. N-Acylamino acids\(^b,c\)

<table>
<thead>
<tr>
<th>NAA</th>
<th>Receptors(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Arachidonoyltaurine</td>
<td>TRPV1 and TRPV4</td>
<td>72</td>
</tr>
<tr>
<td>N-Arachidonoylglycine</td>
<td>GPR18, GPR92</td>
<td>73-75</td>
</tr>
</tbody>
</table>

### D. Primary Fatty Acid Amides

<table>
<thead>
<tr>
<th>PFAM</th>
<th>Receptors(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleamide</td>
<td>GABA(<em>A), 5-HT(</em>{1A}) 5-HT(<em>{2A}), 5-HT(</em>{2C}), and 5-HT(_7), CB(_1)(^d)</td>
<td>76-89</td>
</tr>
</tbody>
</table>

\(^a\)In some cases, the indicated fatty acid amide has not been demonstrated to bind to the listed target by direct binding, but instead has been shown to be an agonist or antagonist to the target using a reporter assay. Details are in the citations listed.

\(^b\)While N-acetylglutamate is not formally a fatty acid amide, this N-acylamino acid binds a protein target as it is an allosteric activator of carbamoylphosphate synthetase I.

\(^c\)Fatty acid conjugation to amino acids serves largely in the detoxification and excretion of xenobiotic carboxylates. Thus, many of the N-acylamino acids are likely to bind to a membrane-bound transporter. For example, Wiles et al.\(^{102}\) have recently shown that N-arachidonoylglycine inhibits the GLYT2a glycine transporter.

\(^d\)The role of oleamide as an endocannabinoid is under debate, as the amount necessary for CB\(_1\) binding is high. Oleamide is known to inhibit antagonist and agonist binding to these receptors, however.\(^88\)
Inhibiting the de-activation of endocannabinoids by FAAH, monoacylglycerol lipase (MGL) and the anandamide transport system have been the subject of studies designed to enhance endogenous repair signaling (see ref 12,19 for recent reviews). FAAH inhibition might serve as a therapeutic strategy for treatment of pain, inflammation, spasticity, some types of cancer, and cardiovascular and psychiatric diseases,19 and the most widely used FAAH inhibitor, URB597, is demonstrated to function without toxicity in preclinical safety studies in rats and monkeys.200 It has been suggested that unlike receptor agonists, which activate receptors everywhere at approximately the same time and might cause opposing effects on the progress or symptoms of certain disorders, selective FAAH (and MGL) inhibitors prolong the activity of cannabinoid receptors only where they’re being produced and degraded, thereby exhibiting higher therapeutic efficacy with less unwanted effects.12 It is also pointed out, however, than non-endocannabinoid FAAH substrates would similarly have prolonged activity, which may or may not produce the desired effect.

Anandamide and 2-AG have been shown to inhibit cancer cell proliferation by acting at CB receptors, as these receptors mediate cell proliferation.201 FAAH inhibitors are therefore potentially useful as anticancer agents as well. As discussed in earlier sections, some FAAH substrates such as oleamide, palmitoleamide, N-palmitoleylethanolamine, and N-oleoylethanolamine are thought to increase the action of other endocannabinoids (i.e. anandamide) by the entourage effect.125

Because the role of MGL as a 2-AG hydrolyzing enzyme was only recently established, the therapeutic significance of this enzyme remains to be clarified, and specific inhibitors remain to be designed.19 As detailed by Felder et al.,195 the potential existence of specific transporters for anandamide and the other fatty acid amides is controversial, but accumulating evidence suggests that the simple passive diffusion of the these hydrophobic compounds across the membrane driven by FAAH-hydrolysis is insufficient to account for published anandamide uptake data. The inhibition of PFAM-producing PAM is shown to have anti-inflammatory effects to prevent edema and during the three phases of adjuvant-induced polyarthritis in rats.202 The mechanism is thought to act by way of reducing endogenous levels of substance P and calcitonin gene-related
peptide, although several endogenous PFAMs have recently been shown to influence inflammation as well (see Chapter 3).

Recent evidence has indicated that cannabinoid receptor antagonists can reduce the self-administration of several commonly addictive drugs. The mechanism of this is currently unknown but proposed to be based on such antagonists blocking the effects of endocannabinoids. The recruitment of such cannabinoid antagonists during increased dopamine neural activity has been shown to ameliorate cravings of several addictive drugs, including marijuana, opiates, alcohol, and tobacco.

In addition to reports of PFAMs binding to well known receptor systems such as serotonin and GABA receptors (Table 1-6), some of the first reports of oleamide function were in the inhibition of gap junction communication. Although this ability of oleamide to interfere with intercellular communication via gap junctions has been demonstrated in a variety of cell systems, it is unknown whether the PFAM acts as an allosteric effector of the gap junction proteins or via specific oleamide/PFAM receptors. Regardless, this modulation of gap junction communication has important implications in cell death. Normal gap junction communication is essential for electrical and chemical syncytium and provides for the delivery of nutrients and growth factors, and for removal of excess metabolites and toxins. Inhibition of this communication by oleamide may preserve cells during apoptotic waves and prevent “bystander killing” such as occurs during ischemia and stroke, and myocardial infarction. In primary hippocampal cultures, oleamide was shown to reduce the spread of apoptosis in response to metabolic depression, and in cerebellar granule cells it acted similarly during K+ deprevation.

The number of diseases (and thus pharmacological targets) associated with acyl amides and their receptors is large. Some of these disorders are outlined in Table 1-7. The fatty acid amides represent an exciting opportunity for the development of new drugs for the treatment of disease. The potential for fatty acid amide-targeted therapeutics is high.
1.6 Conclusion

Fatty acid amides are a large family of structurally-diverse molecules found in humans and other organisms. Because many of these molecules have been shown to be bioactive, particularly in cell signaling, analogs of the fatty acid amides could prove useful as agonists or antagonists for their respective receptors, and a better understanding of how they are synthesized and metabolized will lead to better treatments. The enzymes involved in the biosynthesis and degradation of the various fatty acid amides, many of which are still poorly defined, provide an exciting opportunity for the development of new drugs to treat sleep disorders, anxiety, depression, cardiovascular disease, and neurodegenerative diseases.

Table 1-7: Disorders Associated with Amide-Binding Receptors

<table>
<thead>
<tr>
<th>Receptor Category</th>
<th>Pain</th>
<th>Cardiovascular Disease</th>
<th>Eating/Disorder/Obesity</th>
<th>Inflammation</th>
<th>Addiction</th>
<th>Motor Diseases</th>
<th>Anxiety</th>
<th>Sleep Disorder</th>
<th>Depression/Bipolar Disorder</th>
<th>Cognition/Memory</th>
<th>Refs</th>
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<tbody>
<tr>
<td>CB</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>11-31</td>
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<td>PPAR</td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>15,33-42</td>
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<tr>
<td>TRPV</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>13,15,43-45</td>
</tr>
<tr>
<td>GABA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>✓</td>
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<td>47-53</td>
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<td>5-HT</td>
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<td>✓</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>16,55-60</td>
</tr>
</tbody>
</table>

5-HT, 5-hydroxytryptamine (serotonin) receptor; CB, cannabinoid receptor; GABA, γ-aminobutyric acid receptor; PPAR, peroxisome proliferator-activated receptor; TRPV, transient receptor potential vanilloid type.

*There is a possible connection between serotonin and valvular heart disease.*

*Trial data has been inconsistent regarding 5-HT agonists’ effect on Parkinson’s disease.*
1.7 References


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Development of Quantitative Assays for Long Chain Acyl Amides

2.1 Background of N-Acylglycine and Primary Fatty Acid Amide

Isolation

In 1957, the first (non-sphingosine-based) fatty acid amide was isolated as N-palmitoylethanolamine, a natural anti-inflammatory agent, from egg yolk, soybeans and peanuts.\[^2\] Interest in the \(N\)-acylethanolamines (NAEs) grew after evidence showed that \(N\)-arachidonoylethanolamine (anandamide) was an endogenous ligand for cannabinoid receptors after they were shown to attenuate the electrically evoked twitch response in \(Mus\ musculus\) vas deferens.\[^3\]

Although \(N\)-arachidonoylethanolamine has been studied at length, the identification and characterization of other lipid signaling molecules has lagged behind peptides and polar signaling molecules due to the nonpolar nature of lipids. Not only are they hydrophobic, thus making isolation by traditional methods difficult, but they are in low abundance and in constant chemical flux, often quickly being metabolized to other species. Relative to the NAEs, less is known about long chain primary fatty acid amides (PFAMs), and even less about the \(N\)-acylglycerines (NAGs), more recently discovered members of this group of mammalian signaling molecules. The current literature for extraction and identification of NAGs and PFAMs from mammalian sources is summarized in Table 2-1 and Table 2-2.

Aside from a surge of interest in the 1990’s in short chain NAGs as excretion products that accumulate due to acidurias and other inborn errors of metabolism, little work has been done toward extracting NAGs as mammalian metabolites until very recently (Table 2-1). The signaling NAGs are present in much lower abundance than
found as excretion products in aciduria patients, making their isolation difficult until the advent of sensitive analytical instrumentation combined with efficient extraction techniques. For completeness, a few of the urinary NAG studies have been included in Table 2-1.

Renewed interest in isolating PFAMs from mammalian sources arose in the 1990’s after the discovery that nanomole quantities of oleamide induced physiological sleep in rats.\textsuperscript{12} A survey of the techniques used to isolate PFAMs is presented in Table 2-2. Much of the PFAM research has focused on oleamide, likely because it has a known physiological function (see Chapters 3 for a review of PFAM function).
## Table 2-1: Summary of N-Acylglycine Analysis from Mammalian Sources

<table>
<thead>
<tr>
<th>Initial purification method</th>
<th>Detection method</th>
<th>Detection limit</th>
<th>Endogenous amount</th>
<th>NAG source</th>
<th>NAG(s) identified</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>LC/MS</td>
<td>6 nmol</td>
<td>1-9 mmol/mol creatinine</td>
<td>Human urine</td>
<td>various short chain NAGs</td>
<td>5</td>
</tr>
<tr>
<td>SPE (IE, RP, NP)</td>
<td>LC-QqTOF, MRM</td>
<td>150 fmol, 100 attomol</td>
<td>0.26 to 333 pmol/g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rat tissues</td>
<td>N-palmitoyl, N-stearoyl, N-oleoyl, N-linoleoyl, N-docosa-hexaenoyl</td>
<td>7-9</td>
</tr>
<tr>
<td>SPE (IE, RP)</td>
<td>LC-QqTOF, MRM</td>
<td>Not reported; &lt;500fmol</td>
<td>6-1612 pmol/g&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rat tissues</td>
<td>N-palmitoyl</td>
<td>10</td>
</tr>
<tr>
<td>SPE (IE, RP)</td>
<td>LC-QqTOF, MRM</td>
<td>Not reported</td>
<td>1-140 pmol/g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rat tissues and bovine brain</td>
<td>N-arachidonoyl</td>
<td>11</td>
</tr>
<tr>
<td>NP TLC</td>
<td>LC/MS</td>
<td>Not reported</td>
<td>~50 pmol/g&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rat brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP TLC</td>
<td>GC-MS</td>
<td>1.5 nmol</td>
<td>~45 pmol/10&lt;sup&gt;7&lt;/sup&gt; cells</td>
<td>N&lt;sub&gt;18&lt;/sub&gt;TG&lt;sub&gt;2&lt;/sub&gt; cells</td>
<td>N-oleoyl</td>
<td>this dissertation</td>
</tr>
<tr>
<td>NP HPTLC</td>
<td>GC-MS</td>
<td>Not quantified</td>
<td>N&lt;sub&gt;18&lt;/sub&gt;TG&lt;sub&gt;2&lt;/sub&gt; cells</td>
<td>N&lt;sub&gt;18&lt;/sub&gt;TG&lt;sub&gt;2&lt;/sub&gt; cells</td>
<td>not identified</td>
<td>14</td>
</tr>
<tr>
<td>None</td>
<td>GC-MS</td>
<td>0.2 nmol&lt;sup&gt;15&lt;/sup&gt;</td>
<td>0.024-299 mmol/mol creatinine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Human urine</td>
<td>various short chain NAGs</td>
<td>15-22</td>
</tr>
</tbody>
</table>

Abbreviations: ESI-MS/MS, electrospray ionization tandem MS; GC-MS, gas chromatography-mass spectrometry; HPTLC, high performance TLC; IE, ion exchange; LC/MS, liquid chromatography/MS; LC-QqTOF, liquid chromatography-hybrid quadrupole time of flight MS; MRM, multiple-reaction monitoring on a HPLC-triple quadrupole MS; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NP, normal phase; prep-TLC, preparatory TLC; RP, reversed phase; SPE, solid phase extraction; TLC, thin layer chromatography.

The left hand columns summarize the purification (far left) and detection methods (right) across the studies shown.

<sup>a</sup>Quantity is amount per gram dry tissue.

<sup>b</sup>Quantity is amount per gram wet tissue.

<sup>c</sup>Amount varied based on type of inborn error of metabolism or normal phenotype.
### Table 2-2: Summary of Primary Fatty Acid Amide Analysis from Mammalian Sources

<table>
<thead>
<tr>
<th>Initial purification method</th>
<th>Detection method</th>
<th>Detection limit</th>
<th>Endogenous amount</th>
<th>PFAM source</th>
<th>PFAM(s) identified</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>HPLC</td>
<td>~3.5 nmol</td>
<td>255-660 ng/g³</td>
<td>Squirrel brain</td>
<td>ole</td>
<td>1</td>
</tr>
<tr>
<td>HPLC</td>
<td>ESI-MS, MS/MS, and MS³</td>
<td>Not reported; &lt;0.2 pmol</td>
<td>0.1-5 pmol/100 µl</td>
<td>Cat CSF</td>
<td>ole</td>
<td>4</td>
</tr>
<tr>
<td>HPLC, SPE and TLC</td>
<td>ESI-MS, MS/MS, MS³, IR, NMR, GC-MS, and ozonolysis</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Cat CSF</td>
<td>ole, eruc</td>
<td>12</td>
</tr>
<tr>
<td>Solvent extraction only</td>
<td>ESI-MS (HPLC)</td>
<td>Not reported</td>
<td>Not reported</td>
<td>N₁₈ TG₂ cells</td>
<td>ole</td>
<td>13</td>
</tr>
<tr>
<td>TLC, HPLC</td>
<td>GC-MS</td>
<td>0.1 nmol</td>
<td>55 pmol/10⁻⁷ cells</td>
<td>N₁₈ TG₂ cells</td>
<td>ole</td>
<td>23</td>
</tr>
<tr>
<td>SPE, HPTLC</td>
<td>GC-MS</td>
<td>1.8 nmol</td>
<td>1.4-28 µg/g</td>
<td>Rabbit brain, heart</td>
<td>palmit, stear, ole</td>
<td>14</td>
</tr>
<tr>
<td>SPE</td>
<td>GC-MS</td>
<td>Not reported</td>
<td>0.5-12 ng/g</td>
<td>pig</td>
<td>eruc</td>
<td>28</td>
</tr>
<tr>
<td>SPE</td>
<td>GC-MS</td>
<td>0.5 nmol</td>
<td>112-6042 pmol/10⁻⁷ cells</td>
<td>N₁₈ TG₂ and SCP cells</td>
<td>palmitole, palmit, ole, linole</td>
<td>This dissertation</td>
</tr>
<tr>
<td>Solvent extraction only</td>
<td>GC-MS</td>
<td>0.28 fmol</td>
<td>9.9-44 ng/ml</td>
<td>Rat CSF and serum</td>
<td>ole</td>
<td>29</td>
</tr>
<tr>
<td>Solvent extraction only</td>
<td>GC-MS</td>
<td>Not reported</td>
<td>2-32 µg/ml</td>
<td>Human leutal phase plasma</td>
<td>palmitole, palmit, ole, elaid, linole</td>
<td>45</td>
</tr>
</tbody>
</table>

Abbreviations: GC-MS, gas chromatography-mass spectrometry; ESI-MS, electrospray ionization MS; HPLC, high performance liquid chromatography; HPTLC, high performance TLC; IR, infrared analysis; LC/MS, liquid chromatography/MS; MS, mass spectrometry; MS/MS and MS³, tandem mass spectrometry; NMR, nuclear magnetic resonance; SPE, solid phase extraction; TLC, thin layer chromatography.

The left hand columns summarize the purification (far left) and detection methods (right) across the studies shown.

³Quantity is amount per gram dry tissue.
2.1.1 Lipid Extraction

Although short-chain NAGs are present in certain urine samples and can be analyzed without extraction, the long-chain NAGs and PFAMs are generally in much lower abundance and require more extensive extraction techniques to remove them from the tissues and cells in which they are found. Commonly used extractions are based on those of Folch\textsuperscript{24} and Bligh,\textsuperscript{25} and variations thereof. Usually a 20-fold volume of chloroform:methanol (2:1) or methanol is added to the tissue or cell pellet prior to homogenization, and a small volume of water or brine is added to the supernatant to separate lipids from remaining non-lipid contaminants that remain in the upper, aqueous phase. The lower phase, which contains lipid molecules, including NAGs and PFAMs, is then reduced in volume before initial sample preparation.

2.1.2 Sample Preparation

Older methods of long chain fatty acid-related molecules required purification to homogeneity prior to structural analysis by MS or NMR,\textsuperscript{26,27} a problem which is compounded by the low abundance of lipid signaling molecules such as PFAMs and NAGs. Modern instrumentation can characterize individual lipid components in a more complex, less purified sample. Initial purification is typically done using either preparatory thin layer chromatography (TLC) or solid phase extraction (SPE) after solvent extraction (see Table 2-1 and Table 2-2 for examples). Greater specificity can be achieved using column chromatography (ion exchange or normal phase) or TLC, as the characteristic functional groups (i.e. glycine and primary amide) interact with the solid matrix, and samples can be separated based on those functional groups. Reverse phases are useful for separating compounds based on their acyl chain lengths.

2.1.2.1 Solid Phase Extraction

Normal phase (NP), reversed phase (RP) and ion exchange (IE) chromatography columns have all been used for initial sample purification before MS analysis of NAGs, PFAMs, and other related lipids (see Table 2-1 and Table 2-2). There are several things to consider when attempting to purify a lipid sample by SPE. The different solid matrices
can be used to separate the PFAMs and NAGs from different metabolites depending on the desired outcome. Crude extracts can often contain cholesterol, triacylglycerols, squalenes and other nonpolar lipids, which can be removed by using an ion exchange or normal phase column. When put in acidic conditions, PFAMs gain a positive charge (\(-\text{NH}_3^+\)) and NAGs gain one (\(-\text{NH}_2^+\)) or two (\(=\text{O}^+\)-\(\text{H}\)) positive charges and become good candidates for ion exchange. Reverse phase columns are useful for separating compounds of different acyl chain length. Silica matrix material varies from manufacturer-to-manufacturer and even from lot-to-lot, and the sample purification by SPE should be optimized with a solution of standards. The analytes can become difficult to recover if they bind too tightly with the column material or are in low abundance.

All of these SPE techniques have been put to use by the Bradshaw/Walker group for partial purification of NAGs\(^7\) as well as by many others for selective extraction of different lipid classes.\(^30\)\(^-\)\(^36\) Compounds that contain similar polarities such as PFAMs, NAGs monoacyl glycerols, and \(N\)-acylethanolamines can be difficult to separate on SPE. This may be overcome in the later MS analyses if sufficiently sensitive instruments are available, but TLC is another option that may offer a better pre-analysis separation in these cases.

### 2.1.2.2 Thin Layer Chromatography

TLC has long been used to separate lipid samples from crude extract\(^37\)\(^-\)\(^44\) (see ref. 41 for a review). TLC can purify samples to a greater extent in one step relative to SPE, although amine-based diffusion of the PFAMs and NAGs spreads the amide TLC spot more widely than for other lipid classes. High performance thin layer chromatography (HPTLC) plates use smaller particle size (~15\(\mu\)m), which gives rise to less spot spread and higher resolution, ameliorating the diffusion caused by the amino group. Because NP silica gel is polar, PFAMs and, particularly, the NAGs will not move far from the baseline of the TLC plate and, as plates are developed, the more non-polar lipids will travel away from these amides.

While providing a means to rapidly remove most contaminants at once, TLC can also provide a quick profile of all the types of compounds present in a sample. Larger amounts of lipids can be visualized under UV light, while smaller quantities can be
visualized using a dye such as potassium permanganate (KMnO₄), which is used to visualize as little as 1nmol NAG or PFAM. KMnO₄ is oxidative, so standards are run in tandem with test plates, and position of the target spot is estimated based on the dyed standard before scraping into desorption solvent.

It is important to remove silica and binding agent(s) before the next analytical step to avoid clogging the analytical instrumentation or dampening the signal with excess background noise. A fine syringe filter can remove most of the silica, and careful use of dry solvents can minimize silica solubility. The Merkler and Johnson groups have had some success with desorbing into anhydrous isopropanol (this dissertation and ref. 14); however, certain binding agents can deteriorate GC column material over time. Careful selection of TLC plates or in-house production of the plates will avoid this problem.

2.1.3 Sample Detection

Nuclear magnetic resonance (NMR), although useful for complete atom assignment, is not useful for most PFAM and NAG analyses because of the limited quantities of purified materials obtain from mammalian sources and inability to purify samples to homogeneity. Even when using large animals and large tissue samples, the pmol/g tissue quantities do not provide enough material for 13C-NMR, which requires mg quantities.³ More sensitive techniques are required. Analysis via mass spectroscopy provides excellent sensitivity, and unambiguous assignment of compound can be achieved by mass fragmentation pattern and retention time comparisons to known standards. The two main methods used are GC-MS and LC-MS.

2.1.3.1 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an excellent system for the rapid separation and detection of metabolites. Even with crude preparations, the GC-MS spectrum can be mined for characteristic fragment ions to be quantified, or only those mass to charge ratios (m/z) of interest can be monitored (see section 2.2.12). One advantage of GC over LC is its higher peak capacity due to longer column length. Nonpolar (95-100% dimethylpolysiloxane) columns provide a separation by chain length. Because the typical ionization potential used in most electron ionization systems is 70
eV, much higher than the potential required to ionize organic molecules, fragment ions are observed in much greater abundance than M$^{++}$ ions. However, identification by retention time and fragmentation pattern still allows unambiguous assignment of structure. If a standard is unavailable and determination of molecular weight is desired, chemical ionization (CI) can be used. A protonated molecular ion is formed through a two-step reaction in which a reagent gas is bombarded with electrons to ionize it and then the activated gas is allowed to react with the analyte molecule, forming an intact, ionized compound for mass detection.

Although underivitized NAGs and PFAMs can be seen when in abundance in GC analysis, the amounts of NAGs and PFAMs extracted from a natural source are not sufficiently abundant to allow production of robust standard curves within useful ranges for metabolite analysis without derivitization. Typically, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA),\textsuperscript{19,46-48} tert-butyl dimethylsilyl ether (t-BDMS),\textsuperscript{49} or methylation\textsuperscript{50,51} are used for volatilization, which greatly increases sensitivity over non-volatilized compounds.

### 2.1.3.2 High Pressure Liquid Chromatography-Mass Spectrometry

HPLC-MS/MS has been the method of choice for identifying long chain NAGs and PFAMs over the last 3-5 years. Both HPLC-MS/MS and GC-MS allow for virtually unambiguous structure assignment (when synthetic standards are available) due to both fragmentation pattern analysis and retention times. HPLC-MS/MS often can provide more accurate masses (<10 ppm) than GC-MS (~100 ppm), allowing identification to a higher degree of confidence and with fewer fragment peaks. These methods, in addition to requiring less material, also eliminate the need for pure sample. A modestly purified sample (via SPE) can be further separated on the LC column, and tandem MS can further isolate compounds of interest if their intact molecular weights are known. Tandem mass spectrometers can include triple quadrupole, ion trap, and quadrupole/time-of-flight to isolate fragments of specific mass to charge ratio (m/z) prior to final fragmentation for unambiguous identification.\textsuperscript{26} Sensitivity can be increased further by using a smaller LC column, as the increase in sensitivity is the square of the proportion of the column diameters.\textsuperscript{26} Although Tan \textit{et al.} do not report a 1600-fold increase in sensitivity when
moving from a 4.1 mm to a 75 µm diameter column as theoretically predicted, they did find novel compounds with less than 250 fmol of a fatty acyl amide when using a nano-HPLC QqTOF, and 100 attomol in the multiple-reaction monitoring (MRM) scan on a triple quadrupole MS for quantification of known compounds.\(^9\)

Tan \textit{et. al.} have developed an algorithm designed to assist with the intensive data analysis required to process the roughly 1000 spectra generated from each MS/MS run from crude brain extract. The algorithm searches each MS scan for predicted ions from the masses of acyl groups plus amino acids and other small endogenous amines. Candidate MS/MS must also contain the masses of the acyl and the amino fragments. They recently have used this method to identify 11 novel acyl amino acids in rat tissue homogenates.\(^8\)

\subsection*{2.1.4 Other Considerations}

Unambiguous assignment of unsaturated acyl chains can pose a problem in some samples. Separation of cis/trans isomers is not ideal on a nonpolar matrix such as the dimethylpolysiloxane GC column that is commonly used for lipid analysis, but a highly polar bis(cyanopropyl) polysiloxane phase has been shown to provide this separation.\(^52\) A recent review article describes Ag\(^+\)-HPLC, Ag\(^+\)-TLC, RP HPLC and high-speed counter current chromatography methods for separating cis/trans isomers of fatty acid methyl esters before MS analysis,\(^53\) and could be adopted to fatty acid amides.

Retention times of metabolites can vary due to co-eluting compounds, general sample purity, column deterioration, and changes and general maintenance in chromatography apparatus for both LC- and GC-MS.\(^8\) Retention times are therefore best estimated by running extracts spiked with putative target compounds directly after analysis of unspiked sample rather than running pure NAGs or standards at a later time. This is not always possible when searching for novel metabolites but putative metabolites can be synthesized after the fact. The synthesis of NAGs both for analyte validation and for use as an internal standard is typically done by the reaction of glycine ethyl ester with the desired acid chloride, followed by deprotection of the glycine in base.\(^54\) Primary fatty acid amides are synthesized by amidation of the acyl chloride.\(^55\) (See section 2.2.2)
Internal standards should be added before sample analysis at different steps. A deuterated lipid of similar chain length to the analyte(s) of interest is preferred as it will give similar standard curves for quantitative analysis and have similar retention times but remain distinct from any endogenously found amides. An internal standard should be added before sample extraction, purification, and analysis to determine efficiency of metabolite recovery. Additionally, instrument performance can vary from day to day and from run to run and, therefore, an internal standard of known amount should be included with each GC-MS or LC-MS/MS run if quantitative analysis is desired.

The advent of modern analytical tandem MS/MS instruments has greatly increased our ability to quickly analyze metabolites of low abundance and to search for novel NAGs and PFAMs (as well as other related lipids). Careful selection of extraction solvents, purification matrices, and analytical separation columns allow for the identification and quantification of low-abundant NAGs and PFAMs from complex lipid mixtures. In this chapter, quantitative assays for PFAMs and NAGs are presented, along with some metabolism data for the conversion of oleic acid to oleamide. PFAM purification on NP SPE and identification by GC-MS proved to be a robust method for the quantification of these metabolites. NAGs were found to be more easily separated and recovered on TLC before identification by GC-MS. These methods were put to use in standard solutions, spiked cell and media extracts, and in some model cell lines after incubation with free fatty acids (FFAs).

2.1.5 Oleamide-Producing Cell Selection

The successful isolation of fatty acid amides from mammalian sources, to date, is summarized in Table 2-2. In selecting a cell line for isolation of oleamide, mouse neuroblastoma N18TG2 was an ideal choice because these cells express PAM,56 and are known to produce oleamide and N-oleoylglucose.13,23 These cells could, therefore, serve as a model for validation of extraction methods.

In order to gain a more thorough perspective about long chain PFAM metabolism, two additional cell lines were examined for the production of oleamide from exogenously added oleic acid: sheep choroid plexus (SCP) and human epithelial kidney (HEK-293) cells. These cell lines were chosen because of their potential to synthesize oleamide.
Liver and kidney cells have the highest expression of the short- and medium-chain acyl CoA processing enzyme ACGNAT$^{57-62}$ and, therefore, may have a greater potential to process long chain acyl CoAs, NAGs and PFAMs as well. SCP cells were chosen because this tissue type is known to secrete molecules into CSF where PFAMs have been found.$^{63-65}$

2.2 **Materials and Methods**

2.2.1 **Materials**

Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Donor equine serum was from Thermo Scientific (Waltham, MA). DMEM, EMEM and penicillin/streptomycin were from Mediatech Cellgro (Manassas, VA). Mouse neuroblastoma N$_{18}$TG$_2$ cells were from DSMZ (Deutsche Sammlung von Mikroorganism und Zellkulturen GmBH). SCP and HEK-293 cells were from American Type Culture Collection (Manassas, VA). Primers were from IDT DNA. MicroPoly(A) Pure™ mRNA purification kit and RETROscript® Reverse Transcription kit were from Ambion (Austin, TX). QIAQuick gel extraction kit was from Qiagen (Valencia, CA). PVDF membrane was from Millipore (Billerica, MA). Tween-20, oleic acid and $^{13}$C$_{18}$-oleic acid were from Sigma-Aldrich (St. Louis, MO). PAM (S-16) and FAAH (V-17) antibodies were from Santa Cruz Biotech, Inc (Santa Cruz, CA). FACL5 (N-term), FACL3 (center), FACL6 (center) antibodies were from Abgent (San Diego, CA). Goat anti-rabbit and donkey anti-goat secondary antibodies conjugated with horse radish peroxidase were from ICN Biomedical (Solon, OH). SuperSignal chemiluminescent detection system was from Pierce (Rockford, IL). BSTFA and silica were from Supelco (St. Louis, MO). Deuterated D$_{33}$-heptadecanoic acid was from C/D/N isotopes (Pointe-Claire, Quebec). All other reagents and cell culture supplies were of the highest quality available from commercial suppliers.
2.2.2 Standard Synthesis

2.2.2.1 Acyl Chloride Synthesis

The free fatty acid (FFA) and thionyl chloride were combined in a 1:2 molar ratio neat under nitrogen while stirring (Equation 2-1). The reaction was allowed to proceed under reflux for 30 minutes at 50°C in a heating mantle. Excess unreacted thionyl chloride was removed by heating or under vacuum until the solution no longer produced gas.

\[
\text{R-COOH} + \text{Cl-SO}_2\text{Cl} \rightarrow \text{R-CCL} + \text{SO}_2 + \text{HCl}
\]

Equation 2-1: Synthesis of Acyl Chloride from a Fatty Acid

2.2.2.2 \text{\textsuperscript{13}C}_{18}\text{-Oleamide Synthesis}

\text{\textsuperscript{13}C}_{18}\text{-Oleamide} was synthesized for use as a standard following a procedure similar to Fong et. al.\textsuperscript{55} Undistilled acyl chloride was added dropwise to ice cold concentrated NH\textsubscript{4}OH (29% NH\textsubscript{3}) in a ratio of 1:6 acyl chloride:NH\textsubscript{4}OH (v/v) (see Equation 2-2). The reaction was allowed to continue until the precipitation of \text{\textsuperscript{13}C}_{18}\text{-oleamide} visibly ceased. Excess NH\textsubscript{4}OH was removed from the oleamide crystals by washing with H\textsubscript{2}O in a Büchner funnel. The sample was allowed to dry and stored at -20°C flushed under nitrogen.

\[
\text{R-}^{13}\text{C}_{18}\text{Cl} + \text{NH}_3 \rightarrow \text{R}^{13}\text{C}_{18}\text{NH}_2 + \text{HCl}
\]

\[\text{R} = ^{13}\text{C}_{17}\text{H}_{33}\]

Equation 2-2: Synthesis of \text{\textsuperscript{13}C}_{18}\text{-Oleamide from the Acyl Chloride}
2.2.2.3 Acylglycine Synthesis

Heptadecanoylglycine, D$_{33}$ (HdG) and $^{13}$C$_{18}$-$N$-oleoylglycine were synthesized for use as standards using the procedure slightly modified from Goujard et al.$^{54}$ (Equation 2-3). Glycine ethyl ester hydrochloride and triethylamine were slurried together in a 1:2 molar ratio in a small volume of dichloromethane (DCM). The acyl chloride was taken up in a small volume of DCM and added dropwise to the slurry in a ratio of 1 mmol chloride:10 mmol glycine ethyl ester. The reaction was allowed to proceed, with stirring, at room temperature for 24 hours.

Acidified water (pH ~2) was added to the reaction (1:1 ratio of acidified water to reaction solvent), along with additional DCM (2:1 ratio of new DCM to reaction solvent). The solution was taken into a separatory funnel and washed twice with H$_2$O and once with saturated NaCl solution. The organic phase was dried over anhydrous sodium sulfate and then taken to an oil in vacuo.

The glycine ethyl ester was deprotected by stirring with excess NaOH (~2:1 molar ratio of amide:NaOH) in methanol/water 90:10 (v/v) for 24 hours. The reaction was quenched with HCl (pH ~2) and placed in a separatory funnel with ethyl acetate. The reaction was washed twice with H$_2$O and once with saturated NaCl solution. The organic phase was dried over anhydrous sodium sulfate and taken to a white powder in vacuo. The product was allowed to dry, flushed with nitrogen and stored at -20°C.
2.2.3 Isotope Metabolism Studies

Prior to development of a quantitative PFAM assay, N18TG2 cells were incubated with $^{13}$C$_{18}$-oleic acid (OA) to determine whether these cells would produce $^{13}$C$_{18}$-oleamide from the exogenously added $^{13}$C$_{18}$-OA. In these preliminary incubations, 0.2 mM fatty acid was added directly to the cell media without a BSA carrier. Cells were incubated for 48 hours with $^{13}$C$_{18}$-oleic acid and then collected for PFAM analysis. Metabolite extraction was performed as described in Section 2.2.6, but no solid phase extraction (SPE) was performed. Samples were injected directly onto the GC-MS after derivitization with BSTFA and analyzed for $^{13}$C$_{18}$-oleamide and $^{13}$C$_{18}$-oleonitrile by comparing with chemically synthesized standards.

2.2.4 Oleic Acid-BSA mixture

BSA was used as a carrier for OA in aqueous media because of the low solubility of the long-chain fatty acid in water. OA was dissolved in ethanol, converted to the sodium salt with excess NaOH, dried under vacuum, and dissolved in Dulbecco’s phosphate-buffered saline (PBS) to make a 25 mM solution. The sample was then heated
in 49°C water bath for 5 minutes before adding 2.5 mM BSA. The sample was stirred at room temperature for 4 hours, sterile-filtered, and stored at -20°C.

2.2.5 Cell Culture and Fatty Acid Incubation

All cells were grown in 225 cm² culture dishes. N₁₈TG₂ cells were grown in DMEM supplemented with 100 µM 6-thioguanine. SCP and HEK-293 cells were grown in EMEM (SCP were supplemented with 100 µM sodium pyruvate). SCP and N₁₈TG₂ media had 10% FBS and HEK-293 had 10% horse serum. All cells were grown with 100 I.U./ml penicillin, 1.0mg/ml streptomycin and incubated at 37°C with 5% CO₂ according to supplier instructions. Cultures were grown to 80%-90% confluency, and culture medium removed and replaced with media containing 0.5% FBS or horse serum and 2.5 mM fatty acid/0.25 mM BSA mixture. After 12, 24, or 48 hour incubation, media was collected, cells washed with PBS and trypsinized. An aliquot was taken for counting and determination of cell viability on a hemacytometer with trypan blue. Cells were centrifuged, the supernatant removed, and the pellet stored at -80°C. Spent media was centrifuged to remove any cells and the supernatant also stored at -80°C.

2.2.6 Metabolite Extraction

Metabolites were extracted from cells similar to Sultana and Johnson. Methanol (4 ml) was added to cell pellets and samples were sonicated for 15 minutes at room temperature. Samples were centrifuged at 5000 rpm for 10 min, and the supernatant separated from the pellet, dried under N₂ in a warm water bath at 40°C. The pellet was re-extracted with 4 ml 1:1:0.1 (v/v/v) chloroform:methanol:water, sonicated for 10 min, vortexed for 2 min and centrifuged 10 min at 5000 rpm. Supernatant from this step was added to the dried supernatant from the previous step and dried under N₂ at 40°C. The pellet was re-extracted with 4.8 ml chloroform:methanol 2:1 (v/v) and 800 µL 0.5 M KCl/0.08 M H₃PO₄, sonicated 2 min, vortexed 2 min, and centrifuged 10 min at 5000 rpm. The lower lipid phase was added to the dried supernatant from the previous steps and dried under N₂ at 40°C. These steps are summarized in Figure 2-1.
Metabolite extraction from conditioned media was performed on all the media collected from the flasks (~45 ml per T-225 cm² flask). Extractions were done with 15 ml chloroform:methanol 2:1 (two times) followed by two extractions with 15 ml chloroform:methanol 2:1 plus 2.4 ml 0.5 M KCl/0.08 M H₃PO₄. No sonication was performed, and precipitated protein layers that resulted from solvent addition were condensed by centrifugation for 30-45 min at 5000 rpm. Organic lipid phases were also combined and dried under N₂ at 40°C.

2.2.7 Solid Phase Extraction

Solid phase extraction (SPE) was run for PFAM purification. Silica columns were run as described by Sultana and Johnson.⁴⁶ Each column contained 0.5 g silica. Dried lipid extract samples were taken up in 100 µL n-hexane and added to silica columns after rinsing with n-hexane. The mobile phase was run as follows: 4 ml n-hexane, 1 ml 99:1
hexane:acetic acid, 1 ml 90:10 hexane:ethyl acetate, 1 ml 80:20 hexane:ethyl acetate, 1 ml 70:30 hexane:ethyl acetate, 1.5 ml 2:1 chloroform:isopropanol, 0.5 ml methanol. The last two fractions were combined and dried down under N₂ at 45°C. An internal standard, 3 nmol D₃₃-heptadecanoic acid, was added and dried before derivitization.

2.2.8 Thin Layer Chromatography

TLC was used for NAG purification. Dried lipid extracts were applied to a 10 cm EMD (Gibbstown, NJ) analytical NP TLC plate that had been prewashed with chloroform:acetone 1:1. After spotting the sample onto the TLC plate with both ethyl acetate and ethanol, chloroform:methanol:acetic acid 95:5:1 was used to run up to 5.5 cm. The plate was dried and placed in the second running solvent, hexanes:diethyl ether:acetone 60:40:5 and run up to 8 cm. After drying, the final solvent, hexanes:diethyl ether 97:3, was run up to 9.5 cm. N-Oleolglycine and HdG standards were run alongside the cell extract on a separate plate and visualized using KMnO₄ to test for position of the NAGs. The appropriate area was scraped from the experimental TLC plate containing cell extract and collected into anhydrous isopropanol before water bath sonication for 5 minutes. After initial sonication, the sample was centrifuged to pellet the silica. The supernatant liquid was transferred into a syringe, filtered with a 0.22 µm syringe filter, and set to dry under nitrogen at 40-50°C. The silica was subjected to another round of sonication in anhydrous isopropanol, centrifugation and syringe filtering before adding to the previous supernatant. Sample preparation for analysis of NAGs is summarized in Figure 2-2.

The syringe filter has to be rinsed with isopropanol before running the sample through it to remove the slip additive oleamide. Although oleamide is not the metabolite of interest while performing the TLC purification, there was enough oleamide present to interfere with subsequent analyses. This finding of oleamide in such abundance in the syringe filter may help explain the 8 order of magnitude difference between the endogenous amount of oleamide reported in N₁₈TG₂ cells by this study (119 pmol/10⁷ cells) and Bisogno (55pmol/10⁷cells) (23) Bisogno, T.; Sepe, N.; De Petrocellis, L.; Mechoulam, R.; Di Marzo, V. Biochem Biophys Res Commun 1997, 239, 473. and by Sultana (16.67 pmol/cell). (14) Sultana, T. Doctoral Dissertation, Duquesne University, 2005.
2.2.9 Sample Derivitization

Trimethylsilylation was achieved using BSTFA. Samples were flushed with dry N₂, 100 µL BSTFA added, and the sample allowed to react at 55-60°C for 1 hour before analysis by GC-MS. The reaction scheme of BSTFA with amides to form trimethylsilyl derivatives, nitriles, and enyliminoacetate derivatives (from NAGs) is shown in Figure 2-3.

2.2.10 GC-MS

All analyses were performed using a Shimadzu QP-5000 GC-MS. Separations were achieved on a J & W Scientific (Folsom, CA) DB-5 column (0.25 mm x 30 m). The GC temperature program was 55-150°C at 40°C/min, hold at 150°C for 3.6 min, ramp at 10°C/min to 300°C, and hold for 1 min. The transfer line was held at 280°C and the
injection port at 250$^\circ$C throughout the separation. Helium was used as the carrier gas, at a flow rate of 0.9 ml/min. The mass range was 35-700 amu.

After running two aliquots of the experimental sample through the GC-MS, the remaining sample was dried down, spiked with oleamide, re-derivitized with BSTFA, and re-run on the GC-MS to validate sample retention times

2.2.11 Contamination Controls

As controls, all glassware and plasticware were treated with solvents as described for the cell and media samples and were subjected to sonication, solid phase extraction, and derivitization to test for contaminating plasticizers. Integration of the GC trace was taken over the same time integral as standard amides and for the same set of ions. An averaged number of cells was used to calculate blank amount of amide per cell. Unspent media was also subjected to extraction to check for existing amides. No significant amount of oleamide was found in any of these samples compared to cells and conditioned media samples (data not shown).

2.2.12 Data Analysis

Total ion chromatograms (TIC) were taken to more clearly determine the identities of species present in the sample. Post-run, a set of selected ions unique to oleamide and oleonitrile was overlaid and integrated so that effects of any co-eluting compounds could be minimized. These unique ion sets were 83, 97, 110, 122, 136, 150, 164, 190, 206, 220, 234 and 263 for oleonitrile, and 59, 86, 112, 116, 122, 126, 128, 131, 136, 140, 144, 154, 158, 170, 184, 186, 198, 200, 226, 238, 264, 281, 338 and 353 for oleamide-TMS. Standards were integrated in the same way for production of a standard curve.

Heptadecanoic Acid, D$_{33}$ (HdA), was spiked into each sample as an internal standard to test for performance of the instrument. The amides and nitriles of interest were integrated along with the HdA, both compared to their standard curves, and a correction factor determined based on the integration of HdA compared to its standard curve was used to adjust the amount of amide. Each sample was run on the GC-MS
twice, and each incubation run at least twice to provide replicates. T-tests were performed using GraphPad.

2.3 Results

2.3.1 GC-MS of Standard Spectra

Throughout these experiments BSTFA was used to volatilize the lipids, and the BSTFA derivitization is shown in Figure 2-3A. When derivitizing a primary amide with BSTFA, a side reaction is observed in which a nitrile is formed, as can be seen in the GC-MS of oleamide in Figure 2-4. There is precedence for this type of reaction, and a suggested mechanism based on that proposed by Ichiyama involving L-chloropropionic acid as the electrophile is shown in Figure 2-3B.

Upon derivitization of NAGs, an enyliminoacetate product was also found in addition to the mono- and bi- trimethylsilyl (TMS) products (Figure 2-5). The proposed formation of this enyliminoacetate can be found in Figure 2-3C, and more information regarding the elucidation of this structure is in Appendix A.
Figure 2-3: Mechanism for the Derivitization of Amides with BSTFA

Panel A shows the addition of a trimethylsilyl group to an electronegative atom previously bound to a hydrogen. Panel B shows the competing reaction where a nitrile is formed from a primary amide. Panel C shows an enyliminoacetate product from derivitization with an NAG. For more details on enyliminoacetate identification see Appendix A.
Figure 2-4: GC-MS of Oleonitrile and Oleamide-TMS from Oleamide BSTFA reaction
Panel A (top) shows the entire GC spectrum for this oleamide sample, with a zoom in of the oleonitrile and oleamide-TMS peaks (bottom). Panel B shows the annotated MS of the oleonitrile mass spectrum. Panel C shows the annotated MS of the oleamide-TMS mass spectrum.
Figure 2.5: GC-MS of N-Oleoylglycine Derivitized with BSTFA
Y-axes are intensity. Panel A shows the whole GC chromatogram (top) and closeup of NOG peaks (bottom). Panel B shows the MS spectrum for the enyliminoacetate derivative. Panel B shows the MS of NOG with two TMS groups attached. Panel D shows mono-derivitized NOG.
One of the most common reactions for carboxylated acyl chains is the McLafferty rearrangement. The McLafferty rearrangement and resulting fragments from oleamide and the mono- and bi-derivitized NOG molecules is shown in Figure 2-6. Additional fragmentation schemes for these compounds can be found in Appendices A and B.

\[ \text{ McLafferty Rearrangements for Acyl Amides } \]

The common McLafferty rearrangements for acyl amides are shown here for a primary amide (A), mono-derivitized NAG (B) and di-derivitized NAG (C). The m/z shown are for an oleoyl acyl chain. Additional fragmentation schemes are shown in Appendices A and B.

\[ ^{13}\text{C}_{18}\text{-Oleamide, }^{13}\text{C}_{18}\text{-N-oleoylglycine, and deuterated }D_{33}\text{-N-heptadecanoylglycine were synthesized for use as standards for these analyses. The MS spectra for these samples are shown in Figure 2-7 and Figure 2-9.} \]
Figure 2-7: $^{13}$C$_{18}$-Oleamide-TMS Mass Spectrum

The top panel (A) shows $^{13}$C$_{18}$-oleamide-TMS. Panel B shows $^{13}$C$_{18}$-oleonitrile.
Figure 2-8: \( ^{13}\text{C}_{18}\)-Oleoylglycine Mass Spectra

The top panel (A) shows the enyliminoacetate derivative of \( ^{13}\text{C}_{18}\)-NOG (see Appendix A). Panel B shows \( ^{13}\text{C}_{18}\)-NOG-TMS. Panel C shows \( ^{13}\text{C}_{18}\)-NOG-(TMS)$_2$. 
2.3.2 Isotope Metabolism Studies

In order to determine whether exogenously added OA was directly converted to oleamide, incubations were performed with isotopically labeled $^{13}$C$_{18}$-OA in N$_{18}$TG$_{2}$ cells. These experiments would help determine whether OA is a direct metabolic precursor to NOG and oleamide depending on whether the carbon backbone remained intact. These initial extractions were performed similar to Merkler et. al.$^{13}$ and did not include an initial sample purification before application to GC-MS.

The cells were incubated with 0.2 mM $^{13}$C$_{18}$-oleic acid (sans BSA lipid carrier, see Section 2.2.4), the cells and conditioned media collected after 48 hours incubation before lipid analysis. The GC-MS of $^{13}$C$_{18}$-oleamide extracted from the cells and derivitized with BSTFA is shown in Figure 2-10 and the spectrum of underivitized $^{13}$C$_{18}$-oleamide from the conditioned media is included in Figure 2-11. The GC-MS spectrum

![Figure 2-9: GC-MS of HdG](image)

Panel A shows the MS of the enyliminoacetate derivative of HdG. Panel B shows the MS of HdG-TMS. No HdG-(TMS)$_{2}$ was observed. For more information on the enyliminoacetate derivative, see Appendix A.
of $^{13}$C$_{18}$-NOG isolated from cells and conditioned media and derivitized with BSTFA are shown in Figure 2-12 Figure 2-13, respectively. These results strongly suggest that the chain of custody of the carbon atoms is directly from OA to NOG and oleamide.

![Figure 2-10: GC-MS of N$_{18}$TG$_2$ Cells Incubated with $^{13}$C$_{18}$-Oleic Acid Showing $^{13}$C$_{18}$-Oleamide-TMS](image)

Panel A shows the GC over the entire timecourse (top) and a closeup of the $^{13}$C$_{18}$-oleamide-TMS spectrum (bottom). Panel B shows the annotated MS for $^{13}$C$_{18}$-oleamide-TMS.
Figure 2-11: GC-MS of N18TG2 Conditioned Media Incubated with \(^{13}\)C\(_{18}\)-Oleic Acid Showing \(^{13}\)C\(_{18}\)-Oleamide

Panel A shows the GC over the entire timecourse (top) and a closeup of the \(^{13}\)C\(_{18}\)-oleamide spectrum (bottom). Panel B shows the annotated MS for \(^{13}\)C\(_{18}\)-oleamide.
Figure 2-12: GC-MS of N18TG2 Incubated with 13C18-Oleic Acid Showing 13C18-Oleoylglycine-TMS

Panel A shows the GC over the entire timecourse (top) and a closeup of the 13C18-NOG-TMS peak (bottom). The peak at 20.19 minutes is 13C18-NOG-(TMS)2. Panel B shows the annotated MS for 13C18-NOG-TMS.
Initial isotopic metabolism studies provided qualitative results, but not robust reproducible quantitative PFAM analysis. In order to improve the quantification of PFAMs, an extraction step was added prior to derivitization with BSTFA and analysis by GC-MS. This solid phase extraction on normal phase silica provided an excellent way to remove many interfering compounds such as cholesterol, triacylglycerols, squalenes and other nonpolar lipids. While this extraction was not able to remove all the fatty acids due to the large amount incubated with the cells (2.5 mM), sufficient quantities were removed...
to prevent saturation of the detector on the GC-MS, essential to preserving the life of the
detector system and in removing enough interference to allow the detection of smaller
PFAM peaks.

Extraction efficiency experiments were run for oleamide and other PFAMs, and a
detailed description of the analysis can be found in Chapter 3. Extraction efficiency for
oleamide from cells was 81% ± 7% and in media was 94% ± 9% (average ± standard
deviation).

2.3.3.1 Oleic Acid Time Course Incubations

Once it was established that the N_{18}TG_{2} cell line is capable of converting OA
directly to oleamide and the quantitative assay had been demonstrated with spiked
samples (data not shown), the next step was to quantify the endogenous amount of
oleamide in all three model cell lines and to demonstrate the rate of conversion from OA
to oleamide over time in each of them. (For a discussion on the selection of model cell
lines see section 2.1.5.)

All three model cell lines were found to contain endogenous amounts of
oleamide, where the “endogenous” amount refers to the amount of oleamide extracted
and quantified in normally growing cells without incubation with OA. All three cell lines
also showed an increase in oleamide level after incubation with OA over time. GC-MS
spectra showing oleamide or oleonitrile can be found in Figure 2-14 through Figure 2-19.
Figure 2-14: GC-MS HEK-293 Cell Extract after Incubation with OA for 12h, Showing Oleonitrile

GC-MS of HEK-293 cell extract after incubation with OA for 12 hours. Panel A shows the whole GC and a close-up of the peak of interest. Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for oleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 12 hours in this case before extraction.
Figure 2-15: GC-MS of HEK-293 Conditioned Media Extract after Incubation with OA for 24h, Showing Oleonitrile.

GC-MS of HEK-293 media extract after incubation with OA for 24 hours. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for oleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 24 hours in this case before extraction.
Figure 2-16: GC-MS of N18TG2 Cell Extract after Incubation with OA for 12h, Showing Oleonitrile

GC-MS of N18TG2 cell extract after incubation with OA for 12 hours. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for oleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 12 hours in this case before extraction.
Figure 2-17: GC-MS of N\textsubscript{18}TG\textsubscript{2} Conditioned Media Extract after Incubation with OA for 48h, Showing Oleonitrile

GC-MS of N\textsubscript{18}TG\textsubscript{2} media extract after incubation with OA for 48 hours. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for oleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 48 hours in this case before extraction.
Figure 2-18: GC-MS of SCP cell Extract after Incubation with OA for 48h, Showing Oleamide-TMS

GC-MS of SCP cell extract after incubation with OA for 48 hours. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (oleamide-TMS). Panel C shows the library database MS for oleamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 48 hours in this case before extraction.
Figure 2-19: GC-MS of SCP Conditioned Media Extract after Incubation with OA for 24h, Showing Oleonitrile

GC-MS of SCP media extract after incubation with OA for 24 hours. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for oleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM OA in 0.25 mM BSA for 24 hours in this case before extraction.
The amount of oleamide found in the conditioned media was similar to that found in the cells for all three cell lines at all time points (Figure 2-20). This could mean that these molecules may be in equilibrium with no specific transport protein to move them against a concentration gradient. Due to the high incubation concentration of OA (2.5 mM), there was no observed tailing off of oleamide production in the SCP and N18TG2 cells. The HEK-293 cells, however, did not show a significant increase in oleamide production after the first 12 hours (Figure 2-20). Another finding was that the SCP cells showed significantly higher amounts of oleamide (p<0.001 vs both N18TG2 and HEK-293 cell data).

Figure 2-20: Oleamide Production in SCP, N18TG2, and HEK-293 Cells after Oleic Acid Incubation
For incubation conditions, see materials and methods section. Top panel shows the amount of oleamide found in all three cell lines and media extracts. Bottom panel is close-up showing N18TG2 and HEK-293 only. Error bars shown are ± standard deviation.
N₁₈TG₂ cells had similar endogenous levels of oleamide as the HEK-293 cells, but showed much higher amounts of oleamide after incubation with oleic acid. It is possible that HEK-293 cells are either less capable of taking up oleic acid from its BSA carrier, is less able to convert OA to oleamide, or that these cells process oleamide back into OA through the action of FAAH more quickly than do N₁₈TG₂.

### 2.3.4 N-Acylglycine Assay Development

Initial experiments with separating NAGs via the same SPE method as was used for the primary amide resulted in recovery rates of approximately 35% in the 30 nmol NAG range, and samples below about 10 nmol were not recovered from the SPE columns at all. As a consequence of these findings, TLC was explored as a different method of NAG isolation. In addition, the BSTFA derivitization was optimized because the standard curve for NAGs had a lower slope than for PFAMs and FFAs.

#### 2.3.4.1 Optimization of NAG Derivitization

Because NAGs form four different reaction products: NAG-O-TMS, NAG-N-TMS, NAG-(TMS)₂ and an enyliminoacetate derivative (see Figure 2-3 and Figure 2-5) that make up three distinct peaks on the GC spectrum (NAG-O-TMS and NAG-N-TMS co-elute), the lower limit of detection for these molecules is higher than for molecules that generate fewer products. This is due to a minimal amount of the compound necessary to overcome the background in order to form an integrable GC peak. Therefore, it was necessary to optimize the BSTFA derivitization of NAGs in order to reach the lowest limit of detection for these compounds. Optimization of the reaction of BSTFA with NOG is shown in Figure 2-21. The optimal reaction conditions of those tested were 60°C for one hour in neat BSTFA.²

---

² PFAMs were found to have the same amount of derivitized products after 30 and 60 minutes of reaction time under these conditions (data not shown), but for consistency the reaction times were made the same for all species throughout all ensuing experiments.
Once reaction conditions were optimized, running conditions on TLC were optimized. Different TLC plates were tested (Figure 2-22) and different prewashing solvents tried (Figure 2-23), among other conditions. The best combination was found to be NP analytical TLC plates from EMD (Preparatory plates showed too much background interference from binder; data not shown), prewashed with chloroform:acetone 1:1. Finally, these conditions were used to show separation of HdG in N18TG2 media (Figure 2-24) and SCP cell extracts (Figure 2-25) that were spiked with 15nmol HdG prior to solvent extraction, TLC purification, and GC-MS analysis.

**Figure 2-21: Integration of NOG under Various Reaction Conditions**
Different reaction conditions were tested for NOG derivitization with BSTFA. Left panel shows the integration of each of the 3 NOG species. Right panel shows the species added together. The amount derivitized was 2 nmol NOG. Reaction conditions were in neat BSTFA, BSTFA in toluene, BSTFA spiked with TCMS, and BSTFA spiked with potassium acetate. Times and temperatures are indicated in the legends.
Figure 2-22: Recovery of NAGs on Various TLC Plates
GC chromatogram of 5 nmol HdG run on different normal phase TLC plates. The vertical line passes through the enyliminoacetate derivative of HdG. The peaks around 21 minutes are HdG-TMS. Plates were EMD brand (black; good recovery and low background), Dynamic with a thin plastic backing (pink; decent recovery but higher background), Dynamic with a normal glass backing (below the y-axis of this graph; low background but poor recovery) and Watman (brown; low background but mediocre recovery). Percent recoveries are shown in the bar graph on the right. The EMD plate showed the best recovery with the lowest background.

Figure 2-23: Determination of Best Prewashing Solvent for TLC
GC chromatogram of HdG run on TLC plates prewashed with various solvents. The vertical line passes through HdG-TMS and the left peak at 20.25 minutes is the enyliminoacetate derivative. Prewashing solvents were isopropanol (black) ether:acetic acid 99:1 (pink), chloroform:acetone 1:1 (blue) and none (brown). The recovery was not significantly improved, but background levels were lowered. Chloroform:acetone 1:1 showed the greatest decrease in background while maintaining the highest signal.
Figure 2-24: Recovery of HdG from Spiked N$_{18}$TG$_2$ Media

N$_{18}$TG$_2$ conditioned media was spiked with 15 nmol HdG before running the NAG extraction and quantification assay. For a scheme of the successful NAG assay, see section 2.1.2.2 of methods. Panel A shows the full GC (top) and closeup of HdG-TMS (bottom). Panel B shows the MS of the GC peak indicated with a vertical line. Panel C shows the database MS for HdG-TMS from the synthesized standard. Panel D shows the difference between panels B and C.
Figure 2-25: Recovery of HdG from Spiked SCP Cells
SCP cells were spiked with 15 nmol HdG before running the NAG extraction and quantification assay. For a scheme of the successful NAG assay, see section 2.1.2.2 of methods. Panel A shows the full GC (top) and closeup of HdG-TMS (bottom). Panel B shows the MS of the GC peak indicated with a vertical line. Panel C shows the database MS for HdG-TMS from the synthesized standard. Panel D shows the difference between panels B and C.
2.3.4.2 Extraction Efficiency for NAGs

To test the robustness of our NAG extraction method, NOG and HdG were subjected to the extraction procedure. Percent recoveries were 86% ± 18% and 91% ± 16%, respectively (Figure 2-26).

![Extraction Efficiency of NAGs](image)

**Figure 2-26: Extraction Efficiency of NAGs**

Extraction efficiency experiments were performed the same way as experimental samples (Figure 2-2). NAGs were dried down, extraction solvents added before sonication. Organic layers were combined and dried down. TLC was performed and a second plate run in tandem that was dyed to determine NAG position. Spots were scraped into anhydrous isopropanol, sonicated, centrifuged and the supernatant filtered in a 0.22µm syringe filter. The silica was re-sonicated, spun and filtered. Isopropanol supernatants were combined, spiked with HdA internal standard, and dried down before derivitization with BSTFA and analysis on GC-MS. The error bars are standard deviation from multiple extractions.

2.4 Discussion

Metabolite analysis revealed the presence of endogenous oleamide in all three cell lines tested: HEK-293, N18TG2 and SCP. Oleamide was also detected in conditioned
media (Table 2-3). Finding similar levels of oleamide in cell and conditioned media samples throughout all cell lines means that these molecules may be in equilibrium with no specific transport protein to move them against a concentration gradient. In addition, the production of oleamide increased after incubation with oleic acid. The previously reported amount of oleamide in N\textsubscript{18}TG\textsubscript{2} cells (55 pmol/10\textsuperscript{7} cells)\textsuperscript{23} is in fair agreement with measurements reported here (119 pmol/10\textsuperscript{7} cells). However, the amount reported by Sultana was much higher (16.67 pmol/cell)\textsuperscript{14} and may be due to contamination to slip additives (see footnote on p.34). We have taken precautions to minimize and test for these slip additives, and detailed procedures and discussion of this can be found in Chapter 3.

SCP cells had approximately 45-50 times as much endogenous oleamide as the other two cell lines, and at least 10 times as much lines after incubation with OA. This is consistent with the knowledge that choroid plexus produces cerebrospinal fluid,\textsuperscript{68} and PFAMs have been found in CSF.\textsuperscript{4,64,65} In light of the expression results in which the oleamide-degrading FAAH was not found in SCP cells, the ability of the choroid plexus to harbor and excrete these molecules in higher concentration is commensurate with the lack of detectable FAAH expression (see Chapter 4 for expression results and further discussion).

\textbf{Table 2-3: Endogenous Levels of Oleamide in Cells and Media}

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Endogenous oleamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCP cells media</td>
<td>6367</td>
</tr>
<tr>
<td>N\textsubscript{18}TG\textsubscript{2} cells media</td>
<td>119</td>
</tr>
<tr>
<td>HEK cells media</td>
<td>154</td>
</tr>
</tbody>
</table>

Values are average pmol/10\textsuperscript{7} cells
Endogenous amount refers to oleamide extracted, purified and quantified from cells and media under normal growing conditions without OA.

N\textsubscript{18}TG\textsubscript{2} cells had similar endogenous levels of oleamide as the HEK-293 cells, but showed much higher amounts of oleamide after incubation with OA (Figure 2-20). It is possible that HEK-293 express less fatty acid transport proteins, but another possibility is that these cells might be able to process the amide back into its
corresponding fatty acid through the action of FAAH more quickly than is N<sub>18</sub>TG<sub>2</sub>, a possibility made more likely based on the observed increase in oleamide from 0 to 12 hours. A high level of hydrolysis would prevent accumulation of oleamide, as is seen in the HEK-293 samples. This is consistent with the role the kidney plays in detoxification.

A TLC separation method for NAGs was developed because the SPE method successfully employed to identify PFAMs proved unsuitable for the small amounts of NAGs extracted from cultured cells. The TLC media performed well as a way to separate trace NAGs in spiked cell and media samples for identification, with recovery rates of 79% (HdG) and 84% (NOG, data not shown). Although liquid chromatography-hybrid quadrupole time of flight MS has greater sensitivity and can handle a more complex sample, GC-MS is less expensive and more widely available than the LC-QqTOF and related instruments. No recoverable endogenous amounts were found with the number of cells that were tested for these experiments. For successful use of this assay on a cell sample, refer to Chapter 5.

The extraction efficiencies of both the PFAM and NAG assays were fairly high (82-101%), and the limits of detection 1.5 nmol for NAGs and 0.5 nmol for oleamide. These assays were used to demonstrate the conversion of OA to oleamide in the three model cell lines and recover NAGs from spiked cell and conditioned media extracts.

### 2.5 References


(33) Juaneda, P.; Rocquelin, G. Lipids 1985, 20, 40.


(60) James, M. O.; Bend, J. R. *Biochem J* **1978**, **172**, 285.


(68) Engelhardt, B.; Sorokin, L. *Semin Immunopathol* **2009**.
Metabolic Profiling of Primary Fatty Acid Amides in SCP and N$_{18}$TG$_{2}$ cells

3.1 Review: Primary Fatty Acid Amides as Signaling Molecules

The first five primary fatty acid amides (PFAMs) to be isolated from a mammalian source were found 1989 in luteal phase plasma by Arafat et al: palmitamide, palmitoleamide, oleamide, elaidamide and linoleamide. At the time of their discovery, the biological function of the mammalian PFAMs was unknown. Several years later, oleamide was discovered to accumulate in the CSF of sleep-deprived cats and was misnamed cerebrodiene. Soon after, oleamide was correctly identified in other studies where it was shown to accumulate in the CSF during sleep deprivation and induce physiological sleep and hypomotility in mammals. In addition, brain oleamide levels in the ground squirrel were found to be ~2.5-fold higher in hibernating animals relative to that found in non-hibernating animals. Since these initial reports, more than 300 papers have been published about oleamide and other mammalian PFAMs. The purpose of this review is to summarize what is known about the mammalian endogenous PFAMs, including both in vitro and in vivo studies. An overview of PFAMs isolated from mammalian sources and endogenous amounts is given in Table 3-1.
Due to the early understanding of oleamide’s neurological actions, it has become the model PFAM and has been studied far more extensively than any of the other PFAMs. Oleamide has been found to modulate gap junction communication in glial cells, tracheal epithelial cells, seminiferous tubule cells, and fibroblasts, modulate memory processes, increase food intake, depress body temperature and locomotor...

<table>
<thead>
<tr>
<th>PFAM</th>
<th>Organism</th>
<th>Location in Organism</th>
<th>Amount</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristamide (C14:0)</td>
<td>Human</td>
<td>Meibomian secretion</td>
<td>Not reported</td>
<td>6</td>
</tr>
<tr>
<td>Palmitamide (C16:0)</td>
<td>Human</td>
<td>Meibomian secretion</td>
<td>Not reported</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>13.8 nmol/ml</td>
<td>5</td>
</tr>
<tr>
<td>Palmitoleamide  (C16:1 cis-Δ9)</td>
<td>Human</td>
<td>Plasma</td>
<td>15.6 nmol/ml</td>
<td>5</td>
</tr>
<tr>
<td>Stearamide (C18:0)</td>
<td>Human</td>
<td>Meibomian secretion</td>
<td>Not reported</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Not reported</td>
<td>25</td>
</tr>
<tr>
<td>Elaidamide (C18:1 trans-Δ9)</td>
<td>Human</td>
<td>Plasma</td>
<td>13.1 nmol/ml</td>
<td>5</td>
</tr>
<tr>
<td>Oleamide (C18:1 cis -Δ9)</td>
<td>Cat</td>
<td>CSF</td>
<td>10-50 nmol/ml</td>
<td>17,18</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Plasma</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>N18TG2 cells</td>
<td>55 nmol/10^10 cells</td>
<td>68,69</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Brain</td>
<td>82-99 nmol/g</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>16-26.6 nmol/g</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>CSF</td>
<td>0.16 nmol/ml</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.035 nmol/ml</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squirrel</td>
<td>Brain</td>
<td>0.91-2.3 nmol/g</td>
<td>23</td>
</tr>
<tr>
<td>Linoleamide (C18:2 cis,cis-Δ9,12)</td>
<td>Human</td>
<td>Plasma</td>
<td>7.8 nmol/ml</td>
<td>5</td>
</tr>
<tr>
<td>Eicosenoamide (C20:1cis-Δ13)</td>
<td>Rabbit</td>
<td>Brain</td>
<td>Trace (&lt;1.8 nmol)</td>
<td>13</td>
</tr>
<tr>
<td>Erucamide (C22:1cis-Δ13)</td>
<td>Human</td>
<td>Meibomian secretion</td>
<td>Not reported</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Dorsal air sac</td>
<td>Not reported</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>Plasma</td>
<td>0.011 nmol/g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>0.043 nmol/g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.0089 nmol/g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>0.0036 nmol/g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>0.0018 nmol/g</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Corneal micropocket</td>
<td>Not reported</td>
<td>56</td>
</tr>
</tbody>
</table>

*The validity of this work is under debate. While the MS of meibomian secretions show several PFAMs in the Nichols paper, other commonly observed compounds, including cholesterol esters and wax esters, were not present. In addition, other studies examining the lipid content of meibomian gland secretions did not find PFAMs.*
activity, act as an analgesic, reduce anxiety, and stimulate Ca\(^{2+}\) release. One of its most potent effects is as a vasorelaxant, with an EC\(_{50}\) of 1.2 µM.\(^{50}\)

The targets on which oleamide act have been found to include depressant drug receptors, and it has been found to act as an allosteric activator for serotonin (5-HT) receptor subtypes (5-HT\(_{1A}\), 5-HT\(_{2A}\), 5-HT\(_{2C}\), and 5-HT\(_{7}\))\(^8,9,29,57,58\) and for the GABA\(_A\) receptor.\(^{34-36,49,59,60}\) In addition to these neurotransmitter-type targets, oleamide has been shown to bind to CB\(_1\) cannabinoid receptors directly,\(^{61}\) indicating that its cannabinoid-like actions may not be due to the “entourage effect” alone, as was initially hypothesized.\(^{62}\) The entourage effect arises when one compound increases the effect of another compound by inhibiting its inactivation (in this case, as a competitor for degradation by FAAH hydrolysis or cellular uptake against other cannabinoids such as anandamide). The exact meaning of these data is under debate, as the concentrations required for cannabinoid binding may not be in the physiological range.\(^{43,63}\) Regardless of its role as an endogenous cannabinoid, oleamide does appear to have cannabinomimetic effects by increasing the amount of available anandamide through the entourage effect.\(^{64,65}\) An investigation showed that both oleamide and anandamide impaired memory in FAAH knockout mice,\(^{51}\) indicating that oleamide acts independently of an effect on this enzyme. \(N\)-Oleoylglycine (NOG) was recently found to have oleamide-like effects without affecting the amount of oleamide in blood plasma levels of rats.\(^{66}\) The amount of NOG required to produce a maximum hypothermic response was half the amount of oleamide required to produce the same response. In addition, oleamide-induced vasorelaxation was not affected by the presence of FAAH inhibitors.\(^{67}\) The implications of these findings are unclear.

Although oleamide has served as the model PFAM for analysis as a CNS signaling molecule and much has been uncovered about its physiological action, it is not necessarily the most abundant nor the most metabolically active PFAM. Many of the effects of other PFAM molecules have been found to be similar to those evoked by activation of the cannabinoid receptor systems, which include hypothermia, hypoactivity, analgesia, and catalepsy.\(^{70}\) The known function of PFAMs that have been isolated from
mammalian sources to date is summarized in Table 3-2, many of whose function(s) have mainly been studied *in vitro.*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Organism</th>
<th>Known Significance</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristamide</td>
<td><img src="image" alt="Myristamide Structure" /></td>
<td>Human</td>
<td>Unknown</td>
<td>6</td>
</tr>
<tr>
<td>Palmitamide</td>
<td><img src="image" alt="Palmitamide Structure" /></td>
<td>Human, Rabbit, Mouse&lt;sup&gt;a&lt;/sup&gt;, Sheep&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Unknown, modestly attenuates seizures in mice, modest FAAH inhibitor</td>
<td>5,6,11-13</td>
</tr>
<tr>
<td>Palmitoleamide</td>
<td><img src="image" alt="Palmitoleamide Structure" /></td>
<td>Human, Mouse&lt;sup&gt;a&lt;/sup&gt;, Sheep&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gap junction communication, serotonin receptor binding</td>
<td>5,10</td>
</tr>
<tr>
<td>Stearamide</td>
<td><img src="image" alt="Stearamide Structure" /></td>
<td>Human, Rabbit</td>
<td>Unknown</td>
<td>6,13,25</td>
</tr>
<tr>
<td>Elaidamide</td>
<td><img src="image" alt="Elaidamide Structure" /></td>
<td>Human</td>
<td>Less active than oleamide, but induces sleep, inhibits epoxide hydrolase and phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5,18,24,26</td>
</tr>
<tr>
<td>Oleamide</td>
<td><img src="image" alt="Oleamide Structure" /></td>
<td>Human, Mouse, Rabbit, Rat, Squirrel, Sheep&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sleep, memory, thermal and locomotor regulation, gap junction communication, Ca&lt;sup&gt;2+&lt;/sup&gt; flux, vasodilatation, hunger, anxiety, serotonin receptor binding, erg current inhibition</td>
<td>5,6,13,1 5,17,18,21-23,25,27-52</td>
</tr>
<tr>
<td>Linoleamide</td>
<td><img src="image" alt="Linoleamide Structure" /></td>
<td>Human, Rabbit, Mouse&lt;sup&gt;a&lt;/sup&gt;, Sheep&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sleep and Ca&lt;sup&gt;2+&lt;/sup&gt; flux regulation, erg current inhibition, epoxide hydrolase and phospholipase A&lt;sub&gt;2&lt;/sub&gt; inhibition, gap junction communication, FAAH substrate, serotonin receptor binding</td>
<td>1,10,13-15,20,24 ,26,53</td>
</tr>
<tr>
<td>Eicosenoamide</td>
<td><img src="image" alt="Eicosenoamide Structure" /></td>
<td>Rabbit</td>
<td>Unknown</td>
<td>13</td>
</tr>
<tr>
<td>Erucamide</td>
<td><img src="image" alt="Erucamide Structure" /></td>
<td>Cat, Cow, Human, Rat, Pig, Mouse</td>
<td>Fluid balance, angiogenesis</td>
<td>6,54-56</td>
</tr>
</tbody>
</table>

<sup>a</sup>Demonstrated in this study for N<sub>18</sub>TG<sub>2</sub> (mouse) or SCP (sheep) and not in previous studies.

A recent article by Nichols *et al.* indicated the presence of several PFAMs from human meibomian grand secretions. The validity of these findings is under debate. While the MS of meibomian secretions show several PFAMs in the Nichols paper, other commonly observed compounds, including cholesterol esters and wax esters, were not
present.\textsuperscript{72} In addition, other studies examining the lipid content of meibomian gland secretions did not find PFAMs.\textsuperscript{73} Although oleamide, erucamide and stearamide are known slip additives\textsuperscript{74} added to lubricate plastics during manufacturing, the other PFAMs found in this study have not been reported as plasticizers, one of which is myristamide (C14:0).\textsuperscript{6} This is the first report of myristamide from a mammalian source and its function, other than its potential to promote tear film stability and in maintaining a smooth refractive surface of the eye in meibomian secretions, is completely unknown. It does show some antibacterial properties\textsuperscript{75} and has been demonstrated as a substrate for fatty acid amide hydrolase (FAAH) \textit{in vitro}.\textsuperscript{2,14}

Palmitamide (C16:0) was also reported in human meibomian gland secretions,\textsuperscript{6} but other studies confirm its presence as a mammalian PFAM in human leutal phase plasma in ten of sixteen female subjects\textsuperscript{5} and in rabbit brain and heart\textsuperscript{13} (see Table 3-1). Again, these are the only reports of palmitamide in mammals and its role is mostly speculative. Studies showed that palmitamide is a modest inhibitor of Fatty Acid Amide Hydrolase (FAAH) \textit{in vitro}\textsuperscript{2,7,12} and modestly attenuates seizures in mice,\textsuperscript{11} but more work is necessary to explore its potential role as a mammalian signaling molecule.

Palmitoleamide (C16:1 \textit{cis-}\text{\textDelta}^9) has only been reported in one study as an endogenous compound and its \textit{in vivo} role remains mostly unexplored.\textsuperscript{5} An \textit{in vitro} study showed that palmitoleamide is able to block gap junction communication in mouse glial cells as well as oleamide does,\textsuperscript{15} and can potentiate the 5-HT\textsubscript{1A} serotonin receptor after incubation with serotonin almost as well as can oleamide\textsuperscript{10} (Table 3-3). These roles are not surprising given the known function of oleamide in binding to 5-HT receptors and in blocking gap junction communication. Oleamide and palmitoleamide differ by only two carbons in acyl chain length, and further studies may demonstrate additional similarities in function.

Elaidamide (C18:1 \textit{trans-}\text{\textDelta}^9), while only different from oleamide as a \textit{trans-} isomer at the double bond, does not have as much biological activity as oleamide. Because of its close structural relationship to oleamide, it has been used in many studies to demonstrate specificity of receptors and enzymes for the \textit{cis} isomer oleamide, and an overview of oleamide activity as compared to other PFAMs (where numerical values were available)
is given in Table 3-3. Elaidamide does not attenuate gap junction communication in mouse glial cells as oleamide does, and elaidamide does not serve as an effective binder for 5-HT$_{1A}$ or 5-HT$_{7}$ receptors as palmitoleamide (5-HT$_{1A}$ only), linoleamide, and oleamide do. Elaidamide does not modulate GABA-evoked current and is unable to modulate voltage-gated Na$^+$ channels as oleamide does.

Two enzymatic studies have shown function for elaidamide: it was found to be a potent inhibitor of both human and rat microsomal epoxide hydrolase through simultaneous competitive and noncompetitive inhibition, and it is also an excellent inhibitor of phospholipase A$_2$. Cravatt et.al showed that elaidamide can induce sleep, but only half as long as was observed for oleamide (1 hour as opposed to 2 for a 10 mg dose in rat), even though elaidamide was not found to be an agonist for CB$_1$ receptors transfected into HEK-293T cells. This action may be due to binding to GABA receptor(s). Elaidamide was found to inhibit binding to a voltage-gated Na$^+$ channel of an agonist, but not nearly as effectively as was oleamide. Similarly, elaidamide was found to inhibit repetitive firing of rat embryonic cultured cortical pyramidal cells, but oleamide was 3 times as effective at doing so. Elaidamide is biologically active but not nearly to the extent as its cis isomer. Given the fact that the trans fatty acids are not naturally found except in some dairy products and in the meat of ruminants, the lowered biological activity of elaidamide is concomitant with the fact that the enzymes evolved alongside the cis isomer almost exclusively. There has been only one report of elaidamide as an endogenous mammalian PFAM, and it is likely to have come from a dietary source.
Table 3-3: Relative Activity of PFAMs on Various Targets

<table>
<thead>
<tr>
<th>Target</th>
<th>Oleamide (C18:1 cis-Δ9)</th>
<th>Elaidamide (C18:1 trans-Δ9)</th>
<th>Stearamide (C18:0)</th>
<th>Linoleamide (C18:2 cis,cis-Δ9,12)</th>
<th>Myristamide (C14:0)</th>
<th>Palmitamide (C16:0)</th>
<th>Palmitoleamide (C16:1 cis-Δ9)</th>
<th>Eicosenoamide (C20:1 cis-Δ13)</th>
<th>Erucamide (C22:1 cis-Δ13)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT7</td>
<td>1</td>
<td>0.86</td>
<td>-</td>
<td>0.04</td>
<td>0.91</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>5-HT2C</td>
<td>1</td>
<td>6.67</td>
<td>-17.3</td>
<td>0.44</td>
<td>0.88</td>
<td>-0.16</td>
<td>0.32</td>
<td>0.16</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>5-HT1A</td>
<td>1</td>
<td>0.2</td>
<td>0.32</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>5-HT2A</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.44</td>
<td>0.88</td>
<td>-0.16</td>
<td>0.32</td>
<td>0.16</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>erg inhibition</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>FAAH activity</td>
<td>1</td>
<td>1.54</td>
<td>0.33</td>
<td>0.93</td>
<td>0.47</td>
<td>1</td>
<td>2</td>
<td>7</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>GABAA potentiation</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>Gap junction inhibition</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>5-10 HT potentiation</td>
<td></td>
</tr>
<tr>
<td>mEH inhibition</td>
<td>1</td>
<td>0.98</td>
<td>0.063</td>
<td>1.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>PLA2 inhibition</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Sleep induction</td>
<td>1</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>(time of sleep)</td>
<td>1</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SRF inhibition</td>
<td>1</td>
<td>0.33</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

Relative activity. Activity values for oleamide are set to 1 and activities of other compounds displayed as relative to oleamide activity. Negative values are for negative potentiation (receptor inhibition).

Abbreviations: 5-HT, 5-hydroxytryptamine; erg, ether-à-go-go-related gene; mEH, microsomal epoxide hydrolase; PLA2, phospholipase A2; SRF, sustained repetitive firing (subsequent to a primary action potential) of pyramidal cells.

Values shown are for study with 50 μM PFAM. 20 and 100 μM studies were also done.15

Values shown are for porcine pancreatic PLA2. Several other species’ were also studied.26

Values shown are for mouse N18TG2 cells

d from mouse N18TG2 cells

e rat recombinant

Stearamide (octadecanamide, C18:0) has been found in human blood plasma25 and in rabbit brain and heart,13 but no true in vivo bioactivity has yet been identified; it has mostly been studied in tandem with oleamide to compare the alkyl chain with its unsaturated bioactive cousin much the way elaidamide has been studied as a trans isomer.
of oleamide. Stearamide is not as active as oleamide on 5-HT receptors, and even shows the opposite effect on 5-HT$_{2C}$ receptors to decrease, rather than increase, the efficacy of receptor activation (Table 3-3). It also does not attenuate gap junction communication in mouse glial cells and is 15 times less effective than oleamide as a microsomal epoxide hydrolase inhibitor. Stearamide did not produce a decrease in core body temperature after intraperitoneal administration to rats as did oleamide and N-oleoylglycine. The activity of stearamide relative to other PFAMs is also included in Table 3-3. Elaidamide is mildly biologically active, but stearamide even less so.

Aside from oleamide, linoleamide (C18:2 cis,cis-$\Delta^9,12$) is the most studied endogenous PFAM, and is found to have many of the same activities as oleamide (Table 3-3). It inhibits microsomal epoxide hydrolase as well as oleamide does, and inhibits gap junction communication in glial cells almost as well as does oleamide. Linoleamide was found to induce calcium release from the endoplasmic reticulum of renal tubular cells. Both oleamide and linoleamide were found to block the erg (ether-à-go-go-related gene) current equally well in rat pituitary cells. Linoleamide is an excellent competitive inhibitor for phospholipase A$_2$, as is oleamide, but linoleamide is a better inhibitor and substrate for FAAH than is oleamide. It was found to potentiate the 5-HT$_{1A}$ receptor nearly as well as oleamide, but for the 5-HT$_{2A}$ receptor it demonstrated only 44% of oleamide’s potentiation. It is interesting that one fewer double bond (stearamide) results in very little biological function, while one greater double bond (linoleamide) does not result in significant change in biological function compared to that of oleamide.

The least well-known of the endogenously isolated PFAMs is eicosenoamide (C20:1 cis-$\Delta^{13}$), which was observed in trace amounts in rabbit brain. Eicosenoamide has shown some potentiation of 5-HT receptors, but it does not inhibit gap junction communication by glial cells. Interestingly, the $\Delta^{11}$ isomer is an excellent inhibitor.

Erucamide (C22:1cis-$\Delta^{13}$) does not have a significant effect on the binding affinity of 5-HT$_7$ receptors to 5-hydroxytryptamine and does not inhibit gap junction communication as oleamide does. It does, however, have its own biological function and has been found in many places (Table 3-1), including porcine blood plasma,
where it was found to regulate fluid volumes in various organs.\textsuperscript{55} The major bovine mesentery angiogenic lipid was identified as erucamide, and 2 µg was found to induce angiogenesis.\textsuperscript{56} Mitchell \textit{et al.} showed that a sustained release of erucamide from a polymer matrix has a dose-dependent angiogenic effect on skeletal muscle regeneration after an injury,\textsuperscript{54} and it is speculated that this control of water content combined with repair of circulation may have a synergistic effect on the regenerative process.\textsuperscript{55}

Although many PFAMs have been known for two decades, little is known about their action \textit{in vivo} except for oleamide. What is known, however, points to an interesting role for these molecules in signaling and demonstrates that PFAMs can belong to this class of bioactive molecules known as lipid messengers.

The biosynthetic and catabolic mechanisms of these long-chain PFAMs are discussed briefly in Chapter 1 and at length in Chapter 4. It has already been shown that N\textsubscript{18}TG\textsubscript{2} cells contain oleamide\textsuperscript{69} and convert oleic acid to oleamide,\textsuperscript{68,69} results which were confirmed and expanded by quantifying over a time course in Chapter 2. These kinds of studies on PFAM metabolism have not been expanded to any of the other acyl groups beyond (18:1 \textit{cis}\textsubscript{Δ\text{9}}). Since some of these PFAMs have been found in mammalian sources, it is likely that endogenous amounts of PFAMs other than oleamide could be found in N\textsubscript{18}TG\textsubscript{2} cells. There are many additional questions that might be answered through studies of other PFAMs: are other FFAs converted to their corresponding amides? Can non-natural FFAs be converted to their amides? Do the cells secrete other PFAMs? Do the cells convert certain FFAs more quickly or in more abundance than others, and is there a recognizable trend in chain length, degree of unsaturation, or \textit{cis}/trans isomers? Does the fingerprint of PFAM formation follow the enzyme substrate kinetics of the proposed biosynthetic and hydrolyzing enzymes? Are the related compounds, \textit{N}-acylethanolamines (NAEs) converted to PFAMs? Is there a difference in any of these trends in different cell lines?

In addition to N\textsubscript{18}TG\textsubscript{2} cells, which are known to contain all the enzymatic machinery to convert oleic acid directly to oleamide,\textsuperscript{68,69} Chapter 2 the sheep choroid plexus (SCP) cells were chosen. SCP cells had approximately 45 times more endogenous oleamide than was found in the other two cell lines, N\textsubscript{18}TG\textsubscript{2} and HEK-293. In addition,
after incubation with oleic acid, SCP cells still had at least 10 times as much oleamide as the other two cell lines.

In this study, we examined the metabolism of a panel of fatty acids to their respective PFAMs in N18TG2 and SCP cells. The FFAs chosen correspond to endogenously found PFAMs: palmitic acid (PA), palmitoleic acid (POA) elaidic acid (EA), and linoleic acid (LOA). Data from the oleic acid (OA) incubations presented in Chapter 2 is included herein for discussion purposes. In addition, two non-natural PFAM precursors were studied: tridecanoic acid (TDA) and tridecanoylethanolamine (TDEA) (see Section 3.2)

Some PFAMs, particularly oleamide and erucamide, have been found as slip additives in polyethylenes,\textsuperscript{74,79,80} plasticizers whose presence has brought under debate the finding of PFAMs in meibomian secretions.\textsuperscript{72} A recent article demonstrated the extraction of erucamide from pipette tips by DMSO.\textsuperscript{79} This must be taken into consideration when reviewing older literature and when preparing to perform PFAM extracts. Care has been taken to test for the presence of these slip additives in this study (see Section Error! Reference source not found.).

3.2 Formation of PFAMs from Two Distinct Pathways: FFA and NAE

The formation of PFAMs from an N-acylglycine (NAG) through the action of bifunctional peptidylglycine α-amidating monooxygenase (PAM) has been studied \textit{in vitro}, with kinetic constants comparable to peptide precursor substrates.\textsuperscript{4,81} In addition, chemical inhibition of PAM by \textit{trans}-4-phenyl-3-butenolic acid in N18TG2 cell culture resulted in the accumulation of \textit{N}-oleoylglycine when these cells were grown in the presence of oleic acid\textsuperscript{68} (see also Chapter 5).

The formation of the NAGs, however, is much more controversial. There have been two main pathways proposed, as seen in Figure 3-1. More details on the biosynthetic mechanism(s), including an in-depth discussion of potential enzymes, are discussed in Chapter 4.
Activation of a fatty acid through the formation of a coenzyme A (CoA) intermediate is one obvious and much-hypothesized possibility for acyl chain activation before glycination.\cite{4,68,81,82} There are several acyl-CoA synthetases known to perform this fundamental metabolic chemistry,\cite{83,84} and several candidates to perform glycination of an acyl-CoA, including acyl-CoA:glycine N-acyltransferase,\cite{85,86} bile acid-CoA:amino acid N-acyltransferase,\cite{87} and cytochrome c.\cite{88,89} The glycination pathway is shown in purple in Figure 3-1.

**Figure 3-1: Formation of NAG and PFAM by Two Metabolic Pathways**

The purple arrows show one pathway with successive CoA-ylation and glycination of the FFA; the “glycination pathway.” The brown arrows show successive oxidation of an NAE to form the NAG; the “NAE oxidation pathway.” The NAG is converted to a PFAM by PAM. The two pathways are joined by the action FAAH, which is able convert the NAE into a FFA. More details, including a discussion of potential metabolic enzymes, are in Chapter 4.

Abbreviations: FFA, free fatty acid; FAAH, fatty acid amide hydrolase; NAE, N-acylethanolamine; NAG, N-acylglycine; PAM, peptidylglycine α-amidating monooxygenase; PFAM, primary fatty acid amide.
Sequential oxidation of an \( N \)-acylethanolamine (NAE) is another hypothesized mechanism for NAG biosynthesis, and is indicated in brown in Figure 3-1. In cellulo work has demonstrated the oxidation of NAEs to NAGs in RAW 264.7, C6 glioma and Chang liver cells.\(^{90,91}\) Ivkovic \textit{et al.} showed that an alcohol dehydrogenase, ADH3, is capable of oxidizing an NAE to its acylglycinal,\(^{92}\) which could then be oxidized to the NAG by an aldehyde dehydrogenase, and Aneetha \textit{et al.} showed that ADH7 is capable of oxidizing an NAE fully to a NAG \textit{in vitro}.\(^{93}\) See Chapter 4 for more information on NAE oxidation to NAGs.

These pathways are not mutually exclusive; Bradshaw \textit{et al.} showed that an NAE could be converted to its corresponding NAG either through successive oxidation or through a glycine conjugation (both purple and brown pathways, Figure 3-1) in one cell line.\(^{90}\) In this study, we examined the metabolism of two exogenous acyl compounds, TDEA and TDA, to compare the rates of conversion to tridecanamide in both the SCP and N\textsubscript{18}TG\textsubscript{2} cells and gain some kinetic insight into the competing metabolisms in these two cell lines.

### 3.3 Materials and Methods

**3.3.1 Materials**

Elaidic and palmitoleic acids were from Fisher (Pittsburgh, PA). Linoleic and palmitic acids, 6-thioguanine and fatty acid-free BSA were from Sigma (St. Louis, MO). FBS was from Atlanta Biologicals (Lawrenceville, GA). Penicillin/streptomycin, EMEM and DMEM were from Mediatech Cellgro (Manassas, VA). BSTFA and silica were from Supelco (St. Louis, MO). Mouse neuroblastoma N\textsubscript{18}TG\textsubscript{2} cells were from DSMZ (Deutsche Sammlung von Mikroorganism und Zellkulturen GmBH). SCP cells were from American Type Culture Collection (Manassas, VA). Heptadecanoic acid, D\textsubscript{33} was from C/D/N isotopes (Pointe-Claire, Quebec).
3.3.2 Standard Synthesis

3.3.2.1 Acyl Chloride Synthesis

The free fatty acid (FFA) and thionyl chloride were combined neat in a 1:2 molar ratio under nitrogen while stirring (Equation 3-1). The reaction was allowed to proceed under reflux at 50°C for 30 minutes. Excess unreacted thionyl chloride was removed by heating or under vacuum until the solution no longer produced gas.

\[
\begin{align*}
\text{R-} & \text{O} & \text{Cl-S-O} & \rightarrow & \text{R-} & \text{O} & + & \text{SO}_2 & + & \text{HCl} \\
\text{Equation 3-1: Synthesis of Acyl Chloride from a Fatty Acid}
\end{align*}
\]

3.3.2.2 PFAM Synthesis

For use as a standard, a series of PFAMs were synthesized. Commercially available acyl chlorides were used or, where not available, synthesized as above. The PFAM was prepared similar to Fong et. al.\textsuperscript{94} The undistilled acyl chloride was added dropwise to ice cold concentrated NH\textsubscript{4}OH (29% NH\textsubscript{3}) while stirring and in a ratio of 2.5 ml acyl chloride:15 ml NH\textsubscript{4}OH (see Equation 3-2). Precipitation was allowed to go to completion. Excess NH\textsubscript{4}OH was removed by filtering with cold water. The sample was allowed to dry and stored at -20°C flushed under nitrogen. For GC-MS of synthesized PFAMs, see Appendix B.

\[
\begin{align*}
\text{R-} & \text{O} & \text{Cl} & \rightarrow & \text{R-} & \text{O} & + & \text{NH}_3 & + & \text{HCl} \\
\text{Equation 3-2: Synthesis of PFAM from Acyl Chloride}
\end{align*}
\]
3.3.2.3 TDEA Synthesis

Tridecanoyl chloride was synthesized as in Section 3.3.2.1. The N-tridecanoylthanolamine (TDEA) was synthesized as shown in Equation 3-3 as follows: triethylamine base, acetonitrile, and ethanolamine were combined in a 1.5:1:1 ratio (vol), flushed under nitrogen, and left stirring under nitrogen at room temperature. The acyl chloride taken up in a small volume of acetonitrile and added dropwise to the ethanolamine mixture and left to stir overnight at room temperature.

The N-acylethanolamine reaction was taken to a yellow solid in vacuo, dissolved in a small volume warm ethanol, and recrystallized by adding cold H2O. The crystallization was allowed to continue on ice for approximately 30 min, then the solid was filtered in Buchner funnel and washed with ice cold H2O. The product was allowed to dry, flushed with nitrogen and stored at -20°C. For a GC-MS of the synthesized TDEA, see Appendix B.

![Equation 3-3: Synthesis of 3-13C, 15N-Oleoylethanolamine](image)

3.3.3 Fatty Acid-BSA Mixture

Fatty acids were dissolved in ethanol and saponified with excess NaOH (~2x molar concentration), dried under vacuum and dissolved in phosphate-buffered saline (PBS) to make a 25 mM solution. Sample was then heated in 49°C water bath for 5 minutes before adding 2.5 mM BSA. Sample was stirred at room temperature for 4 hours, sterile-filtered, and stored at -20°C. The fatty acids that were used are listed in

---

© BSA forms globules above 50°C that make the solution impossible to sterile-filter through a 0.22 µm filter
Table 3-4. An N-acylethanolamine, tridecanoyl ethanolamine (TDEA) solution was made similarly, but an additional sonication step was added prior to addition of BSA.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tridecanoic Acid (TDA)</td>
</tr>
<tr>
<td>Palmitic Acid (PA)</td>
</tr>
<tr>
<td>Palmitoleic Acid (POA)</td>
</tr>
<tr>
<td>Linoleic Acid (LOA)</td>
</tr>
<tr>
<td>Oleic Acid (OA)</td>
</tr>
<tr>
<td>Elaidic Acid (EA)</td>
</tr>
</tbody>
</table>

### 3.3.4 Cell Culture and Fatty Acid Incubation

All cells were grown in 225 cm$^2$ culture dishes. N$_{18}$TG$_2$ cells were grown in DMEM supplemented with 100 µM 6-thioguanine. SCP cells were grown in EMEM supplemented with 100 µM sodium pyruvate. Both cell types were grown with 100 I.U./ml penicillin, 1.0mg/ml streptomycin and 10% FBS at 37°C and 5% CO$_2$. Cultures were grown to 75-90% confluency, culture medium removed and cells washed with PBS. After washing, the culture medium was replaced with media containing 0.5% FBS and 10% fatty acid-BSA mixture for a final concentration of 2.5 mM fatty acid in 0.25 mM BSA. Three T-225 cm$^2$ flasks were used for each time point, for a total of 12 flasks for each experiment. After 0, 12, 24, or 48 hour incubation, media was collected, cells washed with PBS and trypsonized. An aliquot was taken for counting on a hemacytometer with trypan blue. Cells were centrifuged and the pellet and conditioned media stored at -80°C.

Zero hour samples were collected without addition of fatty acid-BSA mixture or 0.5% FBS media but collected in 10% FBS media under normal growing conditions. These cells and the conditioned media were also stored at -80°C. Several controls were run with incubation for 48 hours in media containing 0.5% FBS and 0.25 mM BSA (see section Error! Reference source not found.).
3.3.5 Metabolite Extraction

Metabolites were extracted from cells similar to Sultana and Johnson (2006). 4 ml methanol was added to cell pellets and samples were sonicated for 15 minutes at room temperature. Samples were centrifuged at 5000 rpm for 10 min, and the supernatant separated from the pellet, dried under N$_2$ in a warm water bath at 40-50°C. The pellet was re-extracted with 4 ml 1:1:0.1 (v/v/v) chloroform:methanol:water, sonicated for 10 min, vortexed 2 min and centrifuged 10 min at 5000 rpm. Supernatant from this step was added to the dried supernatant from the previous step and dried under N$_2$ at 40-50°C. The pellet was re-extracted with 4.8 ml chloroform:methanol 2:1 (v/v) and 800 µl 0.5 M KCl/0.08 M H$_3$PO$_4$, sonicated 2 min, vortexed 2 min, and centrifuged 10 min at 5000 rpm. The lower lipid phase was added to the dried supernatant from the previous steps and dried under N$_2$ at 40-50°C.

Metabolite extraction from conditioned media was similar. After spinning down to remove any cells, the entire volume of media collected from three flasks (~135 ml) was subjected to solvent extraction. The first two extractions were with 15 ml chloroform:methanol 2:1 and the second two extractions with 15 ml chloroform:methanol 2:1 and 2.4 ml 0.5 M KCl/0.08 M H$_3$PO$_4$. No sonication was performed, and protein layers were condensed by centrifugation for 30-45 min at 10,000 rpm. Organic lipid phases were also combined and dried under N$_2$ at 40-50°C or in vacuo.

3.3.6 Solid Phase Extraction

Silica columns (0.5 g) were washed with n-hexane and run essentially as described by Sultana and Johnson. Lipid extracts were taken up in 100 µL n-hexane and added to washed silica columns. The mobile phase was run without positive pressure as follows: 4 ml n-hexane, 1 ml 99:1 hexane:acetic acid, 1 ml 90:10 hexane:ethyl acetate, 1 ml 80:20 hexane:ethyl acetate, 1 ml 70:30 hexane:ethyl acetate, 1.5 ml 2:1 chloroform:isopropanol, 0.5 ml methanol. The last two fractions were combined and dried down under N$_2$ at 40-50°C. An internal standard, heptadecanoic acid, D$_{33}$ (3 nmol), was added and dried before derivitization.
3.3.7 Sample Derivitization

Trimethylsilylation was achieved using 100µL BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide). Samples were flushed with dry N₂, BSTFA added, flushed briefly again, and allowed to react at 55-60°C for 1 hour before running on GC-MS.

3.3.8 GC-MS

All separations were performed using a Shimadzu QP-5000 GC-MS. Separations were achieved on a J & W Scientific (Folsom, CA) DB-5 column (0.25 mm x 30 m) in splitless mode. The GC temperature program was 55-150°C at 40°C/min, hold at 150°C for 3.6 min, ramp at 10°C/min to 300°C, and hold for 1 min. The transfer line was held at 280°C and the injection port at 250°C throughout the separation. Helium was used as the carrier gas, at a flow rate of 0.9 ml/min. The mass range was 35-450 amu with a scan speed of 2000. The solvent cut time was set to 7 min.

Samples were injected twice; 33.3% of the sample volume, followed by 50% of the remaining sample volume, such that 1 nmol internal sample was in each sample run. After running a set of experimental samples through the GC-MS (0, 12, 24 and 48 hours, each injected twice), one of the 24 or 48 hour samples was dried down, spiked with the PFAM of interest, re-dried down, re-derivitized with BSTFA, and re-run on the GC-MS to validate sample retention times. Figure 3-2 shows an overlay of an experimental and a spiked sample.
Controls

Solvents were run through all the same glassware and plasticware as cell and media samples and were subjected to sonication, solid phase extraction, and derivitization to test for contaminating plasticizers. Integration of the GC trace was taken over the same time integral as standard amides and for the same set of ions (Table 3-5). An averaged number of cells was used to calculate blank amount of amide per cell in the blank samples (see equation below). Unspent media was also subjected to extraction to check for existing amides.

\[
\text{amide/cell} = \frac{\text{amide extracted from cells} - \text{amide in blank}}{\# \text{ cells}}
\]

In addition to unspent media and glass/plasticware, conditioned media and unspent media were incubated with various metabolites and tested to see if PFAMs accumulated over time without any cells present. Unspent media was incubated with either OA, NOG or NOE at 37°C for 48h. After incubation with N18TG2 cells for 48h (sans metabolites), conditioned media was sterile-filtered with a 0.45 µm filter to remove
any cells. The conditioned media was then incubated with OA, NOG or NOE in BSA at 37°C for an additional 48h. Extraction for PFAM analysis was carried out as normal.

Finally, to test for the change in oleamide production over time without the presence of any metabolites (FFAs, NAEs or NAGs), SCP and N₁₅TG₂ cells were incubated for 48h with media containing 0.5% FBS and 0.25 mM BSA but no long chain fatty acyl metabolite. Cells and conditioned media were both collected and subjected to PFAM analysis as normal (Sections 3.3.5-3.3.8).

### 3.3.10 Data Analysis

Total ion chromatograms (TIC) were taken to more clearly determine the identities of species present in the sample. Post-run, a set of selected ions unique to the amides and nitriles under examination was overlaid and integrated so that effects of any co-eluting compounds could be minimized. These unique ion sets are based on electron-impact (EI) fragmentation of the PFAMs and nitriles and can be found in Table 3-5. Fragmentations for nitriles (Figure 3-3) and PFAM-TMS (Figure 3-4) are also given below.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Selected m/z’s</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptadecanoic, D₃₃ (HdA)</td>
<td>119, 135, 149, 360, 375</td>
<td>15.1</td>
</tr>
<tr>
<td>Tridecanonitrile</td>
<td>82, 97, 110, 124, 138, 147, 152, 166, 170, 188, 192, 195</td>
<td>9</td>
</tr>
<tr>
<td>Tridecanamide</td>
<td>93, 100, 128, 131, 158, 170, 186, 200, 213, 270, 285</td>
<td>13.4</td>
</tr>
<tr>
<td>Palmitoleonitrile</td>
<td>122, 136, 150, 164, 178, 192, 206, 235</td>
<td>12.66</td>
</tr>
<tr>
<td>Palmitoleamide</td>
<td>59, 116, 128, 131, 144, 184, 198, 200, 240, 253, 310, 325</td>
<td>16.07</td>
</tr>
<tr>
<td>Palmitonitrile&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69, 110, 124, 138, 152, 166, 180, 194, 208, 237</td>
<td>12.9</td>
</tr>
<tr>
<td>Linoleonitrile&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95, 109, 120, 134, 148, 162, 176, 261</td>
<td>14.8</td>
</tr>
<tr>
<td>Linoleamide</td>
<td>59, 67, 81, 91, 95, 109, 116, 119, 121, 128, 131, 135, 144, 147, 149, 336, 352, 279</td>
<td>15.98</td>
</tr>
<tr>
<td>Oleo/elaido-nitrile</td>
<td>83, 97, 110, 122, 136, 150, 164, 190, 206, 220, 234, 263</td>
<td>14.9</td>
</tr>
</tbody>
</table>

The retention times listed here for each PFAM is the for the –TMS derivative, as opposed to the underivitized amide. These retention times were all accurate for the GC-MS through February 2009, at which point some major repairs were done to the machine. After the repairs, all retention times were shifted about 2 minutes earlier.

<sup>a</sup>Palmitamide-TMS was not integrated due to the coelution of large amounts of oleic acid-TMS and octadecanoic acid-TMS that interfered with even the selected ion integration. Amounts of palmitamide were determined based on the palmitonitrile only, using standard curves of palmitonitrile only

<sup>b</sup>Linoleamide-TMS was not integrated for a similar reason as for palmitamide-TMS, and only linoleonitrile was integrated, using standard curves of linoleonitrile only.
Figure 3-3: Fragmentation Patterns for Long Chain Acyl Nitriles
Shown here are some of the most common fragments for electron impact ionization of the long chain acylnitriles that result from derivitization of a long chain PFAM with BSTFA. Not all the fragments from Table 3-5 are shown here.
Figure 3-4: Fragmentation Patterns of PFAM-TMS Compounds

Shown here are some of the most common fragments for electron impact ionization of PFAM-TMS compounds. Not all the fragments from Table 3-5 are shown here.
Integration of samples was based on the summation of their selected ion chromatograms to reduce interference from any co-eluting contaminants. Figure 3-5 shows how this analysis was performed. The examination of a specific multiple ion chromatogram (MIC) composed of a set of selected ions allows the accurate integration of compounds even with the presence of coeluting substances, and has literature precedence. There is a certain danger that isotopic peaks of coeluting compounds may contribute to the integration of the compound of interest, which is why care is taken to run blanks for all cell and media samples, both as solvents unexposed to any cell or media and media exposed to no cells.

**Figure 3-5: MIC Analysis of a GC Peak**
GC spectrum of tridecanamide-TMS in SCP cells. Panel A shows the TIC. Panel B shows the m/z 131 overlaid in pink on the TIC. Panel C shows the selected characteristic m/z ions for tridecanamide-TMS overlaid in colors over the TIC (black). Panel D shows the MIC, the summation of the individual selected ions. The horizontal red line indicates the integrated area. The y-axis is relative intensity and all graphs are plotted on the same scale. Note that in panel D the background peaks have diminished.
Heptadecanoic Acid, D$_{33}$ (HdA), was added to each sample as an internal standard to test the performance of the instrument. The amides and nitriles of interest were integrated along with the HdA, both compared to their standard curves, and a correction factor determined based on the integration of HdA compared to its standard curve was used to adjust the amount of amide. Once the area under an amide peak was used to determine the number of nmol PFAM, it was divided by the number of cells worth of extract injected (i.e. if 1/3 of the sample was injected, the nmol amide was divided by 1/3 the total number of cells counted for that sample). For media samples, the nmol amide was divided by the cell count from the cell sample taken from the same flasks rather than dividing by volume of media, so that the amount of amide secreted per cell could be estimated. Each extraction sample was run on the GC-MS twice, and each incubation run at least twice to provide replicates. T-tests were run using GraphPad.

3.4 Results

3.4.1 GC-MS of FFA-Incubated Cells and Media

All FFAs in both cell lines were found to be converted to the corresponding PFAM. For brevity, only a panel of GC-MS from cells incubated with linoleic acid is shown in this chapter (Figure 3-6 through Figure 3-9), with one representative time point for each sample. GC-MS for cells and conditioned media after incubation with each FFA and TDEA can be found in Appendix C. Due to a large amount of interfering background in the range of 17.5 – 20 minutes in the conditioned media containing linoleic acid, the amount of amide was calculated using the nitrile only, plotted against a standard curve of linoleonitrile from the linoleamide derivitization reaction. Similarly, oleic acid and octadecanoic acid co-eluted with palmitamide-TMS, so only palmitonitrile was integrated and compared to a standard curve of palmitonitrile only.
Figure 3-6: GC-MS of N18TG2 Incubated with LOA for 12 Hours, Showing Linoleonitrile

GC-MS of N18TG2 cell extract after incubation with LOA for 12 hours, showing linoleonitrile. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (linoleonitrile). Panel C shows the library database MS for linoleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM LOA in 0.25 mM BSA for 12 hours in this case before
Figure 3-7: GC-MS of N18TG2 Conditioned Media Incubated with LOA for 48 Hours, Showing Linoleonitrile.
GC-MS of N18TG2 media extract after incubation with LOA for 48 hours, showing linoleonitrile. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (linoleonitrile). Panel C shows the library database MS for linoleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5mM LOA in 0.25mM BSA for 48 hours in this case before extraction.
Figure 3-8: GC-MS of SCP Cells Incubated with LOA for 48 Hours, Showing Linoleamide-TMS

GC-MS of SCP cell extract after incubation with LOA for 48 hours, showing linoleamide-TMS. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (linoleamide-TMS). Panel C shows the library database MS for linoleamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section. The cells had been incubated with 2.5 mM LOA in 0.25 mM BSA for 48 hours in this case before extraction.
Each PFAM, including the non-natural tridecanamide, was found in both N18TG2 cells and conditioned media. In most cases, the amount of PFAM found in the conditioned media was very similar to the amount of PFAM found in the cells themselves (Figure 3-10).
Figure 3-10: Quantification of PFAMs isolated from N18TG2 cells and media. Continued on the next page.
Blanks showed little interference of PFAMs in unspent media and of slip additives in polyethylene. N18TG2 media blanks were approximately 1-22% of experimental amounts found in conditioned media, except for linoleamide, which was
44%. Solvent blanks were even lower: between 0.5-8% of cell sample amounts, except for background interference in the region of tridecanamide, which gave background levels of 17-18% of experimental levels.

**Figure 3-11: Endogenous PFAMs in N_{18}TG_{2} Cells**
The amount of PFAMs found in normally growing N_{18}TG_{2} cells without incubation with a FFA is shown here. Error bars are ± standard deviation.

**Figure 3-12: Primary Fatty Acid Amides Produced in N_{18}TG_{2} Cells Incubated with Various Fatty Acids**
Comparison of PFAMs found in N_{18}TG_{2} cells after incubation with various FFAs and one NAE. Only cell data are shown here. Media data are available in Table 3-6 and Figure 3-10.
Endogenous amides are quantified in Figure 3-11, and the amounts of amides found in N\textsubscript{18}TG\textsubscript{2} cells and conditioned media after 0, 12, 24 or 48 hours incubation with the corresponding fatty acids are co-graphed in Figure 3-12 and in Table 3-6. In terms of endogenously found amides, palmitamide was by far the most abundant, followed by palmitoleamide, oleamide and linoleamide.

Palmitamide was the most abundant PFAM after incubation with its corresponding FFA (0.25 mM) in N\textsubscript{18}TG\textsubscript{2} cells. Aside from TDA, which is not a natural FFA and whose corresponding PFAM was found in very low amounts, PA was the only saturated FFA used for this experiment. Palmitoleamide was the next most abundant PFAM, followed by elaidamide, oleamide, linoleamide, and tridecanamide. Based on these findings, N\textsubscript{18}TG\textsubscript{2} cells appear to convert 16-carbon chain FFAs to their PFAMs in greater abundance than 18-carbon chain or perhaps the hydrolysis of the 18-carbon PFAMs occurs preferentially. Elaidamide was found in greater abundance than oleamide, but only after 48 hours. Trans fatty acids are not normally found in appreciable amounts in nature.\textsuperscript{77} Most of the incubations revealed a linear increase in PFAM over time, but a much greater amount of elaidamide production was seen in the 48 compared to the 12 and 24 hour samples.

3.4.3 Quantitative Analysis of PFAMs in SCP Cells

Each PFAM, including the non-natural tridecanamide, was found in both SCP cells and conditioned media. In most cases, the amount of PFAM found in the conditioned media was very similar to the amount of PFAM found in the cells themselves (Figure 3-13).
Figure 3-13: Quantification of PFAMs isolated from SCP cells and media, and blanks. Continued on the next page.
Controls showed even less interference of PFAMs in SCP samples than was found in N18TG2 samples. SCP media blanks were approximately 1-6% of experimental amounts found in conditioned media. Solvent blanks were between 1-4% of cell sample amounts, except for POA, which was 11% of the experimental value.

Figure 3-13: Quantification of PFAMs isolated from SCP cells and media, and blanks
In each graph, cells are shown in blue, media in green, unspent media in pink and solvent blank in orange (left to right). Samples were incubated for 0, 12, 24 or 48 hours with corresponding fatty acid-BSA mixture. Error bars are ± standard deviation.
Endogenous PFAMs are quantified in Figure 3-14, and the amounts of amides found in SCP cells and conditioned media after 0, 12, 24 or 48 hours incubation with
corresponding fatty acids are co-graphed in Figure 3-15 and in Table 3-6. SCP cells had a different profile of endogenously found amides than did N₁₈TG₂. Oleamide was much more abundant than any other endogenous PFAM, followed by palmitamide, palmitoleamide, and linoleamide. Again, there was a much greater abundance of PFAMs in SCP cells than in N₁₈TG₂ cells, as was found for oleamide in Chapter 2.

In SCP cells, as in N₁₈TG₂, elaidamide was found in greater amounts than oleamide, but the punctuated result after 48 hours in N₁₈TG₂ is replaced here with a more gradual increase in PFAM. Palmitamide and linoleamide, however, show a more punctuated production with a significant increase in amount after 48 hours. Linoleamide was again the least abundant PFAM endogenously, and again tridecanamide was synthesized the least quickly.
<table>
<thead>
<tr>
<th></th>
<th>SCP cells</th>
<th>SCP media</th>
<th>SCP unspent media</th>
<th>N18TG2 cells</th>
<th>N18TG2 media</th>
<th>N18TG2 unspent media</th>
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<td></td>
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<tr>
<td>12h</td>
<td>2033 ± 308</td>
<td>2239 ± 922</td>
<td>143</td>
<td>102 ± 10</td>
<td>284 ± 225</td>
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<td>2653 ± 747</td>
<td>3228 ± 1245</td>
<td>211</td>
<td>261 ± 120</td>
<td>503 ± 162</td>
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<tr>
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<td>2289 ± 314</td>
<td>5132 ± 426</td>
<td>291</td>
<td>750 ± 317</td>
<td>1276 ± 367</td>
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<tr>
<td>12h</td>
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<td>2029 ± 1387</td>
<td>144</td>
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<tr>
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<tr>
<td>12h</td>
<td>1162 ± 208</td>
<td>857 ± 442</td>
<td>2028 ± 1021</td>
<td>5821 ± 136</td>
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<td>21369 ± 6710</td>
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<td>208 ± 156</td>
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<td>207 ± 1021</td>
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<tr>
<td>48h</td>
<td>33044 ± 3012</td>
<td>13170 ± 3873</td>
<td>2137 ± 424</td>
<td>1734 ± 134</td>
<td>423</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12h</td>
<td>6367 ± 2276</td>
<td>4087 ± 1852</td>
<td>119 ± 53</td>
<td>208 ± 156</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>12255 ± 3147</td>
<td>17458</td>
<td>735 ± 198</td>
<td>1656 ± 199</td>
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<td></td>
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<tr>
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<td>29336 ± 10433</td>
<td>53650 ± 29800</td>
<td>1258 ± 372</td>
<td>853 ± 268</td>
<td>454</td>
<td></td>
</tr>
</tbody>
</table>

Amount of PFAMs found in cell and media samples endogenously and after incubation for 12, 24 or 48 hours with the corresponding FFA. Endogenous amount is listed without a time. Values are pmol/10^7 cells ± standard deviation. Samples have blanks subtracted from them: solvent blank for the cell samples, and unspent media blank for the media samples. The TDA/TDEA samples already have the 0h integration (negative control) subtracted from them. Endogenous 18:1Δ⁹ PFAM is assumed to be oleamide.¹⁶
3.4.4 Comparison of SCP and N<sub>18</sub>TG<sub>2</sub> PFAM Production

In every case except with palmitoleamide, more of the PFAM was found in SCP cells than in N<sub>18</sub>TG<sub>2</sub> after incubation with FFA (Figure 3-16 and Figure 3-17), although nearly equal amounts of palmitamide were found in the two cell lines. In fact, there was 23 times as much linoleamide in SCP than in N<sub>18</sub>TG<sub>2</sub> cells after 48 hours incubation with LOA, but only 3 times as much elaidamide (Table 3-6), so the amount of variation was large and the two cell lines had distinct PFAM “fingerprints.”

The much higher amount of endogenous oleamide in SCP cells (54 times compared to N<sub>18</sub>TG<sub>2</sub> levels) that was discovered in Chapter 2 did not hold true for other endogenous PFAM levels (Figure 3-16). Other endogenous PFAMs ranged from just 1.2 times higher (palmitoleamide) to 2.4 times higher (linoleamide) in the SCP as compared to N<sub>18</sub>TG<sub>2</sub> cells.

![Graph showing endogenous PFAMs in SCP and N<sub>18</sub>TG<sub>2</sub> Cells](image)

**Figure 3-16: Endogenous PFAMs in SCP and N<sub>18</sub>TG<sub>2</sub> Cells**

The amount of endogenous amides in N<sub>18</sub>TG<sub>2</sub> (left) and SCP cells (right). Cells were grown in normal growth media with 10% FBS until 80-95% confluent, collected and extracted as described in the methods section. Quantification was done by GC-MS.
Figure 3-17: Comparison of PFAMs in SCP and N18TG2 Cells. Continued on the next page.
Figure 3-17: Comparison of PFAMs in SCP and N₁₈TG₂ Cells

The amount of amides in SCP cells (brown) and media (blue), and in N₁₈TG₂ cells (purple) and media (green) plotted side by side here for comparison purposes. Cells were grown in media containing 0.5% FBS, 0.25 mM BSA and 2.5 mM FFA for 0, 12, 24 or 48 hours, collected and extracted as described in the methods section. Quantification was done by GC-MS.
3.5 Controls

In addition to the solvent blanks and unspent media controls (see sections 3.4.2 and 3.4.3), controls were run to test for the formation of PFAMs in unspent media and in spent media upon incubation with various metabolites. A recent report by Mueller and Driscoll\textsuperscript{52} identified oleamide-synthesizing activity in FBS, a component used in culture media in these experiments. In the Mueller and Driscoll experiments, incubation with oleoyl-CoA and ammonia or glycine and resulted in the formation of oleamide or N-oleoylglycine, respectively.

To test for oleamide-forming activity in the media used in these experiments, both conditioned and unspent media were incubated for 48 hours with various oleoyl metabolites. These results are summarized in Figure 3-18, and show no detectable difference between the amount of oleamide found in unspent media and media (spent or unspent) incubated with one of the metabolites. This shows that there is no PFAM-forming metabolism occurring in the media with NOG, OA and NOE as precursors, and that the cells are not excreting enzymes capable of metabolizing these acyl amides.
To test for the change in oleamide production in the cells without the presence of oleic acid or other oleoyl metabolites (but in the presence of all other components), both N18TG2 and SCP cells were incubated for 48h with media containing 0.5% FBS and 0.25 mM BSA carrier. There was no detectable difference between the amount of oleamide found in the normally growing “0h” cells and those incubated for 48h with BSA and low serum media (Figure 3-19 Figure 3-20). This shows that the incubation conditions (0.5% FBS and 0.25 mM BSA) have no measurable effect on PFAM production in the absence of the FFA.

Figure 3-18: N18TG2 Media Incubated with Metabolites with or without Previous Exposure to Cells.
Media containing 0.5% FBS was incubated with OA, NOE or NOG for 48 hours. Half of the samples were conditioned media (48 hours of cell exposure) with any cells removed by sterile filtration. Each sample was repeated. Error bars are ± standard deviation. Conditioned media and extracted cells after 48 hours incubation with OA are also shown on the left for comparison.
Abbreviations: NOE, N-oleylethanolamine; NOG, N-oleoylglycine; OA, oleic acid
**Figure 3-19: N_{18}TG_{2} Incubated with BSA and Normal Cells**

Amount of oleamide in cells and media extracted after incubation with BSA alone, under normal growing conditions (“0h”), and in unspent media and solvent blank. Cells were incubated as normal for 48 hours and extracted as described in sections 3.3.5-3.3.8.

**Figure 3-20: SCP Incubated with BSA and Normal Cells**

Amount of oleamide in cells and media extracted after incubation with BSA alone, under normal growing conditions (“0h”), and in unspent media and blank. Cells were incubated as normal for 48h and extracted as described in sections 3.3.5-3.3.8.
To test the robustness of this extraction method, cells and media were spiked with each PFAM and subjected to the standard extraction procedure. Percent recoveries ranged from 82-101% (Figure 3-21).

![Figure 3-21: Extraction Efficiency for PFAMs](image)

N<sub>18</sub>TG<sub>2</sub> cells and media were spiked with 5 nmol of each amide and subjected to the normal solvent extraction, SPE, and derivitization for GC-MS. PFAM peaks were integrated using a set of characteristic ions as in Table 3-5 and compared to a standard curve. Error bars are ± standard deviation.

### 3.6 Discussion

This is the first known report of the conversion of FFAs other than oleic acid to their respective PFAM in N<sub>18</sub>TG<sub>2</sub> cells. Although oleamide is the best studied PFAM, it is only one of many PFAMs that are known to be produced by this cell line and other tissue types. In addition, this is the first report of the PFAMs being found both endogenously and upon incubation with a FFA in SCP cells.
In comparing the two cell lines, there are several obvious differences. Aside from the finding that SCP cells had more of each PFAM except palmitoleamide, the pattern of PFAM abundance differs between these two cell lines in terms of amount of each PFAM species. N\textsubscript{18}TG\textsubscript{2} cells showed a higher turnover of 16-carbon fatty acids to primary amides than did SCP cells, and there was no discernable pattern of PFAM abundance in SCP cells relating to acyl chain length of degree of saturation. To qualify, the amount of corresponding PFAMs found in SCP cells was approximately PA ≥ LOA > EA > OA > POA > TDEA > TDA, whereas in N\textsubscript{18}TG\textsubscript{2} it was PA > POA > EA > OA > LOA > TD ≥ TDEA after 48 hours of incubation time. Oleamide was the most abundant of the five PFAMs found in meibomian secretions,\textsuperscript{6} but still the unexpected findings that oleamide is far more abundant than the other SCP endogenous PFAMs, and that conversion of most FFAs to their corresponding PFAM occurs in much greater amounts in the SCP cells bodes further investigation.

It was not possible to detect the difference between the cis/trans isomers oleamide and elaidamide due to the impurities in the complex cellular lipid extract, and the endogenous 18:1 amide found is reported here as oleamide because oleamide has been reported in N\textsubscript{18}TG\textsubscript{2} cells and various other sources\textsuperscript{43,68,69,96} while elaidamide has only been found in human plasma to date.\textsuperscript{5} Trans fatty acids are not found in appreciable amounts in higher organisms, aside from some in dairy fat and in the meat of ruminants, whose stomachs contain bacterial isomerases capable of performing this conversion.\textsuperscript{77} (The elaidamide found in human leutal phase plasma\textsuperscript{5} likely came from a dietary source, as do most trans-fatty acids.\textsuperscript{16}) It is interesting, therefore, that both N\textsubscript{18}TG\textsubscript{2} and SCP cells had greater amounts of elaidamide than oleamide after incubation with EA and OA, respectively.

The kinetic data for peptidylglycine α-amidating monooxygenase (PAM) with various NAGs as substrates is shown in Figure 3-22. The presence of low endogenous amounts of linoleamide may be partially accounted for by the lower V\textsubscript{max}/K\textsubscript{M} of the doubly unsaturated N-linoleoylglycine. However, once LOA incubation began, SCP cells showed a much higher amount of linoleamide. This may reflect a low availability of endogenous LOA. The N\textsubscript{18}TG\textsubscript{2} cells, however, maintained a lower amount of linoleamide
per cell than other PFAMs after incubation with the corresponding FFA. This could reflect any number of possibilities: N18TG2 may express fewer FFA transport proteins that are specifically able to transport the doubly unsaturated FFA across cell membranes, LOA may be preferentially shuttled to a different metabolic route, or the different species may show different substrate specificities for any of the metabolic enzymes, including PAM, acyl CoA synthetase(s), cytochrome c, and FAAH.

Although the degradative enzyme, FAAH, has been studied extensively,97,98 there is not a great deal known about substrate PFAMs with less than 18 carbons. Reported kinetic constants vary, but linoleamide is likely a better substrate than oleamide (Table 3-8 Table 3-7). This helps explain why linoleamide was found in lower abundance in N18TG2 cells, since FAAH was not found to be expressed in SCP (see Chapter 4). Based on the other PFAM data in N18TG2 cells, 16-carbon PFAMs can be predicted to be worse substrates for FAAH than 18, since those 16-carbon PFAMs were found in greater abundance. Palmitamide was found to be a worse FAAH substrate than oleamide in vitro2,7 (see Table 3-7). Lower degrees of unsaturation may also be favorable against FAAH degradation because, in the N18TG2 trend, the unsaturated PFAM was most abundant, followed by the mono-unsaturated species and finally the bi-unsaturated linoleamide, which was found in lowest abundance. FAAH hydrolyzes NAEs more
slowly with decreasing numbers of double bonds. It is interesting that oleamide is a better substrate for FAAH than is myristamide (Table 3-7) but tridecanamide is in far lower abundance than oleamide and any of the other PFAMs.

It is interesting that oleamide is a better substrate for FAAH than is myristamide (Table 3-7) but tridecanamide is in far lower abundance than oleamide and any of the other PFAMs.

<table>
<thead>
<tr>
<th>FAAH source</th>
<th>N₁₈TG₂</th>
<th>Rat recombinant</th>
<th>Human recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anandamide</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Linoleamide</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleamide</td>
<td>42</td>
<td>73</td>
<td>70</td>
</tr>
<tr>
<td>Palmitamide</td>
<td>10</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Myristamide</td>
<td>24</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

The activity of anandamide is taken to be 100%, and the activity of the other substrates compared to anandamide. Data taken from

<table>
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<th>Acyl Chain</th>
<th>Km (µM)</th>
<th>Vmax (µmol/min·mg)</th>
<th>V/K</th>
<th>Source</th>
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<td>Linoleoyl</td>
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<tr>
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<td>8.3</td>
<td>3317</td>
<td>400</td>
<td>Rat brain</td>
<td>3</td>
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<td>941</td>
<td>105</td>
<td>N₁₈TG₂</td>
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<td>14</td>
<td>340</td>
<td>243</td>
<td>N₁₈TG₂</td>
<td>1</td>
</tr>
</tbody>
</table>

' enzymatic activity is from a 10,000xg pellet
' enzymatic activity is from the microsomal fraction

No enzymatic assays have been done for FAAH with the substrate elaidamide to the author’s knowledge, but as the relative levels of PFAMs in N₁₈TG₂ thus far have completely mirrored FAAH substrate preference, one might predict that elaidamide is a poorer FAAH substrate than oleamide. This would explain why elaidamide was found in greater abundance than oleamide even though EA is not a naturally occurring FFA.

In addition to testing for the conversion of endogenously found FFAs to their respective amides, a non-natural FFA and its corresponding NAE was also incubated with the cells to test for its conversion to a PFAM in an attempt to determine not only whether a non-natural acyl chain (aside from elaidic acid) could be converted to its primary amide, but also to gain some insight into the mode of formation of PFAMs (see Figure 3-1). This is the first report of conversion of an NAE to PFAM in these cell lines, as well as the first report of non-natural fatty acid and ethanolamines being converted to...
their corresponding PFAM. Although both cell lines were able to perform this conversion, the rates of conversion were much lower than those found for natural FFAs (Figure 3-12 Figure 3-15), and the patterns of metabolic flow-through were not the same in the N_{18}TG_{2} and SCP cells (Figure 3-23).

![Figure 3-23: Tridecanamide in SCP and N_{18}TG_{2} Cells after TDA and TDEA Incubations.](image)

The rate of conversion of TDEA is steeper than the rate of conversion of TDA in SCP cells. In N_{18}TG_{2} cells, however, there is no discernable difference in substrate conversion.

N_{18}TG_{2} cells metabolized TDA and TDEA with equal discrimination, whereas SCP cells preferred the TDEA substrate to the TDA. Bradshaw et al. found that incubation of labeled N-arachidonoyl-D_{4}-ethanolamine produced labeled N-aracydonoylglycine in both RAW 264.7 and C6 glioma cells, indicating the oxidative formation of NAG. In addition, the unlabeled N-arachidonoylglycine was found in C6 glioma cells, indicating they also hydrolyze the NAE to its FFA for glycination. Upon addition of a FAAH inhibitor, the NAG was no longer formed in the glioma cells, suggesting that the preferential mode of action of NAG formation is hydrolysis of the NAE to its FFA in the glioma, but not in the RAW 264.7 cells. The Bradshaw data and the findings herein demonstrate that not only is there more than one way to produce a
NAG, but that the mode of NAG production can vary from cell line to cell line (and likely tissue type to tissue type). This bodes further exploration to determine if there are differences in expression of the metabolizing enzymes in these cell lines, and in Chapter 4 it is determined that FAAH is not expressed in SCP cells and an alcohol dehydrogenase was found in SCP, but not N18TG2, cells. These and other differences in enzyme expression have many implications for the metabolism of FFAs and NAEs in these cell lines (for more discussion see Chapter 4).

3.7 Conclusion

The assay for PFAM quantification in cells and media that was developed in Chapter 2 was used here to show the conversion of a panel of long chain fatty acids to their corresponding amides in two model cell lines: SCP and N18TG2 cells. In addition to five endogenously found FFAs, two non-natural, odd-chain compounds were found to be converted to their corresponding PFAM in both cell lines and their respective conditioned media.

The amount of endogenous PFAMs was much higher in the SCP cells, particularly for oleamide. After incubation with a fatty acid, the profile of PFAMs was different between the two cell lines. The PFAM profile in N18TG2 cells mirrors the known substrate specificity for FAAH, with the PFAMs having the lowest $V_{max}/K_M$ being in greatest abundance and those with the most favorable kinetic data being in least.

In addition to the differing profile of PFAMs after FFA incubation, SCP and N18TG2 cells showed different usage of TDA and TDEA. While N18TG2 cells used both at the same rate to form tridecanamide, SCP showed a preference for ethanolamine metabolism. Once again, the long chain fatty acid amide metabolism is shown to differ in different cell lines. These findings bode further exploration, and an investigation into their enzyme expression may yield some answers to these discrepancies.

3.8 References

(22) Herrera-Solis, A.; Vasquez, K. G.; Prospero-Garcia, O. *Pharmacol Biochem Behav* **2010**.


(52) Mueller, G. P.; Driscoll, W. J. *Vitam Horm* 2009, 81, 55.
(95) Sultana, T.; Johnson, M. E. J Chromatogr A 2006, 1101, 278.
4 Amide-Processing Enzyme Expression in Oleamide-Producing Cells

4.1 Location and Biological Significance of Primary Fatty Acid Amides and N-Acylglycines

Primary fatty acid amides (PFAMs) and N-acylglycines (NAGs) are important bioactive lipids found across many species. PFAMs have been shown to be responsible for various effects in the central nervous system, including induction of sleep,

stimulation of Ca\(^{2+}\) release,\(^{79,92}\) inhibition of the erg current in pituitary cells,\(^{93}\) and activation of specific serotonin receptor subtypes\(^{97-99}\) and the GABA\(_A\) receptor.\(^{80-83}\) (For a complete review of endogenous PFAMs see Chapter 3.)

Less is known about the NAGs, but a recent surge of interest in these molecules and the development of better separations and more sensitive mass spectrometry instruments has allowed for the identification of many more N-acylamino acids than were known just a few years ago.\(^{100-103}\) N-Conjugation of long-chain fatty acids to glycine is a known method of detoxification and elimination, although recently N-oleoylglycine has been shown to have bioactivity as it serves in regulation of body temperature and locomotion.\(^{104}\) Other long-chain N-acylglycines have also been reported to have additional function. For example, N-arachidonoylglycine is an endogenous ligand for the orphan GPR18 receptor,\(^{108}\) is analgesic,\(^{20}\) inhibits FAAH\(^{20}\) and the GLYT2a glycine transporter.\(^{109}\) A summary of known mammalian long-chain PFAM and NAG metabolites can be found in Table 4-1.
Table 4-1: Occurrence and Role of Endogenous Long-Chain PFAMs and NAGs in Mammals

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<tr>
<th>Class</th>
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<td>Myristamide (C14:0)</td>
<td>Human</td>
<td>Unknown</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Palmitamide (C16:0)</td>
<td>Human, Mouse</td>
<td>Unknown, modestly attenuates seizures in mice, modest FAAH inhibitor</td>
<td>56, 58 (this dissertation)</td>
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<tr>
<td></td>
<td>Palmitoleamide (C16:1 cis-Δ³)</td>
<td>Human, Rabbit, Mouse</td>
<td>Gap junction communication, serotonin receptor binding</td>
<td>58, 60 (this dissertation)</td>
</tr>
<tr>
<td></td>
<td>Stearamide (C18:0)</td>
<td>Human, Rabbit</td>
<td>Unknown</td>
<td>56, 60</td>
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<tr>
<td></td>
<td>Elaidamide (C18:1 trans-Δ³)</td>
<td>Human</td>
<td>Less active than oleamide, but induces sleep, inhibits epoxide hydrolase and phospholipase A₂</td>
<td>58, 70</td>
</tr>
<tr>
<td></td>
<td>Oleamide (C18:1 cis-Δ³)</td>
<td>Human, Mouse, Rabbit, Rat, Squirrel</td>
<td>Sleep, memory, thermal and locomotor regulation, gap junction communication, Ca²⁺ flux, vasodilatation, hunger, anxiety, serotonin receptor binding,  erg current inhibition</td>
<td>23, 58, 71-87, 58, 60, 88-91 (this dissertation)</td>
</tr>
<tr>
<td></td>
<td>Linoleamide (C18:2 cis-Δ⁹,12)</td>
<td>Human, Rabbit, Mouse</td>
<td>Sleep and Ca²⁺ flux regulation,  erg current inhibition, epoxide hydrolase and phospholipase A₂ inhibition, gap junction communication, FAAH substrate, serotonin receptor binding</td>
<td>92, 93, 56, 60 (this dissertation)</td>
</tr>
<tr>
<td></td>
<td>Eicosenamide (C20:1 cis-Δ⁰)</td>
<td>Rabbit</td>
<td>Unknown</td>
<td>28, 60</td>
</tr>
<tr>
<td></td>
<td>Erucamide (C22:1 cis-0Δ¹³)</td>
<td>Cat, Cow, Human, Rat</td>
<td>Fluid balance, angiogenesis</td>
<td>56, 105-107</td>
</tr>
<tr>
<td>NAG</td>
<td>N-palmitoylglycine (C16:0)</td>
<td>Rat</td>
<td>Sensory neuronal signaling, antinociception, Ca²⁺ influx</td>
<td>17, 101</td>
</tr>
<tr>
<td></td>
<td>N-stearoylglycine (C18:0)</td>
<td>Mouse, Rat</td>
<td>Unknown</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>N-oleoylglycine (C18:1 cis-Δ⁹)</td>
<td>Mouse, Rat</td>
<td>Temperature and locomotion control</td>
<td>88, 101, 102, 104 (this dissertation)</td>
</tr>
<tr>
<td></td>
<td>N-linoleoylglycine (18:2 cis-Δ⁹,12)</td>
<td>Rat</td>
<td>Anti-inflammatory</td>
<td>101, 102, 110</td>
</tr>
<tr>
<td></td>
<td>N-arachidonoylglycine (20:4 cis-Δ³,8,11,14)</td>
<td>Cow, Rat</td>
<td>Antinociceptive and inflammatory suppression, control of T-cells proliferation, suppression of IL-1β secretion, reduction rectal carcinoma growth, Ca²⁺ and insulin release, GLYT2a inhibition</td>
<td>3, 110-114</td>
</tr>
<tr>
<td></td>
<td>N-docosahexaenoylglycine (22:6 cis-Δ³,7,10,13,16,19)</td>
<td>Rat</td>
<td>Anti-inflammatory</td>
<td>102, 110</td>
</tr>
</tbody>
</table>
4.2 PFAM and NAG Biosynthesis

The pathway(s) for PFAM and NAG biosynthesis are currently under study in several laboratories, including the Bradshaw (formerly Walker), Merkler, and Mueller groups. The consensus is that there are two main routes to NAG biosynthesis, although the regulation of each route, the prevalence of each route in different cell and tissue types, and the exact enzymes involved are still under investigation. One of the proposed biosynthetic mechanisms is the activation of a long-chain free fatty acid (FFA) to an intermediate, the coenzyme A (CoA) thioester being a likely candidate, followed by glycination to form the NAG. A second proposed route for NAG biosynthesis involves sequential oxidation of an N-acylethanolamine (NAE) first to the N-acylglycinal and then to the NAG.

The route(s) to PFAM biosynthesis are better understood. The primary proposed mechanism of PFAM biosynthesis is by oxidative cleavage of the NAG by the bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM). Another mechanism for which recent in vitro studies have given some evidence involves the direct amidation of an acyl-CoA with ammonia. The current proposed pathways for the synthesis and degradation of the NAGs and PFAMs is shown in Figure 4-1.
Considerable evidence has been gathered to support the action of peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3) in converting the NAGs to PFAMs. PAM is a bifunctional enzyme composed of peptidylglycine α-hydroxylating monooxygenase (PHM, EC 1.14.17.3) that catalyzes the stereospecific hydroxylation of the glycine α-carbon of glycine-extended substrates. The second functional unit of PAM
is peptidyl-α-hydroxyglycine α-amidating lyase (PAL, EC 4.2.3.5), which releases glyoxylate from the α-hydroxylated acyl or peptidyl-glycine to form a PFAM or an amidated peptide (Equation 4-1). PAM is expressed in neuroendocrine tissues where it is known to amidate peptide messengers from their glycine-extended precursors. In vitro studies have shown that many non-peptides are PAM substrates, and that $V_{\text{max}}/K_M$ for NAGs are comparable to those for peptide substrates. The equations for the stepwise transformation are shown below in Equation 4-1.

![Equation 4-1: Amidation of an N-Acylglycine by PAM](image)

Chemical inhibition of PAM by trans-4-phenyl-3-butenoic acid in the PAM-expressing N$_{18}$TG$_2$ cells resulted in the accumulation of N-oleoylglycine when these cells were grown in the presence of oleic acid. The main area of debate for this metabolic network has been in the formation of NAGs. Bradshaw et al. and Burnstein et al. demonstrated the conversion of NAEs to NAGs in three cell lines, and Merkler et al. demonstrated the conversion of $^{14}$C-oleic acid to a $^{14}$C-compound with the same retention time as N-oleoylglycine. These experiments demonstrate not only that NAGs are cellular metabolites but that they can be synthesized from two distinct precursors:
NAEs and FFAs. These two distinct metabolic precursors also indicate two distinct biosynthetic mechanisms: oxidation of an NAE and glycination of an activated intermediate, such as acyl-CoA.

**4.2.1 Anabolic Route to the NAGs I: Glycination of a Free Fatty Acid**

The ATP-dependent formation of acyl-CoA from fatty acids via acyl CoA ligase (ACS, a.k.a. acyl-CoA synthetase, EC 6.2.1.x) is a well-established reaction that is found throughout the evolutionary tree, from Achaea to complex eukaryotes.\(^{59,117,118}\) Overexpression in hepatic cells results in an increase in the amount of cellular long-chain acyl-CoAs.\(^{119}\) All the common fatty acyl-CoAs are known mammalian metabolites, including palmitoyl, palmitoleoyl, stearoyl, oleoyl, linoleoyl and arachidonoyl-CoA.\(^{120,121}\) This two-step reaction is diagrammed in Equation 4-2.

\[
\text{Equation 4-2: Fatty Acyl-CoA Lygase Reaction}
\]

Exactly which ACS isozyme is responsible for the CoA-ylation of particular FFAs *in vivo* has not been resolved completely, but much *in vitro* evidence has been amassed. The very long-chain ACSs, known both as ASCVLs and SLC27As (for solute carrier family 27A), metabolize fatty acids of 18-26 carbons in length, and the long chain ACSs, known as ACSLs, metabolize fatty acids of 12-22 carbons in length.\(^{59,118}\) Relative fatty acid preference for ACSL isoforms 1 and 3-6 is summarized in Table 4-2. (Isoform 2 was found to be the same as isoform 1.)
Acyl CoA:glycine N-acyltransferase (ACGNAT, a.k.a. GAT, GLYAT, EC 2.3.1.13) is one possible glycination enzyme found in the liver, kidney and brain.\textsuperscript{13,122,123} There are two isoforms of this enzyme; isoform b is composed of the first 163 amino acids residues of isoform a, although kinetic studies have not been done to distinguish substrate preference between the two isomers. The ACGNAT reaction is shown in Equation 4-3, and proceeds via a ping pong mechanism.\textsuperscript{124}

\begin{equation}
\text{R-S-CoA + H}_2\text{N-CH}_2\text{COOH} \rightarrow \text{R-NHCH}_2\text{COOH} + \text{CoASH}
\end{equation}

\textbf{Equation 4-3: Formation of N-Acylglycine by ACGNAT}

ACGNAT is known to form short-chain and branched-chain N-acylglycines from the corresponding acyl-CoA thioesters.\textsuperscript{122,125} However, ACGNAT is not likely to be the enzyme responsible for long-chain NAG formation because The $V_{max}/K_M$ for acyl-CoAs dramatically decreases as the length of the acyl chain increases.\textsuperscript{40,122,126,127} Long-chain acyl-CoA thioesters are not ACGNAT substrates,\textsuperscript{125} with decanoyl-CoA being the longest acyl-CoA substrate accepted by mammalian ACGNAT.\textsuperscript{128,129} The Merkler laboratory verified these results using purified bovine liver ACGNAT. After a 4-hour incubation with high concentrations of ACGNAT, there was approximately 4% conversion of oleoyl-CoA to N-oleoylglycine (unpublished data), conditions under which benzoyl-CoA or butanoyl-CoA were completely converted to their respective NAG in less than 30 seconds. In addition, incubation of bovine liver ACGNAT PAM with

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Isoform} & \textbf{Fatty acid preference} \\
\hline
ACSL1 & 16:0 $\approx$ 16:1 $\approx$ 18:1 $\approx$ 18:2 $\approx$ 14:0 $\approx$ 18:3 $\approx$ 20:4 $\approx$ 20:5 $\gg$ 20:0 $\approx$ 22:0 $\approx$ 22:6 $\approx$ 24:0 \\
ACSL3 & 14:0 $\approx$ 20:4 $\approx$ 20:5 $\gg$ 16:1 $\approx$ 18:1 $\approx$ 18:2 $\approx$ 18:3 $\approx$ 16:0 $\approx$ 18:0 $\gg$ 20:0 $\approx$ 22:0 $\approx$ 22:6 \\
ACSL4 & 20:4 $\approx$ 20:5 $\gg$ 16:0 $\approx$ 18:0 $\approx$ 18:2 $\approx$ 16:1 $\approx$ 18:3 $\approx$ 22:6 $\gg$ 14:0 $\approx$ 18:1 $\approx$ 20:0 $\gg$ 22:2 \\
ACSL5 & 16:0 $\approx$ 16:1 $\approx$ 18:1 $\approx$ 18:2 $\approx$ 18:3 $\approx$ 20:4 $\approx$ 14:0 $\approx$ 18:0 $\gg$ 20:0 $\approx$ 22:0 $\approx$ 24:0 \\
ACSL6 & 16:0 $\approx$ 18:1 $\approx$ 20:4 $\approx$ 20:5 $\approx$ 14:0 $\approx$ 22:6 $\approx$ 16:1 $\approx$ 18:0 $\approx$ 18:2 $\approx$ 18:3 $\approx$ 20:0 $\approx$ 22:0 $\approx$ 24:0 \\
\hline
\end{tabular}
\caption{In Vitro Substrate Preferences of Purified ACSL Isoforms}
\end{table}

This table is taken from Soupene and Kuypers (2008).\textsuperscript{59}
octanoyl-CoA, glycine, and ascorbate, yielded the formation of octanamide and glyoxylate, but a similar experiment using oleoyl-CoA in place of octanoyl-CoA yielded neither oleamide nor glyoxylate. Based on these data from the Merkler lab combined with the previous literature, ACGNAT is very unlikely to be the in vivo enzyme responsible for long-chain NAG production.

Two more enzymes that perform related chemistry have been proposed to transfer a glycine to an acyl-CoA: one that normally transfers myristoyl groups onto proteins and one that transfers amino acids to bile acids. N-Myristoyltransferase (NMT, EC 2.3.1.97) is responsible for the N-terminal acylation of proteins and has a strong preference for myristoyl-CoA and an absolute requirement for an N-terminal glycine residue (Equation 4-4). Although palmitoyl-CoA is a reasonable inhibitor, free glycine and proteins with an N-terminal other than glycine are not substrates.

\[
\text{myristoyl-CoA} + \text{gly-peptide} \rightarrow \text{myristoyl-gly-peptide}
\]

\textit{Equation 4-4: NMT Glycination Reaction}

Bile acid-CoA:amino acid N-acyltransferase (BAAT, a.k.a. BACAT, EC 2.3.1.65) catalyzes the conjugation of glycine or taurine to bile acid-CoA thioesters, and may also catalyze NAG biosynthesis in vivo. O’Byrne \textit{et al.} showed that BAAT could conjugate glycine to fatty acyl-CoA thioesters in vitro. However, \(V_{\text{max}}/K_M\) values were relatively low compared to the bile acid-CoA thioesters, with the values for the fatty acyl CoAs being only 20% of that reported for bile acids, and there was competing thioesterase activity that resulted in FFA formation even in the presence of glycine. While the in
vitro data does support a role for BAAT in the biosynthesis of certain saturated long-chain (C_{16}-C_{20}) and very long chain (C_{22}-C_{26}) NAGs, the specific activity of BAAT actually decreases for oleoyl-CoA when glycine is added as a co-substrate, and the specific activity for unsaturated acyl-CoAs was a fourth to a third of that for saturated acyl-CoAs. Therefore, if BAAT is an in vivo NAG biosynthesizing enzyme, it would likely function in limited capacity to form only the long-chain unsaturated NAGs. The BAAT glycination reaction is shown in Equation 4-5.

\[
\begin{align*}
\text{R-S-CoA} & \quad + \quad \text{H}_2\text{N-CH}_{2}\text{COOH} \\
\text{bile acyl-CoA} & \quad \text{glycine}\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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The finding that cyt c will catalyze the conjugation of several other oleoylamino acids (and N-arachidonylglycine) as well as anandamide and several PFAMs could indicate that this chemistry is an artifact of the redox behavior of cyt c and that it may or may not be the *in vivo* enzyme regulating the biosynthesis of NAGs. The aminolysis of acyl-CoAs in the presence of ammonia occurs non-enzymatically at pH 9 and above without H$_2$O$_2$. It is possible that H$_2$O$_2$ causes the reduction of Fe$^{3+}$ in cyt c, making it a better nucleophile to attack the carboxyl of acyl-CoA and increase the already high leaving potential of coenzyme A such that glycine or another nucleophile could more easily displace it to form the NAG. In fact, anandamide is formed spontaneously in the presence of arachidonoyl-CoA and ethanolamine, and the inclusion of H$_2$O$_2$ further increased the yield of anandamide. Addition of cyt c to this mixture actually reduced the amount of anandamide formed.

Heating of the cyt c-containing enzyme extract to 95°C was shown to increase the amount of product made. Incubation with different proteolytic enzymes, chymotrypsin, endoproteinase glu-C and elastase, did not decrease enzymatic activity. Incubation with trypsin and thermolysin was shown to increase enzymatic activity even after 30 minutes of incubation, although the commercially available cyt c also displayed resistance to proteolysis and heat denaturation. These treatments may be removing proteins that form inactivating complexes with cyt c or acyl-CoAs.

Although the optimal pH for these reactions was shown to be 7.5, the other conditions under which these reactions were demonstrated are not biologically favorable. Optimal conditions were 100 µM oleoyl-CoA, 300 mM glycine and 2 mM H$_2$O$_2$, and

---

**Equation 4-6: Cyt C-Mediated Formation of N-Acylglycine**

Amino acids other than glycine are also substrates.¹⁰
even under these conditions the maximum percent conversion to \(N\)-oleoylglycine was <15\%.\textsuperscript{55} Because of this requirement of such high concentrations of ammonia and hydrogen peroxide, and the fact that cyt c co-purifies with superoxide dismutase (SOD) and fatty acyl-CoA binding protein (ACBP), an oleamide “synthesome” was proposed (Figure 4-3 in Section 4.2.4) for the synthesis of PFAMs. This model may or may not be adaptable to the formation of NAGs if the amino transferase (AT) could also be a glycyl- or aminoacyl- transferase. More \textit{in vivo} or \textit{in cellulo} work is required to determine the exact significance of cyt c in terms of NAG formation. The subcellular localization of cyt c and other putative NAG biosynthetic enzymes is shown in Table 4-3.
Table 4-3: Subcellular Localization of Putative Biosynthetic Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reported Subcellular Localization</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA:glycine N-acyltransferase (ACGNAT)</td>
<td>cytoplasm, mitochondrion</td>
<td>13,14</td>
</tr>
<tr>
<td>Long Chain Fatty Acyl-CoA Ligasea (ACSL, all isoforms)</td>
<td>cytoplasm, cytosol, endoplasmic reticulum, membrane</td>
<td>22,31</td>
</tr>
<tr>
<td></td>
<td>(mitochondria-associated)</td>
<td>-33</td>
</tr>
<tr>
<td>Bile Acid-CoA:amino acid N-acyltransferase (BAAT)</td>
<td>cytosol, peroxisome</td>
<td>40,41</td>
</tr>
<tr>
<td>N-Myristoyltransferase (NMT)</td>
<td>cytosol, membrane (minor location), mitochondrion (minor location)</td>
<td>42-44</td>
</tr>
<tr>
<td>Fatty acid amide hydrolase (FAAH-1)bcde</td>
<td>membrane</td>
<td>46,47</td>
</tr>
<tr>
<td>Peptidylglycine α-amidating monooxygenase (PAM)</td>
<td>secretory granule, cytoplasm, membrane (integral)</td>
<td>48,49</td>
</tr>
<tr>
<td>Cytochrome C (Cyt c)</td>
<td>mitochondrion, secretory granule</td>
<td>50,51</td>
</tr>
<tr>
<td>Alcohol dehydrogenase (ADH, all long-chain isoforms)bcde</td>
<td>cytosol, membrane (outer)</td>
<td>52,53</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase (AlDH, all long-chain isoforms)bcde</td>
<td>microsome</td>
<td>61</td>
</tr>
<tr>
<td>Cytochrome P450 (CYP4F)</td>
<td>microsome</td>
<td>66</td>
</tr>
</tbody>
</table>

*aSome studies show that different isoforms display different subcellular localizations. For example, ACS1 was identified in endoplasmic reticulum, mitochondria-associated membrane and cytosol but not in mitochondria. In the same study, ACS4 was found primarily in the mitochondria-associated membrane and in the mitochondrial membrane but not the endoplasmic reticulum.

*bThere are reports of NMT in cytosol but these were done in Helicobacter pylori and Arabidopsis thaliana.

*cFAAH-2 was shown to be located extracellularly.

*dNAAA was located in the peroxisome.

*eAlthough both FAAH isoforms possess transmembrane domains, FAAH-1 has its catalytic domain facing the cytoplasm and FAAH-2 the luminal compartment.

*fDuring apoptosis, cytochrome c leaves the mitochondria.

*gMus musculus lactate dehydrogenase was found extracellularly.

*hStudies in various yeast isoforms reveal other subcellular localization, including membrane, microbody, microsome, mitochondrion and peroxisome.
4.2.2 Anabolic Route II: N-Acylethanolamine Oxidation

While it is clear that NAGs are produced in vivo (or in cellulo) from glycine and fatty acids or acyl-CoAs, it is also likely that there is more than one biosynthetic route to NAG formation, just as there is more than one biosynthetic route for the NAEs. In particular, a second metabolic pathway leading to the NAGs has been proposed: the oxidation of NAEs to NAGs by either an alcohol dehydrogenase alone or the sequential actions of an alcohol dehydrogenase and an aldehyde dehydrogenase (Equation 4-7). It is possible that both NAG biosynthetic pathways operate either in tandem, or one may be the favored biosynthetic route in specific cells, tissues, and disease states.

The first evidence for the NAE-dependent pathway came in 2000 when Burstein et al. showed that anandamide is converted to N-arachidonoylglycine in cultured liver cells and conditioned media. More recently, Bradshaw et al. showed the conversion of deuterated anandamide to N-arachidonoylglycine in both RAW 264.7 and C6 glioma cells (Figure 4-2, NAE oxidation pathway). These two cell lines seem to have different types of metabolisms. In addition to the labeled N-arachidonoylglycine, the unlabeled

---

**Equation 4-7: Sequential Oxidation of an N-Acylethanolamine by ADH or ADH and AlDH**

The first reaction would be done by an alcohol dehydrogenase. The second reaction would be done by either an alcohol dehydrogenase or an aldehyde dehydrogenase.
product was also found in the C6 glioma (but not RAW 264.7) cells, and upon addition of a FAAH inhibitor, URB 597, the unlabeled NAG was no longer formed in the glioma cells, suggesting that the glioma cells also hydrolyze NAE to its FFA for glycination (Figure 4-2, glycination pathway). This metabolism was not observed in the RAW 264.7 cells, which only made the NAG through the oxidative pathway and generated the deuterated NAG, demonstrating that not only is there more than one way to make a NAG, but that different cell types show different metabolic profiles.

![Figure 4-2: Two Pathways of Formation for NAGs](image)

Work of Bradshaw demonstrates both pathways are at work in C6 glioma cells. Both deuterated and undeuterated N-arachidonoylglycine were observed after incubation with D₄NAE. Upon inhibition of FAAH, only D₂NAG was observed. The glycination pathway is abbreviated here as an unknown. The authors suggest direct glycination of the FFA but their data does not rule out the activation of the FFA to a CoA or similar intermediate. Abbreviations: ADH, alcohol dehydrogenase; AlDH, aldehyde dehydrogenase; D₂/D₄NAG, deuterated N-acetylglycine; FAAH, fatty acid amide hydrolase; NAG, N-acetylglycine

The specific dehydrogenase enzymes responsible for these oxidations have not been determined. An alcohol dehydrogenase (ADH) would be responsible for the first oxidation of the NAE to an N-acylglycinal (top reaction, Equation 4-7). The second oxidation involving conversion of an N-acylglycinal to the NAG (bottom reaction, Equation 4-7), may either be catalyzed by an aldehyde dehydrogenase (AlDH), or and ADH, as some ADHs are known to oxidize NAEs and N-acylglycinals. Very recently,
Aneetha et al. demonstrated the NAD$^+$-dependent oxidation of N-arachidonoylethanolamine (a.k.a. anandamide) with purified human ADH4 (a.k.a. ADH 7, EC 1.1.1.1) in vitro, and showed that the unstable N-arachidonoylglycinal was then dismutated to the NAG. Another compelling reason to examine this isozyme is that ADH4 was found in the cornea, where PFAMs have been found in meibomian$^\circledast$ gland secretions.\textsuperscript{56}

Ivkovic et al. demonstrated that a panel of NAEs were substrates for bovine ADH3 with the $V_{max}/K_M$ increasing as the acyl chain increased in length.\textsuperscript{141} Despite this trend, the best NAE substrates exhibited a $V_{max}/K_M$ value that was >5% of the values obtained for alcohol substrates. This group determined that ADH3 did not catalyze aldehyde dismutation, meaning that the product was the N-acylglycinal, not the NAG. In sum, the work of Aneetha et al. and Ivkovic et al validate that the NAEs can serve as substrates for ADH, but it seems unlikely that either enzyme has a role in NAG biosynthesis in vivo, although solubility issues only allowed for study of NAEs up to 12 carbons in length.

A potential role for aldehyde dehydrogenase to catalyze reaction 2 in Equation 4-7 remains unexplored. While enzymes involved in NAE oxidation in vivo remain to be definitively identified, work from Burstein and Bradshaw demonstrate that this chemistry does occur in cultured cells.\textsuperscript{25,102} The aldehyde dehydrogenases (AlDH) in this study were selected based on their affinity to bind long-chain substrates. AlDH3A1 (E.C. 1.2.1.14) is selective for medium-to long-chain aldehydes.\textsuperscript{142} Additionally, AlDH3A1 is found in large abundance in the mammalian cornea (5-50% of water-soluble protein depending on species),\textsuperscript{143} alongside ADH4, and several PFAMs have been found in meibomian\textsuperscript{1} gland secretions.\textsuperscript{56} AlDH3A2 (E.C. 1.2.1.48) is known to have activity towards saturated and unsaturated aliphatic aldehydes ranging from 6-24 carbons in length, with NAD$^+$ as a cofactor.\textsuperscript{61}

\textsuperscript{56} The validity of this work is under debate. While the MS of meibomian secretions show several PFAMs in the Nichols paper, other commonly observed compounds, including cholesterol esters and wax esters, were not present. (139) Butovich, I. A. Prog Retin Eye Res 2009, 28, 483. In addition, other studies examining the lipid content of meibomian gland secretions did not find PFAMs. (140) Butovich, I. A.; Uchiyama, E.; Di Pascaule, M. A.; McCulley, J. P. Lipids 2007, 42, 765.
4.2.3 Degradation of Amides

Fatty acid amide hydrolase (FAAH, EC 3.5.1.4), an integral membrane enzyme capable of hydrolyzing PFAMs, NAGs and NAEs, has been well characterized both in vivo and in vitro\textsuperscript{15-21} (see Figure 4-1, bottom right). Cravatt et al. used an oleyl trifluoromethylketone column to perform affinity chromatography on rat liver homogenate. Once isolated, they cloned the cDNA for the enzyme, and expression in Chinese hamster ovary (CHO) cells resulted in high levels of hydrolysis, both of oleamide and anandamide, thus proving that this enzyme was responsible for their degradation in cellulo.\textsuperscript{18} Orthologs were soon identified for human,\textsuperscript{15} mouse,\textsuperscript{15} and pig.\textsuperscript{144} In fact, a FAAH knockout study showed 50- to 100-fold reductions in the hydrolysis rates for anandamide and other fatty acid amides in mice, indicating that FAAH is the primary enzyme responsible for their hydrolysis in vivo.\textsuperscript{21} Pharmacological blockade of FAAH activity also led to highly elevated levels of endogenous PFAMs,\textsuperscript{21,145-147} leading to PFAM-associated analgesia,\textsuperscript{21,145,148} lowered anxiety,\textsuperscript{145} and decreased inflammation.\textsuperscript{147,149} This enzyme adds another dimension to the possible control and route to NAG and PFAM biosynthesis and degradation.

There are two known isoforms of FAAH, FAAH-1 and FAAH-2, that show distinct expression patterns.\textsuperscript{47} Though the two human isoforms share only 20% sequence identity\textsuperscript{47} the murid and human FAAH-1 orthologues share >80% sequence identity,\textsuperscript{15} indicating that it is well-conserved. FAAH-1 and -2 show similar rates of hydrolysis for PFAMs and similar inhibitor sensitivity profiles, but FAAH-2 exhibits greater activity with NAEs and N-acyltaurines.\textsuperscript{47} The catalytic domain of FAAH-1 is predicted to reside on the cytoplasmic side, whereas that of FAAH-2 is predicted to be oriented in the luminal compartment of the cell by computer modeling and detergent sensitivity experiments.\textsuperscript{47} FAAH-2 has been identified in primates, marsupials, elephants, and more distantly related vertebrates but not in mouse, rat, sheep, dogs, cows or pigs, and it is believed that there may have been multiple independent losses of the ancient FAAH-2 gene during mammalian evolution.\textsuperscript{47} Incidentally, the FAAH isoform for which we probed in N\textsubscript{18}TG\textsubscript{2} and SCP cells in this study is FAAH-1.
In addition to degradation by FAAH, there are several other known possibilities to increase the hydrophilicity of these molecules for urinary excretion and other purposes. Cyclooxygenase-2 (COX-2, EC 1.14.99.1),\textsuperscript{62,63} lipoxygenase (LO, EC 1.13.11.12).\textsuperscript{62} A bacterial cytochrome P450 (P450BM-3, a.k.a. CYP102A1) has recently been shown to monooxygenate the acyl chain of \textit{N}-palmitoylglycine and other \textit{N}-palmitoylamino acids.\textsuperscript{65} This enzyme is widely studied due to its similarity to mammalian P450s, and the expression of mammalian cytochrome P450 (CYP4F, EC 1.14.13.30) was examined. NAGs contain a peptide bond, and therefore could be susceptible to proteolytic degradation as well.\textsuperscript{54} These degradative reactions are summarized in Table 4-4.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM</td>
<td>NAG + ASC + O\textsubscript{2} → PFAM + SDA + H\textsubscript{2}O + glyoxylate</td>
</tr>
<tr>
<td>FAAH\textsuperscript{a}</td>
<td>NAG/PFAM + H\textsubscript{2}O → FFA + glycine/NH\textsubscript{3}</td>
</tr>
<tr>
<td>Protease</td>
<td>NAG + H\textsubscript{2}O → FFA + glycine</td>
</tr>
<tr>
<td>LO</td>
<td>NAG + O\textsubscript{2} → HETE-Gly (oxidized NAGs)</td>
</tr>
<tr>
<td>COX-2\textsuperscript{b}</td>
<td>NAG + AH\textsubscript{2} + 2O\textsubscript{2} → A + H\textsubscript{2}O</td>
</tr>
<tr>
<td></td>
<td>+ PGH\textsubscript{2}-Gly and HETE-Gly (oxidized NAGs)</td>
</tr>
<tr>
<td>CYP4F\textsuperscript{b,c}</td>
<td>NAG + AH\textsubscript{2} + O\textsubscript{2} → A + H\textsubscript{2}O + (ω-1, ω-2, and ω-3-monohydroxylated NAGs)</td>
</tr>
</tbody>
</table>

Degradative reactions for NAGs.

\textsuperscript{a}This has only been shown for certain NAGs.

\textsuperscript{b}AH\textsubscript{2} represents an electron acceptor such as NAD\textsuperscript{+}

\textsuperscript{c}The coenzyme used here is NADP\textsuperscript{+} because reactions were performed \textit{in vitro} with bacterial CYP450BM-3 which used NADP\textsuperscript{+}. This bacterial P450 is used as a model of mammalian flavoprotein-reductase-utilizing class II P450s, and the mammalian P450 would presumably use NAD\textsuperscript{+}.  

### 4.2.4 Alternate Proposed Metabolism

In addition to the metabolism described above, a number of less well supported hypotheses for PFAM biosynthesis have been proposed (recently reviewed in references 55,135), and are summarized in Table 4-5.

The direct amidation of oleic acid by ammonia catalyzed by either FAAH working in reverse or by a glutamine synthetase-like enzyme has been proposed by more
than one group.\textsuperscript{23,57} Monocarboxylates, like fatty acids, are not substrates for either glutamine synthetase\textsuperscript{150} or asparagine synthetase.\textsuperscript{151} The conversion of oleic acid to oleamide by rat brain microsomes is inhibited by both glutamine and ATP-Mg\textsuperscript{2+},\textsuperscript{57} data arguing against a glutamine synthetase-like synthesis of oleamide and the other PFAMs.

The direct amidation of a FFA by the action of FAAH working in reverse was demonstrated \textit{in vitro}.\textsuperscript{16,57} However, oleamide production in the N\textsubscript{18}TG\textsubscript{2} cells \textit{increases} upon the inhibition or inactivation of FAAH,\textsuperscript{16,57} and FAAH knockout mice were found to have increased levels of \textit{N}-palmitoylglycine,\textsuperscript{17} providing evidence against a role for this enzyme for PFAM production \textit{in vivo}. In addition, the conditions used to achieve these amidations were not biological: 50 mM ammonia and an optimal pH above 9.\textsuperscript{16,57}

Another proposed mechanism for oleamide biosynthesis is by aminolysis of phospholipids\textsuperscript{16} or catabolism of sphingomyelin.\textsuperscript{73} There is no current evidence for sphingomyelin catabolism to a PFAM.\textsuperscript{55} Incubation of [\textsuperscript{14}C]-oleic acid-containing phospholipids with NH\textsubscript{4}OH in the presence of N\textsubscript{18}TG\textsubscript{2} cell homogenates did not result in the formation of [\textsuperscript{14}C]-oleamide, arguing against phospholipid aminolysis as a route to the formation of intracellular oleamide.\textsuperscript{16}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Proposed Amidation} & \textbf{Reaction} & \textbf{ref} \\
\hline
Cytochrome c & Acyl-CoA + NH\textsubscript{3} + H\textsubscript{2}O\textsubscript{2} + 2H\textsuperscript{+} \rightarrow PFAM + CoA-SH + 2H\textsubscript{2}O & 11,12 \\
Gln Synthase & FFA + ATP + Gln \rightarrow PFAM + ADP + P\textsubscript{i} + Glu & 23 \\
FAAH (in reverse) & FFA + NH\textsubscript{3} \rightarrow PFAM + H\textsubscript{2}O & 57 \\
Aminolysis & Phospholipid + NH\textsubscript{3} \rightarrow PFAM + Lyso-Phospholipid & 16,35,36 \\
Catabolism & Sphingomyelin \rightarrow PFAM + phosphosphingolino-choline or -ethanolamine & 64 \\
Serum synthase & Acyl-CoA + NH\textsubscript{3} (or gly) \rightarrow PFAM (or NAG)+ H\textsubscript{2}O & 55 \\
NAG synthase & 1. FFA + gly \rightarrow NAG + H\textsubscript{2}O \\
& 2. NAG \rightarrow PFAM via PAM reaction & 25 \\
\hline
\end{tabular}
\end{table}

Mueller and Driscoll (2009) recently reported oleamide synthesizing activity in fetal bovine serum (FBS).\textsuperscript{55} This “serum synthase” uses oleoyl-CoA and ammonia to form oleamide, or oleoyl-CoA and glycine to form \textit{N}-oleoylglycine. The 65 kDa enzyme is reported to be inactivated by normal enzyme denaturing conditions (heat, solvent,
trypsinization) and hydrogen peroxide, whereas cyt c is not inactivated under these conditions.\textsuperscript{55}

Acyl-coenzyme A is not the only activated intermediate possible. Other possible activated fatty acids for attack by glycine could include a fatty acyl adenylate similar to the amino acid adenylates involved in charging tRNAs or in the biosynthesis of mycothiol. Long-chain acyl adenylates are not known, but this does not rule out a potential role for them as activated intermediates. Another possibility could be a fatty acid thioester linked to a cysteine in a carrier protein, similar to intermediates observed in fatty acid biosynthesis.

Bradshaw \textit{et al.} recently proposed direct glycination of the fatty acid through a nucleophilic attack of the glycyl $\alpha$-amino group at the carboxylate of the fatty acid by an unknown NAG synthase.\textsuperscript{25} Direct glycination seems chemically unreasonable at biological pH since the amino group would be protonated and, thus, would be a poor nucleophile to attack the carboxyl. One solution could be a decrease in the pK\textsubscript{a} of the $\alpha$-amino group of the attacking glycine in the active site of the putative NAG synthetase. However, there is no direct evidence for such a novel NAG synthetase. Although the data presented from the Bradshaw group do not rule out such a unique chemistry, it also does not rule out the presence of an activated intermediate such as an acyl-CoA.

Recent evidence has been given to show cyt c is responsible for the formation of oleamide as well as $N$-oleoylglycine from oleoyl-CoA in the presence of H\textsubscript{2}O\textsubscript{2} \textit{in vitro}.\textsuperscript{11,24,132} Cyt c was first identified as the oleamide-synthesizing enzyme in rat liver and kidney extracts.\textsuperscript{11} The cyt c reaction has a pH optimum of 7.5, and although it’s stimulated greatly by the presence of H\textsubscript{2}O\textsubscript{2}, it can proceed at a low rate in the absence of H\textsubscript{2}O\textsubscript{2} and has a K\textsubscript{M} of 21 $\mu$M for oleoyl-CoA with NH\textsubscript{3}. Radiolabel studies showed that other long-chain acyl CoAs could inhibit the formation of $^{14}$C-oleamide from $^{14}$C-oleoyl-CoA, showing that other long-chain acyl CoAs could be substrates for cyt c.
However, the \textit{in vivo} significance of these findings is unclear. Cells lacking both somatic and testicular forms of cyt c failed to upregulate oleamide biosynthesis during apoptosis as expected. Upon protein purification, the oleamide-synthesizing activity remained after heat denaturation and exposure to proteolytic enzymes, and maximal conversion under optimal conditions was only 39.5\%. The turnover rate is also very slow: 1 s$^{-1}$ in optimal conditions. Neither ammonia nor H$_2$O$_2$ demonstrated saturating kinetics, and became inhibitory above their optimal concentrations. Again, the optimum amounts of H$_2$O$_2$ (2 mM) and ammonia (125 mM) are not in biological range, a finding that has prompted the proposal of an “oleamide synthesome” in which cyt c, SOD, ACBP and an AT would together act to house these high concentrations of substrates or keep them...
in a configuration to lower the optimal concentrations necessary (shown in Figure 4-3). Driscoll et al. has co-purified an ACBP and SOD with cyt c from rat kidney, providing support for such a synthesome.\textsuperscript{11} In this hypothetical oleamide synthesome SOD would produce H\textsubscript{2}O\textsubscript{2}, the ACBP would orient they acyl-CoA for action by cyt c, and the AT would generate the amide nitrogen, possibly from arginine or glutamine, although the amino transferase has not yet been identified.\textsuperscript{55}

The studies presented above provide for several hypotheses regarding the biosynthesis of NAGs and PFAMs. A summary of the evidence gathered \textit{in vivo} and \textit{in vitro/in cellulo} is shown in Figure 4-4. Most notably, there is likely more than one biosynthetic mechanism for the formation of NAGs \textit{in vivo}: oxidation of an NAE and glycination of a FFA. Enzyme expression studies in NAG- and PFAM-producing cells should shed some light into which enzymes may be involved in their metabolism. In this study we examined the expression of putative NAG and PFAM biosynthetic enzymes, including ACGNAT, ACSL3-6, BAAT, NMT, FAAH-1, PAM, cyt c, ADH3-4, AlDH3A1-2 and CYP4F.
4.3 Model Cell Systems

$N_{18}TG_2$ cells are an excellent model system for PFAM biosynthesis. When cultured with $^{14}$C-oleic acid, they produce $^{14}$C-oleamide and $^{14}$C-oleoylglycine.$^{28}$ These cells must, therefore, contain all the necessary enzymatic machinery to produce PFAMs from FFAs. In order to gain a more thorough perspective about long-chain PFAM metabolism, two additional cell lines were examined: SCP and HEK-293 cells, both of
which were shown to produce oleamide after incubation with oleic acid (Chapter 2). Several PFAMs have been found in the cerebrospinal fluid (CSF), another reason to suggest that the CSF-producing choroid plexus cells are a good candidate for the study of PFAM biosynthesis. Glycination is a common method to facilitate urinary detoxification of acyl compounds (see also Chapters 1 and 2), explaining why these cells have been included in our studies. In addition, SCP and N_{18}TG_{2} cells were shown to produce PFAMs from a variety of FFAs and from an NAE (Chapter 3), making them good candidates for analysis since they must both contain the enzymatic machinery for both the FFA → PFAM conversion and the NAE → PFAM conversion.

In addition to the PFAM-producing cell lines, commercially available human cDNA was used for PCR analysis. Liver and kidney cells exhibit the highest expression levels of the short-and medium-chain acyl-CoA processing enzyme ACGNAT and were chosen not only to serve as positive control for expression of that enzyme but also for their potential to process long-chain acyl CoAs. Liver is also known to express BAAT. Since several PFAMs have been found in the cerebrospinal fluid (CSF), whole brain cDNA was also used as another positive control to find any proteins that may be expressed in SCP cells.

4.4 Materials and Methods

4.4.1 Materials

Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Donor equine serum was from Thermo Scientific (Waltham, MA). DMEM, EMEM and penicillin/streptomycin were from Mediatech Cellgro (Manassas, VA). Mouse neuroblastoma N_{18}TG_{2} cells were from DSMZ (Deutsche Sammlung von Mikroorganism und Zellkulturen GmBH). SCP and HEK-293 cells were from American Type Culture Collection (Manassas, VA). Primers were from Integrated DNA Technologies (Coralville, IA). MicroPoly(A) Pure mRNA purification kit and RETROscript Reverse Transcription kit were from Ambion (Austin, TX). First strand human cDNA was from Origene (Rockville, MD). QIA-Quick gel extraction kit was from Qiagen (Valencia, CA). PVDF membrane was from Millipore (Billerica, MA). Tween-20, protease inhibitor
cocktail (P8340) and Bradford reagent were from Sigma-Aldrich (St. Louis, MO). PAM (S-16) and FAAH (V-17) antibodies and their blocking peptides were from Santa Cruz Biotech, Inc. (Santa Cruz, CA). FACL5 (N-term), FACL3 (center), and FACL6 (center) antibodies (against ACSL proteins) and their corresponding blocking peptides were from Abgent (San Diego, CA). Goat anti-rabbit and donkey anti-goat secondary antibodies conjugated with horse radish peroxidase were from ICN Biomedical (Solon, OH). The SuperSignal chemiluminescent detection system was from Pierce (Rockford, IL). All other reagents and cell culture supplies were of the highest quality available from commercial suppliers.

4.4.2 Cell Culture

All cells were grown in 225 cm$^2$ culture dishes. $N_{18}$TG$_2$ cells were grown in DMEM supplemented with 100 µM 6-thioguanine. SCP and HEK-293 cells were grown in EMEM. All cell types were grown with 100 I.U./ml penicillin, 1.0 mg/ml streptomycin and 10% FBS (SCP and $N_{18}$TG$_2$) or horse serum (HEK-293) at 37°C and 5% CO$_2$ according to supplier instructions. Cultures were grown to 80% confluency, cells washed with PBS and trypsinized. Cells were collected by centrifugation, rinsed with PBS, and re-pelleted before removal of supernatant and lysis.

4.4.3 Protein Sample Preparation

Cell pellets were taken up in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% (v/v) Triton X-100, and 1% (v/v) of a commercially available protease inhibitor cocktail. After incubation at 4°C for 10 minutes, samples were sonicated on ice with a microprobe sonicator for 5 minutes. Samples were then centrifuged and the protein concentration of the supernatant was determined via a Bradford assay using BSA as the standard according to manufacturer’s instructions. Samples were then diluted to 1-2 mg/ml with running buffer containing 5% β-mercaptoethanol and boiled for 5 minutes before storing at -20°C.

$^\text{2}$ From Sigma-Aldrich (P8340). Contained 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin.
4.4.4 Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run with 10% (resolving) and 4% (stacking) acrylamide at 170 V until the dye front ran off the bottom of the gel (40-70 min). Gels were electroblotted to a PVDF membrane at 80 V for 1 hour, and membranes were soaked for 1 hour in a blocking solution (Tris-buffered saline [TBS] containing 5% nonfat dry milk (NFDM) and 0.5% Tween-20 (TBS-T)), and incubated overnight at 4°C in the presence of one of the following antibodies: PAM (S-16), FAAH (V-17), FACL5 (N-term), FACL3 (center), or FACL6 (center) in TBS-T with 1% NFDM. Antibodies were not commercially available for all enzymes of interest. Western blots were run for all available antibodies.

After incubation, the membrane was washed 5 times with TBS-T, and then incubated for 1 hour with goat anti-rabbit or donkey anti-goat conjugated with horse radish peroxidase (diluted 1:10,000) in TBS-T with 3% NFDM at room temperature. A final set of 5 rinses was performed in TBS-T before visualizing antibody-antigen complexes using the SuperSignal chemiluminescent detection system on radiographic film.

A molecular weight ladder was loaded to a lane on each SDS-PAGE gel. Initial studies were with an unlabeled ladder, Bio-Rad (Hercules, CA) prestained standards, cat# 161-0318, and had to be drawn manually from the PVDF membrane to the film after exposure. Later studies utilized a commercial set of molecular weight standards (MagicMark™ XP from Invitrogen, Carlsbad, CA) and contained an IgG binding site, allowing direct visualization with downstream immunodetection. Bands were measured from an origin to make a standard curve and the unknown molecular weight assigned based on that standard curve. One such example of a standard curve is shown in Figure 4-14.

Blocking experiments were also performed with each antibody to verify that the proteins being detected were specific for the antibodies being used. Antibodies were incubated with 50x molar excess of blocking peptide for 2.5 hours at 36°C with agitation, followed by 2-24 hours at 4°C in a total volume of 500 µl in PBS before continuing with normal Western blot procedure. For FACL5, several bands disappeared in the N18TG2
and SCP samples, leaving behind the bands of appropriate MW for ACSL5. This indicated the location of the true band for ACSL5 and showed that it was being expressed in the cells, as it outcompeted the blocking peptide for FACL5 antibody. The HEK-293 cells did not show reduction of band number but had fewer bands after original incubation with FACL5 antibody. The bands disappeared for the remaining blocking experiments, but all previously detected bands had been the correct MW.

4.4.5 RT-PCR and Sequencing

Cell samples (N18TG2 and SCP) were isolated from culture (one T-75cm² flask of ~90% confluency), lysed, and their mRNA isolated using the MicroPoly(A) Pure™ mRNA purification kit. The mRNA sample was then reverse transcribed into cDNA using the RETROscript® Reverse Transcription kit. Human cDNA from liver, kidney and whole brain was used as commercially available. PCR primers were designed based on available sequences from GenBank, and a list of primers used can be found in Appendix D. Typical PCR conditions were an initial 3 min denaturation at 95°C, followed by 25-45 cycles of denaturation at 95°C (1 min), annealing at 45°C (1 min) and elongation at 72°C (1 min) with a final elongation cycle of 7 min at 72°C. The PCR product was gel-purified using a QIAQuick gel extraction kit, and the product sequenced on a Beckman sequencer or sent out for sequence verification at Moffitt Cancer Research Center.

Primers were designed from the available sequences using the OligoPerfect™ primer designer from Invitrogen. Many sequences for sheep are unknown and primers were designed from multiple sequence alignments of other mammalian sequences from regions of high homology. Upon verifying sequenced results, Basic Local Alignment Search Tool (BLAST) results for multiple related species with a similarity score of ≥96% were considered positive results for sheep.
4.5 Results

4.5.1 RT-PCR Gels

The results of RT-PCR and PCR are shown below. For all of these samples, bands were subjected to sequencing for verification. BLAST hits of the sequencing results were used to confirm the identity of the putative mRNA sequence. A summary of these results can be found in Table 4-7 and Figure 4-20.

![Image of RT-PCR Gels](image1)

**Figure 4-5: PCR in Human Kidney**
*Both ACGNAT bands were sequenced and both were matches to ACGNAT. There are two isomers of ACGNAT.

![Image of N18TG2 RT-PCR for ACSL4](image2)

**Figure 4-6: N18TG2 RT-PCR for ACSL4**
Band on left shows N18TG2 cDNA with ACSL4 primers (268 bp)

![Image of N18TG2 cDNA with Nested PAM primers](image3)

**Figure 4-7: N18TG2 cDNA with Nested PAM primers**
Nested primers were used to detect the expression of PAM. No band was expected after the first round of PCR.
Figure 4-8: PCR for FAAH

Lane 1: SCP cDNA (no band)
2: human whole brain cDNA
3: human kidney cDNA
4: human liver cDNA
5: MW ladder
All lanes with FAAH primers, and produce bands with 276 bp

Figure 4-9: PCR in Human Whole Brain and Liver cDNA
*Note that band is wrong size in lane 13. BLAST search of the sequencing results showed no significant similarity to database sequences

Figure 4-10: SCP PCR for PAM
SCP cDNA with PAM2 primer (nested) (431 bp)
The PCR for ACGNAT in human kidney revealed two PCR bands (Figure 4-5), both of which were sequenced and both of which were positive matches to human ACGNAT after BLAST analysis. There are two isozymes of ACGNAT, consistent with the finding of two bands. No other ACGNAT PCR revealed two distinct bands. Another anomalous finding was that a band appeared as a product of PCR with human liver cDNA and FAAH primers that was smaller than the expected size (Figure 4-9). Sequencing and BLAST analysis revealed no significant similarity to database sequences.

No positive result could be obtained for FAAH in SCP cells. Four different primer sets were designed and tested, each with no positive results. PAM was found in every sample tested, as were each ACS isoform and cyt c, as would be expected. In cases where enzyme expression could not be found in the SCP and $N_{18}TG_2$ model cell lines, human cDNA acted as a means to test the efficacy of the system for detection of those enzymes. ACGNAT, BAAT and AlDH3A2 were all found in the human cDNA but not the model cell lines. CYP4F was not found in SCP cells which may reflect a low abundance or
expression of a different isoform(s) in those cells, as there are many. There were three enzymes whose mRNA could not be detected in any samples: ADH4, AlDH3A1 and NMT (although NMT was only tested in SCP and N15TG2 cDNA). However, other isoforms of ADH and AlDH were detected. Table 4-7 provides a summary of PCR results.

4.5.2 Western Blots

Sample Western blots are shown below. Not all enzymes had commercially available antibodies, and Western blots were performed for all available antibodies. A summary of these results can be found in Table 4-7 and Figure 4-20. A sample graph for determination of molecular weight from a protein ladder is shown in Figure 4-14.

![Standard Curve for a MW Ladder in SDS-PAGE](image)

Figure 4-14: Standard Curve for a MW Ladder in SDS-PAGE
**Figure 4-15: FACL3 Western Blots**

Western blot for N\textsubscript{18}TG\textsubscript{2} and HEK-293 cells with FACL3 antibody against ACSL3. The amount of protein loaded is listed at the top of each well. Published MW for ACSL3 include 79-80 kDa, 73 kDa, and 64 kDa\textsuperscript{9} (from rat and mouse brain and human liver). The N\textsubscript{18}TG\textsubscript{2} sample on the left was lysed with a protease inhibitor cocktail with dithiothreitol, PMSF, Na\textsubscript{3}VO\textsubscript{4} and aproprotinin instead of the cocktail from Sigma.

<table>
<thead>
<tr>
<th>HEK-293</th>
<th>N\textsubscript{18}TG\textsubscript{2}</th>
<th>SCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5µg</td>
<td>5µg</td>
<td>5µg</td>
</tr>
<tr>
<td>22.5µg</td>
<td>15µg</td>
<td>15µg</td>
</tr>
<tr>
<td>83</td>
<td>74</td>
<td>64</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4-16: FACL5 Western Blots**

Western blot for N\textsubscript{18}TG\textsubscript{2}, HEK-293 and SCP cells with FACL5 antibody against ACSL5. The amount of protein loaded is listed at the top of each well. Published MW for ACSL5 include 73, 74.5 and 76 kDa (rat liver)\textsuperscript{22,37}

<table>
<thead>
<tr>
<th>HEK-293</th>
<th>N\textsubscript{18}TG\textsubscript{2}</th>
<th>SCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5µg</td>
<td>5µg</td>
<td>10µg</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>73</td>
</tr>
</tbody>
</table>

**Figure 4-17: FACL6 Western Blots**

Western blot for N\textsubscript{18}TG\textsubscript{2}, HEK-293 and SCP cells with FACL6 antibody against ACSL6. The amount of protein loaded is listed at the top of each well. Published MW for ACSL6 include 75 kDa (human K562 cells)\textsuperscript{30}, 78 kDa (human erethrocyes)\textsuperscript{26,27} A cross-reacting protein around 55 kDa (PC12 cells) was also reported\textsuperscript{29}
The Western blot bands detected matched literature-reported molecular weights fairly well (see Table 4-6, and captions of Figure 4-15). Although the molecular weights in this study that are displayed in Table 4-6 are derived from measuring the distance traveled over the length of the gel and plotting this ratio against a standard curve, inconsistencies in the gel and interference from overabundant protein in a single well can change the position of the band, as seen in Figure 4-15. In this case, the two different lanes containing HEK-293 proteins show different molecular weights due to loading a greater amount of protein in the “15µg” well. To help alleviate this, multiple wells were usually run with different amounts of protein from the same source. In each Western blot sample, the molecular band patterns across the three species were very similar, with the exception of PAM. There are many splice variants of PAM.
different tissues display different isoform expression. Expression data gathered through Western blotting matched that gathered via RT-PCR.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MW detected</th>
<th>Published MW</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSL3</td>
<td>83</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>ACSL5</td>
<td>75</td>
<td>76</td>
<td>74.5</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td>ACSL6$^a$</td>
<td>78</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAM</td>
<td>124</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>84-86</td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>59</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ACSL, long-chain acyl-CoA synthetase; FAAH, fatty acid amide hydrolase; MW, molecular weight; PAM, peptidylglycine α-amidating monooxygenase

Color key: mouse; sheep; human; rat; monkey

The tissue/cell source of each published MW is given in the caption below the corresponding Western blot figure.

$^a$A cross-reacting 55kDa protein was reported to bind to anti-ACSL6 antibodies

### 4.5.3 Summary

Using RT-PCR (or PCR) and DNA sequencing supplemented and verified by Western Blot analysis where possible, the expression of several purported PFAM biosynthetic enzymes was assayed in a series of cells and cDNA libraries, including the $N_{18}$TG2, SCP and HEK-293 cells, and in cDNA libraries from human liver, kidney, and whole brain cDNA. These expression data for all DNA sources are summarized in Table 4-7, and a visual representation of how these data fit with amide metabolism in $N_{18}$TG2 and SCP cells is shown in Figure 4-20. Not all enzymes had commercially available
antibodies, and where it was possible to perform both a Western Blot and RT-PCR, the results were in good agreement with each other.

Cyt c was found in all cell lines, as would be expected due to its important role in oxidative phosphorylation. The presence of ACSL(3-6), PAM, and FAAH, and not BAAT, ACGNAT or NMT in the oleamide-producing N18TG2 cells supports the hypothesis that cyt c or an enzyme yet to be discovered catalyzes N-fatty acylglycine

<table>
<thead>
<tr>
<th>Table 4-7: Summary of Expression Data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Acyl-CoA:glycine N-acyltransferase (ACGNAT)</td>
</tr>
<tr>
<td>Long Chain Fatty Acyl-CoA Ligase 3 (ACSL3)</td>
</tr>
<tr>
<td>Long Chain Fatty Acyl-CoA Ligase 4 (ACSL4)</td>
</tr>
<tr>
<td>Long Chain Fatty Acyl-CoA Ligase 5 (ACSL5)</td>
</tr>
<tr>
<td>Long Chain Fatty Acyl-CoA Ligase 6 (ACSL6)</td>
</tr>
<tr>
<td>Bile Acid-CoA:amino acid N-acyltransferase (BAAT)</td>
</tr>
<tr>
<td>N-Myristoyltransferase (NMT)</td>
</tr>
<tr>
<td>Fatty acid amide hydrolase (FAAH-1)</td>
</tr>
<tr>
<td>Peptidylglycine α-amidating monoxygenase (PAM)</td>
</tr>
<tr>
<td>Cytochrome C (Cyt c)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase 4 (ADH4)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase 3 (ADH3)</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase 3A1 (ADH3A1)</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase 3A2 (ADH3A2)</td>
</tr>
<tr>
<td>Cytochrome P450 (CYP4F)</td>
</tr>
</tbody>
</table>

P+/-, expression was/was not found by (RT-)PCR; W+/-, expression was/ was not found by Western Blot.

For human kidney samples, the cDNA was used for PCR and HEK-293 cells were used for Western blotting. PCR and Western blot results agree. At least one positive result was obtained for each enzyme except ADH3A1 and NMT.
production by the proposed glycination route \textit{in vivo}. Control experiments showing the expression of ACGNAT and BAAT in liver and ACGNAT in kidney are consistent with literature reports,\textsuperscript{40,125,128,129} and demonstrate the ability to find those enzymes at least in those cells. The expression of metabolic processing enzymes as found in these studies may contribute to the small amounts of NAGs isolated throughout tissue and cell types,\textsuperscript{17,101-103,111} since any NAG formed would be quickly metabolized by PAM, FAAH or CYP4F, or any of the other processing enzymes not probed for in this study (Table 4-4). No ADH4 expression was found in any cells, but those tissues probed are not known to contain ADH4, which is characteristically expressed in epithelial tissues of the aerodigestive tract and a few other tissues.\textsuperscript{167} The lack of expression of the specific ADH and AIDH enzymes for which we probed denotes that either other ADH and possibly AIDHs may be responsible for NAE oxidation in N\textsubscript{18}TG\textsubscript{2} cells, or that glycination of the FFA is the only pathway for NAG formation in these cells (aside from possible direct amidation of acyl-CoA by cyt c, or some unknown possibility).
The expression of ADH3 alongside PAM, ACSL and cyt c in the SCP cells shows that there are multiple possible mechanisms to synthesize NAGs in these cells, and confirms that the metabolic network may be more complicated than a linear scheme (refer to Figure 4-1 Figure 4-20). SCP cells may be able to synthesize NAGs both through glycination of the FFA and oxidation of an NAE.

FAAH had previously been found in N_{18}TG_2 cells,^{168,169} and those findings were confirmed in this report. FAAH-1 was also found in human liver, kidney and brain,^{47,170} findings which were also replicated here. SCP cells had approximately 45 times as much endogenous oleamide and at least 10 times as much after incubation with oleic acid (see Figure 4-20: Metabolic Diagram of Expression Data for SCP and N_{18}TG_2 Cells Overview of expression data in SCP (pink) and N_{18}TG_2 (orange) expression data as it relates to the proposed metabolic pathways.)
Chapters 2 and 3). The lack of detectable levels of FAAH expression in SCP cells is commensurate with the higher levels of PFAM that were detected in previous chapters, and makes sense given the known role of choroid plexus in producing CSF where PFAMs have been found.\textsuperscript{72-74,172}

The literature precedence for FAAH expression is mixed. While there has been a noted lack of FAAH expression in mouse choroid plexus (CP),\textsuperscript{173} FAAH has been found in rat CP.\textsuperscript{173} Since oleamide has been found in rat plasma (35-56nM)\textsuperscript{89,172} and rat cerebrospinal fluid (156nM),\textsuperscript{172} enzymologic studies may reveal the affinity of rat FAAH for oleamide to be lower than other species’ or for the active oleoyl amide in rats to be primarily N-oleoylglycerine, since it can act independently of oleamide.\textsuperscript{104} The possibility that different mammalian FAAH shows different substrate specificity may have some precedence. Although human and rat FAAHs share 82% amino acid identity, they demonstrate distinguishable enzymological properties.\textsuperscript{15} Rat FAAH shows a much lower rate of hydrolysis of myristamide and palmitamide than does human FAAH; human FAAH hydrolyzes these substrates about three times faster.\textsuperscript{15} N-arachidonoylglycerine is a much more potent inhibitor of rat FAAH than of human or mouse, and N-arachidonoylalanine is a much more potent inhibitor of human and rat FAAH than of mouse.\textsuperscript{20}

The level of each PFAM found in Chapter 3 after incubation with the corresponding FFA in N\textsubscript{18}TG\textsubscript{2} cells was inversely proportional to substrate preference for FAAH, but this pattern was not observed in SCP. With no FAAH to put pressure on the degradation of these PFAMs, the SCP cells showed a different profile. This difference in PFAM fingerprint, along with the large difference in PFAM amount in the two cell lines, demonstrates the importance of FAAH in determining endogenous levels of PFAMs.

One of the central questions in NAG biosynthesis is that of the prevalence of the two competing pathways hypothesized: NAE oxidation of FFA glycination (Figure 4-2). In Chapter 3 it was shown that SCP and N\textsubscript{18}TG\textsubscript{2} cells show different affinities for FFAs and NAEs in converting them to the PFAM. N\textsubscript{18}TG\textsubscript{2} cells were indiscriminant with respect to the formation of tridecanamide from its two metabolic precursors: N-tridecanoylethanolamine (TDEA) and tridecanoic acid (TDA). SCP cells, however, were able to convert TDEA to the corresponding PFAM at a higher rate than for the FFA. One
possibility is the differential expression of discriminating fatty acyl amide transport proteins in the two cell lines. The expression data presented here provides a hypothesis. N18TG2 cells lacked expression of all ADH and AlDH’s examined. Thus, these cells likely metabolized TDEA to TDA by way of FAAH before conversion to tridecanamamide. This may explain why TDA and TDEA were equal metabolic substrates in N18TG2 in terms of PFAM formation, as the acyl chains would all proceed through the same metabolic pathway, and the action of FAAH would not impede this metabolism due to its high turnover rate.15 Another NAE-hydrolyzing enzyme, N-acylethanolamine-hydrolyzing acid amidase (NAAA) may also hydrolyze TDEA to its corresponding FFA.46 The expression of NAAA was not examined.

SCP cells were shown to express ADH3. While ADH3 is not known to perform the N-acylglycinal → NAG reaction, it could be responsible for the synthesis of the aldehyde intermediate and an unidentified enzyme could finish the oxidation to an NAG. Studies have also shown that this dismutation of the relatively unstable long-chain aldehyde intermediate to its NAG can proceed at low rates non-enzymatically.141 Therefore, it is hypothesized that sequential oxidation of an NAE is the preferred metabolic route in SCP cells.

HEK-293 cells were used for the Western blot expression studies, but commercially available human whole kidney cDNA was used for PCR. All the enzymes found in the whole human kidney cDNA were also found in HEK-293 cells even though HEK-293 contained only epithelial cells of the kidney.

**4.6 Conclusions**

The expression data presented here helps confirm several hypotheses diagrammed in Figure 4-1. Sequential oxidation of an NAE is a possible alternate route to the synthesis of N-acylglycines since dehydrogenases were found to be expressed in oleamide-producing SCP cells, cells that also showed a differential pattern of FFA and NAE conversion to PFAMs than N18TG2 cells. Perhaps the most significant finding was in regards to putative enzymes involved in the acyl-CoA → NAG reaction. Cyt c or a novel enzyme is likely to be involved in conversion from acyl-CoA to the NAG intermediate since no other CoA:glycine transferring candidate enzymes were found in
the N18TG2 cells (Figure 4-20 and Table 4-8). More analysis is required to determine the exact in vivo role of cyt c and regulatory mechanisms of this pathway.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction Catalyzed</th>
<th>Expressed in N18TG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACGNAT</td>
<td>R-CO-S-CoA + Gly → R-CO-NH-CH2-COOH + CoA-SH</td>
<td>No</td>
</tr>
<tr>
<td>Cyt C</td>
<td>R-CO-S-CoA + Gly → R-CO-NH-CH2-COOH + CoA-SH</td>
<td>Yes</td>
</tr>
<tr>
<td>NMT</td>
<td>Myristoyl-CoA + Gly-Peptide → Myristoyl-Gly-Peptide + CoA-SH</td>
<td>No</td>
</tr>
<tr>
<td>BAAT</td>
<td>Bile Acyl-CoA + Gly → Bile Acyl-CO-NH-CH2-COOH + CoA-SH</td>
<td>No</td>
</tr>
<tr>
<td>ADH3</td>
<td>R-CO-NH-CH2-CH3OH + NAD⁺ → R-CO-NH-CH2-CHO + NADH</td>
<td>No</td>
</tr>
<tr>
<td>ADH4</td>
<td>R-CO-NH-CH2-CH3OH + NAD⁺ → R-CO-NH-CH2-CHO + NADH</td>
<td>No</td>
</tr>
<tr>
<td>FAIDH3A1</td>
<td>R-CO-NH-CH2-CHO + NAD⁺ → R-CO-NH-CH2-COOH + NADH</td>
<td>No</td>
</tr>
<tr>
<td>FAIDH3A2</td>
<td>R-CO-NH-CH2-CHO + NAD⁺ → R-CO-NH-CH2-COOH + NADH</td>
<td>No</td>
</tr>
</tbody>
</table>

4.7 References


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5 Metabolic Elucidation of PFAM and NAG Biosynthetic Pathways

5.1 Introduction

The findings in Chapters 2-4 provide new information regarding the biosynthesis of NAGs and PFAMs in model cell lines. Both N-acylethanolamines (NAEs) and free fatty acids (FFA) can serve as precursors for the formation of primary fatty acid amides (PFAMs), and although the list of candidate enzymes involved in these pathways has been narrowed, their identities are still unclear. Enzyme expression assays provided no alcohol dehydrogenase (ADH) candidate in the N18TG2 cells, and the only putative glycination enzyme found was cytochrome c (cyt c), which one would expect to find in every cell line performing oxidative phosphorylation.

Herein the role of cyt c in PFAM formation is investigated. We also examine the kinetics and mechanism of PFAM formation from NAE and FFA precursors using isotope labeling. In addition, we examine further the roles of fatty acid amide hydrolase (FAAH) in an additional model cell line, aortic endothelium, to further our understanding of not only the degradative enzyme but also the potential role of PFAMs in the cardiovascular system. Additionally, we explore the role of peptidylglycine α-amidating monoxygenase (PAM) with specific RNAi targeting combined with isotopic labeling to explicitly define its role in PFAM biosynthesis.
5.2 Primary Fatty Acid Amides and FAAH in Aortic Endothelial Cells

Peptidylglycine α-amidating monooxygenase (PAM) is widely distributed throughout the body, and has been found in the cardiovascular system in cardiac ventricles,\(^5\) blood,\(^6\) atria,\(^5\) and aortic endothelium.\(^7\) Other α-amidating peptide biosynthetic enzymes, prohormone convertase 1 and 2 and carboxypeptidase E, are also found in the atria.\(^8\)\textsuperscript{-11} These enzymes, including PAM, are known to play important roles in regulating amidated peptides in the cardiovascular system,\(^7\)\textsuperscript{,12,13} but amidated acyl chains (PFAMs) have only been isolated from whole rabbit heart,\(^14\) so the individual tissue component(s) responsible for PFAM biosynthesis are unknown,\(^15\) although PFAMs have been found in human\(^16\) and rat,\(^17,18\) plasma and rat cerebrospinal fluid.\(^17\) It has been suggested that these molecules are rapidly degraded by fatty acid amide hydrolase (FAAH), making their isolation difficult.

FAAH has been established as the enzyme responsible for hydrolyzing oleamide and other primary fatty acid amides (PFAMs) and \(N\)-acylethanolamines (NAEs) \textit{in vitro} and in other cell systems.\(^19\) FAAH has been well characterized in two endothelial cell lines: rat kidney\(^20\) and human umbilical vein.\(^21\) Although FAAH-2 has been found in primate heart, FAAH-2 is not a murid enzyme and to date there has been no evidence for PFAM hydrolysis via FAAH in the heart where PAM has been found.\(^15,22\)

Phenylmethylsulfonyl fluoride (PMSF) is a known FAAH inhibitor,\(^15\) and it has been used to prevent anandamide degradation in rat brain membrane samples.\(^23\) In this study, we attempt to determine whether oleamide can be isolated from human aortic endothelial cells (HAEC) endogenously, and whether FAAH is actively degrading oleamide in these cells to better understand the role of oleamide and other PFAMs in the cardiovascular system. Aortic endothelial cells were chosen because of their role in vasorelaxation,\(^24\) where anandamide-regulated vasodilation is endothelium-dependent.\(^25\)
5.2.1 Materials and Methods

5.2.1.1 Materials

HAEC cells and endothelial cell basal medium with growth supplements were from Lonza (Walkersville, MD). Oleic acid (OA) was from Sigma-Aldrich (St. Louis, MO). BSTFA and silica were from Suppelco (St. Louis, MO). All other reagents and cell culture supplies were of the highest quality available from commercial suppliers.

5.2.1.2 Cell Culture and Fatty Acid Incubation

Cells were grown in 75 cm² culture dishes at 37°C and 5% CO₂ according to manufacturer’s instructions. Cultures were grown to 75-90% confluency and 200 µM oleic acid was added alone or in combination with either 1% dimethylsulfoxide (DMSO) or 100 µM phenylmethylsulfonyl fluoride (PMSF). Note that BSA was not used as a carrier protein for OA. PMSF (100 µM) was added to flasks containing OA for the last hour of incubation (except for the flask already containing PMSF). (For a sample list see Table 5-1). Cells were then collected by scraping, centrifuged, the conditioned media decanted, and the resulting cell pellet and conditioned media stored at -80°C. Cells were too small to count on the available microscopes so a cell volume was taken instead. Cell count was estimated based on the assumptions of cell density being 1 g/ml and the weight of a human cell being 10⁻⁹ g.⁴

5.2.2 Metabolite Extraction

Metabolites were extracted from cells using protocols similar to Sultana and Johnson.⁵ Methanol (10 ml) was added to the cell pellets and samples were sonicated for 15 minutes at room temperature. Samples were centrifuged at 5000 rpm for 10 min and the supernatant decanted and dried under N₂ in a warm water bath at 40-50°C. The pellet was re-extracted with 4 ml 1:1:0.1 (v/v/v) chloroform:methanol:water, sonicated for 10 min, vortexed 2 min and centrifuged 10 min at 5000 rpm. The supernatant from this step was added to the supernatant from the previous step and continued drying under N₂ at 40-50°C. The pellet was re-extracted with 4.8 ml chloroform:methanol 2:1 (v/v) and 800 µL
0.5 M KCl/0.08 M H₃PO₄, sonicated 2 min, vortexed 2 min, and centrifuged 10 min at 5000 rpm. The lower lipid phase was added to the supernatants from the previous steps and taken to dryness under N₂ at 40-50°C.

Metabolite extraction from conditioned media was carried out similarly. The entire media contents of each experimental flask were centrifuged to pellet any cells and the supernatant submitted to solvent extraction. The first two extractions were with 15 ml chloroform:methanol 2:1 and the second two extractions with 15 ml chloroform:methanol 2:1 and 2.4 ml 0.5 M KCl/0.08 M H₃PO₄. No sonication was performed, and protein layers were condensed by centrifugation for 30-45 min at 10,000 rpm. Organic lipid phases were also combined and dried under N₂ at 40-50°C.

5.2.3 Solid Phase Extraction

Silica columns (0.5g) were washed with n-hexane and run essentially as described by Sultana and Johnson. Lipid extracts were taken up in 100 µL n-hexane and added to washed silica columns. The mobile phase was run without positive pressure as follows: 4 ml n-hexane, 1 ml 99:1 hexane:acetic acid, 1 ml 90:10 hexane:ethyl acetate, 1 ml 80:20 hexane:ethyl acetate, 1 ml 70:30 hexane:ethyl acetate, 1.5 ml 2:1 chloroform:isopropanol, 0.5 ml methanol. The last two fractions were combined and dried down under N₂ at 40-50°C.

5.2.4 Sample Derivitization

Trimethylsilylation was achieved using 100 µL BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide). Samples were flushed with dry N₂, BSTFA added, flushed briefly again, and allowed to react at 55-60°C for 1 hour. More nitrogen was flushed to reduce the sample volume, and the entire sample was injected onto the GC-MS.

5.2.5 GC-MS

All separations were performed using a Shimadzu QP-5000 GC-MS. Separations were achieved on a J & W Scientific DB-5 column (0.25 mm x 30 m) in splitless mode.
The GC temperature program was 55-150°C at 40°C/min, hold at 150°C for 3.6 min, ramp at 10°C/min to 300°C, and hold for 1 min. The transfer line was held at 280°C and the injection port at 250°C throughout the separation. Helium was used as the carrier gas, at a flow rate of 0.9 ml/min. The mass range was 35-750 amu with a scan speed of 2000. The solvent cut time was set to 5 min.

**5.2.6 Results**

The amount of oleamide in each HAEC cell and conditioned media sample was successfully determined and can be found in Table 5-1. The amount of oleamide found after 24 and 48 hours of incubation with OA (and no BSA vehicle) was approximately the same, but both values were much higher than that observed in the normal cell sample. An interesting note is that incubation for 48 hours with OA and a delivery vehicle, DMSO, resulted in an even greater amount of oleamide per cell than did inhibition of FAAH.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Oleamide in Cells</th>
<th>Oleamide in Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h 1% DMSO + OA</td>
<td>11.6</td>
<td>215</td>
<td>227</td>
</tr>
<tr>
<td>24h OA</td>
<td>11.2</td>
<td>52</td>
<td>63.2</td>
</tr>
<tr>
<td>48h PMSF + OA</td>
<td>10.1</td>
<td>101</td>
<td>111</td>
</tr>
<tr>
<td>48h OA</td>
<td>2.5</td>
<td>61</td>
<td>63.5</td>
</tr>
<tr>
<td>normal</td>
<td>1.2</td>
<td>0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Values are average pmol oleamide per 10⁷ cells. Cell count was estimated based on cell volume and an estimated cell weight of 10⁻⁹g.⁴
Inhibition of FAAH by PMSF has resulted in a marked increase in oleamide production after 48 hours as compared to cells incubated with OA and no FAAH inhibitor (Figure 5-1), indicating that FAAH or another protease targeted by PMSF contributes to the degradation of oleamide or one of its precursors. Another possibility is that a protease that would normally hydrolyze N-oleoylglycine is being targeted by PMSF, although a protease responsible for this hydrolysis has not yet been identified (see Chapter 4).

The finding of oleamide in endothelial cells is consistent with their function in control of vasodilatation, as oleamide is a potent vasorelaxant,\textsuperscript{24} and indicates that PFAMs may have a function in the cardiovascular system. Another function of endothelial cells is in angiogenesis, and erucamide is known to play a role in blood vessel growth.\textsuperscript{27,28} It would not be unexpected to find erucamide and other PFAMs in this tissue type.

Although PMSF is a known FAAH inhibitor, it and many other chemical inhibitors are not necessarily specific to the enzymes of interest. The development of RNA interference (RNAi) allows for direct targeting of the enzyme of interest by taking advantage of the natural defense system of the cell against double-stranded bacterial
RNA, and is used herein to explore the functions of two other potential PFAM-forming enzymes: PAM and cytochrome c.

5.3 siRNA optimization

When double stranded RNA (dsRNA) is detected in a eukaryotic cell containing this defense system, the enzyme Dicer cleaves long dsRNA into shorter fragments of dsRNA about 20-25 nucleotides in length. Many modern RNAi experiments start by incubating with these shorter dsRNA pieces delivered by some vehicle through the media. One of the strands of these small interfering RNA (siRNA) pieces is then bound to an endonuclease-containing RNA-induced silencing complex (RISC) to act as a template to search for complimentary mRNA within the cell. Once a complimentary mRNA sequence is detected by the RISC complex, it is cleaved by the Argonaute portion of the complex, preventing its translation and effectively knocking down expression of that gene.

In order to use siRNA for gene silencing, experimental conditions had to be optimized for the N18TG2 cells. Typical methods of siRNA delivery include transfection reagents and, for more problematic samples, electroporation. Exact structures of transfection reagents are proprietary, but most are lipid- or amine-based. Electroporation is used in samples for which standard transfection methods are problematic. Two transfection reagents were used in this study.

5.3.1 Materials and Methods

5.3.1.1 Materials

Transfection was done with Silencer® siRNA transfection II optimization kit from Ambion (Austin, TX) containing both siPORT™ NeoFX™ and siPORT™ Amine transfection reagents, GAPDH siRNA and scrambled siRNA. DMEM and penicillin/streptomycin were from Mediatech Cellgro (Manassas, VA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Mouse neuroblastoma N18TG2 cells were from DSMZ (Deutsche Sammlung von Mikroorganism und Zellkulturen
GmbH). PVDF membranes were from Millipore (Billerica, MA). Tween-20, protease inhibitor cocktail (P8340), and Bradford reagent were from Sigma-Aldrich (St. Louis, MO). GAPDH (FL-335) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) Goat anti-rabbit secondary antibody conjugated with horse radish peroxidase was from ICN Biomedical (Solon, OH). SuperSignal chemiluminescent detection system was from Pierce (Rockford, IL). All other reagents and cell culture supplies were of the highest quality available from commercial suppliers.

5.3.2 Cell Culture

During normal growth, N11TG2 cells were grown in DMEM supplemented with 100 µM 6-thioguanine, 100 I.U./ml penicillin, 1.0 mg/ml streptomycin and 10% FBS at 37°C and 5% CO₂ according to supplier instructions. During RNAi optimization experiments, 2.5x10⁴ cells were incubated in each well of a 6-well plate; DMEM + pen/strep (normal media with no FBS) was used (instead of OPTI-MEM I media as suggested by the manufacturer. The concentration of each siRNA was used as suggested by the manufacturer, and two different delivery reagents: siPORT Amine and siPORT NeoFX, were used according to manufacturer’s instructions. After 72 hours incubation, cells were collected by scraping, centrifuged in a sterile 1.5 ml Eppendorf tube, rinsed with PBS, and re-pelleted before removal of supernatant and cell lysis.

5.3.3 Protein Sample Preparation

Cell samples were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, and 1% of a commercially available protease inhibitor cocktail. After incubation at 4°C for 10 minutes, samples were sonicated on ice with a microprobe sonicator for 5 minutes. Samples were then centrifuged, and the protein content of the supernatant determined by Bradford assay. The supernatant was diluted to 1-3 mg/ml with running buffer containing 5% β-mercaptoethanol and boiled for 5 minutes before storing at -20°C.

© From Sigma-Aldrich (P8340). Contained 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin.
5.3.4 Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run with 10% (resolving) and 4% (stacking) acrylamide at 170 V until the dye front ran off the bottom of the gel. Gels were electroblotted to a PVDF membrane at 80 V for 1 hour, and membranes were soaked for 1 hour in a blocking solution (Tris-buffered saline, TBS, containing 5% nonfat dry milk (NFDM) and 0.5% Tween-20 (TBS-T)) and incubated overnight at 4°C in the presence of the antibody (diluted 1:500) in TBS-T with 1% NFDM.

After incubation, the membrane was washed 5 times with TBS-T, and then incubated for 1 hour with goat anti-rabbit conjugated with horse radish peroxidase (diluted 1:10,000) in TBS-T with 3% NFDM at room temperature. A final set of 5 rinses was performed in TBS-T before visualizing antibody-antigen complexes using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL) on radiographic film.

5.3.5 Results

GAPDH knockdown was observed in N18TG2 cells incubated with siRNA in the siPORT® Amine, but not the siPORT® NeoFX®, transfection reagent, as seen in Figure 5-2. Published MW for GAPDH (37 kDa) is similar to that found here.1-3 In addition to the successful demonstration of siPORT® Amine as a transfection reagent for N18TG2 cells, the demonstration that no knockdown occurred after incubation with scrambled siRNA indicates no off-target effects in this system.
5.4 PAM knockdown studies

One of the criticisms of PFAM metabolic elucidation is that, while pharmacological inhibitors have been used to target PAM \textit{in vitro} \cite{30,31} and \textit{in cellulo} \cite{32}, these inhibitors lack specificity and high efficacy. \cite{33,34} Inhibition of PAM by \textit{trans}-4-phenyl-3-butenoic acid (PBA) after injection was very short lived, with serum PHM activity being restored after just 24 hours. \cite{34} In addition to targeting PAM, 1 mM PBA was also shown to inhibit cyclooxygenase-2 activity by 82\%, \cite{35} which may cause undesired off-target effects \textit{in cellulo}.

Although pharmacological inhibitors have been used to demonstrate the accumulation of $^{14}$C-$N$-Oleoylglycine after incubation with $^{14}$C-oleic acid, \cite{32} strongly suggesting that PAM is involved in converting $N$-oleoylglycine to oleamide, an experiment designed to specifically target PAM is required to determine the extent of its \textit{in cellulo} significance in this pathway. There are more specific and effective pharmacological inhibitors of PAM, \cite{35} but the most effective and specific inhibitor by modern standards is targeted siRNA. Another layer of specificity is added by the use of a labeled substrate: $^{13}$C$_{18}$-oleic acid. Incubation with labeled OA and with successful PAM knockdown using the NAG assay method developed in Chapter 2 would represent
definitive proof of a role played by PAM in this biochemistry in cellulo if the corresponding labeled NAG could be isolated from the cells in appreciable amounts.

5.4.1 Materials and Methods

TriFECTa™ RNAi kit containing three anti-PAM siRNA was a gift from Integrated DNA Technologies (Coralville, IA). The siPORT™ Amine transfection agent was from Applied Biosystems (Foster City, CA). PAM (S-16) antibody was from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA). Donkey anti-goat secondary antibodies conjugated with horse radish peroxidase was from ICN Biomedical (Solon, OH). BSTFA and silica were from Supelco (St. Louis, MO). Heptadecanoic acid, D_{33} was from C/D/N isotopes (Pointe-Claire, Quebec). Fatty acid-free bovine serum albumin (BSA) and \(^{13}\text{C}_{18}\)-oleic acid were from Sigma-Aldrich (St. Louis, MO). The remaining materials are as described in Section 5.3.1.1.

The siRNA time course was run as described in Section 5.3.1 but with 24 and 48 hour incubations to determine optimal siRNA incubation time. \(^{13}\text{C}_{18}\)-N-Oleoylglycine and \(N\)-heptadecanoylglycine, D_{33}, (HdG) were synthesized as described in Chapter 2, Section 2.2.2.3. The fatty acid-BSA mixture was made as described in Chapter 3, Section 3.3.3. Based on time course study results, the cells were incubated for 56 hours with siRNA “A,” 48 of those hours with 2.5 mM \(^{13}\text{C}_{18}\)-oleic acid in 0.25 mM BSA. Growing and collection conditions of N_{18}TG_{2} cells are described in Chapter 2, Section 2.2.5.

After collection, cells were extracted as described in Section 2.2.6. An aliquot of cell extract was removed for oleamide analysis, and metabolites were separated via SPE as described in Section 2.2.7. The remaining sample was run on prep TLC as described in Section 2.2.8 for N-oleoylglycine analysis. Before prep-TLC and after solvent extraction, samples were spiked with 15 nmol HdG. GC-MS was run as described in Section 2.2.10, including running a spiked sample after two aliquots of the original sample were run.
5.4.2 Results

Two of the siRNA tested in the timecourse study, “A” and “B,” showed good knockdown for PAM in the N_{18}TG_{2} cells (Figure 5-3). siRNA “A” showed better lasting effects over 48 hours so it was used in subsequent experiments. The time course studies show that the siRNA-mediated knockdown for PAM lasts for at least 48 hours with this sequence, covering the entire time of the incubation with fatty acid. After 24 hours there is less PAM than there is after 48 hours with the siRNA, as the cell slowly returns to its normal metabolism. This means that any lingering PAM should interfere minimally with this experiment when OA is added to the culture medium (Figure 5-3).

![Figure 5-3: PAM Knockdown by siRNA in N_{18}TG_{2} Cells](image)
The Western blot shows the amount of PAM in N_{18}TG_{2} cells after incubation with various siRNA for 24 and 48 hours.

Once it was determined which siRNA produced the best knockdown results, incubations were performed with siRNA “A” followed by 2.5 mM {^{13}C_{18}}-oleic acid in 0.25 mM BSA 8 hours later for a total incubation time of 56 hours. The average viability after 48 hours of PAM siRNA was 54%. An aliquot of each sample was removed for oleamide quantification, and the remaining sample used for N-oleoylglycine (NOG) quantification. Normal samples were run alongside as negative controls. The results of the prep-TLC isolation and GC/MS are shown in Figure 5-4, and the quantified amounts of N-oleoylglycine are shown in Figure 5-5.
Figure 5-4: $^{13}$C$_{18}$-N-Oleoylglycine in N$_{18}$TG$_2$ Cells Incubated with $^{13}$C$_{18}$-Oleic Acid and PAM siRNA
Panel A shows the GC of N$_{18}$TG$_2$ cell extract over the whole time scale (top) and a close-up of the $^{13}$C$_{18}$-N-oleoylglycine-TMS (bottom). Panel B shows the MS of $^{13}$C$_{18}$-N-oleoylglycine-TMS, and panel C shows the fragmentation of this molecule. Note that $^{13}$C$_{18}$-N-Oleoylglycine is N-Oleoylglycine-3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20-$^{13}$C$_{18}$. There are no $^{13}$C on the glycine portion of the molecule.
The amount of N-oleoylglycine found in the PAM-knockdown N₁₈TG₂ cells was higher than expected at 7510 (± 697) pmol/10⁷ cells, compared to the normal cells with 50 (± 29) pmol/10⁷ cells (Figure 5-5). This is the first quantitative measurement of endogenous N-oleoylglycine in N₁₈TG₂ cells. A prior experiment showed an accumulation of a peak with the same retention time as N-oleoylglycine with the addition of a PAM inhibitor, PBA, but that peak was neither verified nor quantified, and an endogenous amount was not determined.

In comparing the amount of N-oleoylglycine (NOG) to oleamide found after incubation with oleic acid (OA), there is much more NOG than oleamide found when PAM expression is suppressed (Figure 5-6). The average amount of oleamide (both labeled and unlabeled) found in these cells after 56 hours of PAM siRNA and 48 hours of 2.5 mM ¹³C₁₈-OA incubation was 7.5 pmol/10⁷ cells, much smaller than the normal endogenous amount of 119 pmol/10⁷ cells in the absence of OA. These data explicitly define PAM as the in cellulo enzyme responsible for the oxidative cleavage of NOG to oleamide.

The amount of oleamide found after 0, 12, 24, and 48 hour incubations with OA sans PAM siRNA from Chapter 2 is shown here in Figure 5-6 for comparison alongside the NOG quantification. The amount of NOG isolated after PAM knockdown and OA incubation (7510 pmol/10⁷ cells) is much higher than the amount of oleamide isolated
after OA incubation (2250 pmol/10^7 cells). This may indicate other fates of NOG beyond amidation by PAM. Indeed, a recent study revealed that NOG can function independently of oleamide; oleamide-like effects were observed after injection of NOG but serum levels of oleamide did not increase.36 Another possibility is that the greater amount of NOG may indicate simply that oleamide is a better substrate for FAAH than NOG.

**Figure 5-6: Oleamide and N-Oleoylglycine in N18TG2 cells with and without PAM RNAi**
The left graph shows oleamide isolated from N18TG2 cells and media incubated with OA over a time course (red and blue). Co-graphed is labeled NOG and oleamide isolated after 56 hours incubation with anti-PAM siRNA (turquoise and orange), as well as the endogenous amount of NOG (light blue). The graph on the right is a close-up of endogenous levels of oleamide and NOG, as well as oleamide after knockdown of PAM and incubation with OA. Despite the higher level of NOG found after PAM knockdown and OA incubation, the endogenous amount of NOG found was lower than endogenous oleamide. The low levels of endogenous NOG reported here may partially explain the lack of literature in this field until more recently (see Chapter 2). The advent of more sensitive analytical techniques was required to identify compounds of such low abundance. In addition to quantifying NOG after PAM knockdown and OA incubation, the amount of oleamide was also quantified.
5.5 Cytochrome C Knockdown Studies

Several recent articles by the Mueller group have implicated cytochrome c (cyt c) in the biosynthesis of oleamide. *In vitro* studies have shown the formation of oleamide and N-oleoylglycine upon incubation of oleoyl-CoA with cyt c and either ammonia or glycine, respectively.\(^{37-39}\) In addition, it was found to be expressed in oleamide-producing N\(_{18}\)TG\(_{2}\) cells whereas other potential NAG-forming enzymes were not (Chapter 4). The potential role of cyt c in PFAM biosynthesis is elaborated in Figure 5-7.

Due to its co-purification with a superoxide dismutase (SOD) and fatty acyl-CoA binding protein (ACBP), an oleamide “synthesome” was hypothesized in which the ACBP would orient an incoming acyl-CoA so that its thioester is proximal to the heme of cyt c. SOD would provide the H\(_2\)O\(_2\) that was observed to increase catalytic activity.

The oleamide- and N-oleoylglycine-forming activity of cyt c increases in the presence of H\(_2\)O\(_2\), although some activity is observed without the peroxide. The reported optimal level of H\(_2\)O\(_2\) for these reactions is very high (2 mM H\(_2\)O\(_2\)). Base levels of cyt c activity were explored sans H\(_2\)O\(_2\) and found to be 5.4% conversion at best with 0.5 mg/ml cyt c, 5 mM NH\(_3\) and 1 mM oleoyl-CoA after 75 minutes of incubation time (see Appendix E). Other acyl-CoAs were not found to be cyt c substrates under these conditions (data in Appendix E). If cyt c is involved in PFAM biosynthesis it is likely to use H\(_2\)O\(_2\) or some other means of reoxidizing its Fe\(^{2+}\).

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Figure 5-7: Putative Role of Cyt C in PFAM Biosynthesis
1 = Acyl CoA synthetase (ACS)
2 = unknown enzyme or cytochrome c (cyt c)
3 = Peptidylglycine \(\alpha\)-amidating monooxygenase (PAM)
To best study the role of cyt c in vivo/in cellulo, its removal by RNAi is the most targeted approach to isolate its role in PFAM biosynthesis. In these experiments, N₁₈TG₂ cells were incubated with cyt c siRNA and oleic acid (OA) to determine the effect of cyt c knockdown on oleamide biosynthesis as compared to our standard incubation with OA from Chapter 2.

5.5.1 Materials and Methods

Silencer® Select Pre-designed siRNA against mouse somatic cytochrome c and siPORT™ Amine transfection agent were from Applied Biosystems. Mouse anti-cytochrome c and goat anti-mouse-HRP antibodies were from ZYMED® Laboratories (San Francisco, CA). BSTFA and silica were from Supelco (St. Louis, MO). Heptadecanoic acid, D₃₃ was from C/D/N isotopes (Pointe-Claire, Quebec). Fatty acid-free bovine serum albumin (BSA) and oleic acid were from Sigma-Aldrich (St. Louis, MO). The remaining materials are as described in Section 5.3.1.1.

The siRNA timecourse was run as described in Section 5.4.1. The fatty acid-BSA mixture was made as described in Chapter 3, Section 3.3.3. The cells were incubated for 56 hours with siRNA “A,” with 12, 24 or 48 of those hours with 2.5 mM OA in 0.25 mM BSA. Growing and collection conditions for N₁₈TG₂ cells are described in Chapter 2, Section 2.2.5.

After collection, cells were extracted as described in Section 2.2.6 and metabolites separated via SPE as described in Section 2.2.7. GC-MS was run as described in Section 2.2.10, including running a spiked sample after two aliquots of the original sample were run. Samples were analyzed as described in Chapter 3, Section 3.3.11.

5.5.2 Results

The results of cyt c knockdown studies are shown in Figure 5-8. Two different siRNA sequences were used, and both showed equal effectiveness at knocking down expression of cyt c.
The timecourse studies show that the siRNA-mediated knockdown of cyt c lasts for at least 48 hours, covering the entire time of the incubation with fatty acid. Before the 48 hour time period, existing cyt c may still be present, and this may be reflected in the oleamide timecourse extraction, but the 48 hour samples show almost complete knockdown of the enzyme (Figure 5-8).

One concern with using RNAi against cyt c is the toxicity to the cells, given its central role in oxidative phosphorylation. After 24 hours incubation with cyt c siRNA, the average viability of the N18TG2 cells was 88%. After 56 hours it was still 61%, comparable to what was observed in the PAM knockdown studies.

To compare the amount of oleamide made in N18TG2 cells with and without cyt c knockdown, the same incubation was performed as in Chapter 2 in which the cells were incubated for 0, 12, 24 or 48 hours with 2.5 mM OA in 0.25 mM BSA under the same conditions, except that each flask was also incubated for 56 hours with cyt c siRNA, including the “0h” sample. The results of this incubation are shown in Figure 5-9 (top). For comparison, these results are plotted alongside the normal OA incubation results as well (Figure 5-9, bottom).
There is no significant difference between N$_{18}$TG$_{2}$ cell incubated with OA and cyt c siRNA and the same cells without cyt c siRNA (p = 0.6941, Figure 5-9). The 48 hour time point shows more oleamide per cell in the cyt c knockdown cells than in the normal cells. A recent publication by Mueller and Driscoll$^{40}$ presents the concern of off-target oleamide knockdown that may be due to dramatic adaptive shifts in whole cell physiology caused by the knockdown of cyt c. However, experimental observations reveal more, not less, oleamide.

These findings argue against the role of cyt c in oleamide biosynthesis in vivo, although it is possible that other cell lines show different metabolism and use cyt c as it has been shown in vitro$^{40}$ as has also been suggested by Mueller and co-workers. A
similar experiment on additional cell lines would determine whether cyt c plays this role in other cell lines.

5.6 **Interrogation of the N-Acylglycine Biosynthetic Pathways through Isotopically Labeled Precursors**

Two main pathways have been proposed for N-acylglycine (NAG) biosynthesis: sequential oxidation of an N-acylethanolamine (NAE) and glycination of the free fatty acid (FFA) through an activated intermediate (see the introduction to Chapter 4 for more details). Evidence presented in Chapter 3 has demonstrated that $N_{18}TG_2$ cells are capable of converting N-tridecanylethanolamine (TDEA) into tridecanamide, but it is unknown whether the TDEA is oxidized to N-tridecanoylglycine or hydrolyzed to tridecanoic acid (TDA) before glycination. Strategic use of $^{15}$N and $^{13}$C isotope labels would allow for the elucidation of these different biosynthetic possibilities, as outlined in Figure 5-10. The appearance of one or two isotopic labels in the final oleamide product would provide key insight into the mechanism of NAG formation.
Figure 5-10: Elucidation of the Biosynthesis of a PFAM from Two Distinct Pathways

Two competing pathways for N-acylglycine biosynthesis. Oxidation of N-acylethanolamine is shown in brown and FFA glycination in purple. $^{13}$C is shown in green and $^{15}$N is shown in red.

ASC, ascorbate; SDA, semihydroascorbate

5.6.1 Materials and Methods

Heptadecanoic acid, D$_{33}$ and 1-$^{13}$C-Potassium oleate were from C/D/N isotopes (Pointe-Claire, Quebec). Fatty acid-free bovine serum albumin (BSA), oleic acid and $^{15}$N-

Ethanolamine were from Sigma-Aldrich (St. Louis, MO). The remaining materials are as described in Section 5.3.1.1.

The fatty acid-BSA mixture was made as described in Chapter 3, Section 3.3.3. The cells were incubated for 0, 12, 24 or 48 hours with 0.4 mM 3-$^{13}$C, $^{15}$N-

oleoylethanolamine in 0.4 mM BSA. Growing and collection conditions are described in Chapter 2, Section 2.2.5.

After collection, cells were extracted as described in Section 2.2.6 and metabolites separated via SPE as described in Section 2.2.7. GC-MS was run as described in Section 2.2.10, including running a spiked sample after two aliquots of the original sample were run. Samples were analyzed as described in Chapter 3, Section 3.3.11.

5.6.1.1 Synthesis of Labeled N-Oleoylthanolamine

1-$^{13}$C-Oleoyl chloride was synthesized as shown in Equation 5-1 as follows: 2 g potassium oleate was suspended in approximately 50 ml dichloromethane as a slurry. Thionyl chloride (1.8 ml) was added dropwise under nitrogen while stirring at 50°C under reflux. The oleate dissolved upon addition of SOCl$_2$. The solution was allowed to react under reflux at 50°C for 1 hour.

TLC was performed after 60 minutes. The normal phase TLC was run in chloroform:methanol 98:2 and stained with potassium permanganate solution (Figure 5-11).

The acid chloride in DCM was added to a separatory funnel with approximately 75 ml H$_2$O to wash. After collecting the bottom organic layer, the pH of the aqueous layer was approximately 0.55. A second wash was performed with approximately 0.5 g sodium bicarbonate in the water. The pH of the aqueous layer was approximately 2.7. A
third wash was performed with approximately 0.5 g sodium bicarbonate again in the water, after which the aqueous layer had a pH of approximately 8.2. A fourth wash was performed without bicarbonate and the aqueous layer had a pH of 6.6. The organic DCM layer was then poured over MgSO₄ to dry, decanted into a round-bottom flask and taken to a yellow oil in vacuo.

The N-oleylethanolamine was synthesized as shown in Equation 5-1 (bottom) as follows: 15 ml triethylamine base, 10 ml acetonitrile, and 0.5 ml ¹⁵N-ethanolamine were combined in a 50 ml round-bottom flask, flushed with N₂, and left stirring under N₂ at room temperature. The volume of the acyl chloride was reduced to approximately 2 ml in vacuo and then diluted to 10 ml with acetonitrile. This acyl chloride mixture was added dropwise to the ethanolamine mixture and left to stir overnight at room temperature.

The resulting crude N-acyl ethanolamine was taken to a yellow solid in vacuo, dissolved in approximately 25 ml warm ethanol, and recrystallized by adding cold H₂O. The crystallization proceeded on ice for approximately 30 min, and the solid filtered in a Büchner funnel and washed with ice cold H₂O. A white flaky, shiny solid (0.118 g) was obtained (5.8% yield) and was 94% pure by GC-MS analysis (Figure 5-12).

\[
\begin{align*}
\text{Equation 5-1: Synthesis of } & \text{3-}^{13}\text{C},^{15}\text{N-Oleylethanolamine} \\
\text{The isotopic labels are shown in color (}^{13}\text{C, green and }^{15}\text{N, red)}
\end{align*}
\]
Figure 5-12: GC-MS of $3^{13}$C, $15^N$-Oleoyl-ethanolamine

GC-MS of $3^{13}$C, $15^N$-oleoyl-ethanolamine over entire timecourse (panel A) and zoom in of the appropriate peak (B). The MS of the indicated GC peak is shown in panel C. The peak at 15.3 minutes is doubly-derivitized $N$-oleoyl-ethanolamine-(TMS)$_2$ (MS not shown).
5.6.2 Results

The quantification of oleamide in the N18TG2 cells incubated with labeled NOE over time is shown in Figure 5-13 (top). For reference, the results of incubation with OA and with TDEA are also shown (Figure 5-13 bottom left and right, respectively). A sample GC-MS of one of the cell extractions is shown in Figure 5-14.

Figure 5-13: Oleamide in N18TG2 Incubated with Labeled Oleoylethanolamine
Oleamide isolated from N18TG2 cells after incubation with labeled N-oleoylethanolamine (top). These data are also plotted alongside oleamide isolated from conditioned N18TG2 media incubated with OA (bottom left) and cells incubated with TDEA (bottom right) for comparison. NOE, 13C,15N-oleoylethanolamine.
Figure 5-14: GC-MS of Labeled Oleonitrile from N\textsubscript{18}TG\textsubscript{2} Cell Extract

GC-MS of N\textsubscript{18}TG\textsubscript{2} cell extract after incubation with \textsuperscript{13}C, \textsuperscript{15}N-oleylethanolamine for 48 hours, showing oleonitrile. Panel A shows the whole GC (top) and zoom in of the oleonitrile peak (bottom). Panel B shows the MS of the indicated GC peak (oleonitrile). Panel C shows the library database MS for unlabeled oleonitrile. Panel D shows the difference between panels B and C. Note that in the cell-extracted oleonitrile, there are more isotopic peaks in the fragments containing the labeled carbon and/or nitrogen.
There was more oleamide isolated in the conditioned media than in the cells, something not observed in any of the incubations with FFAs and TDEA, as the amounts isolated in cells and conditioned media were very similar. The significance of this is unclear. The amount of oleamide found in the conditioned media after incubation with NOE is comparable to that found in the conditioned media after incubation with OA (Figure 5-13, bottom left). The amount of oleamide found in the cells after incubation with NOE is comparable with the amount of tridecanamide found in the cells after incubation with TDEA (Figure 5-13, bottom right).

Oleonitrile derived from the reaction of BSTFA with oleamide isolated from N18TG2 cells after incubation with 3-13C,15N-oleylethanolamine is shown in Figure 5-14. The MS for the oleonitrile derived from cell-extracted oleamide (panel B) shows many more isotopic peaks than the library MS for unlabeled oleonitrile (panel C). Averaged spectra were taken over the time interval in which oleonitrile could be found, and averaged background spectra were subtracted from the oleonitrile spectra. Shown in Figure 5-15 is a set of the isotopic m/z’s that were examined for this study overlaid as selected ion traces in color. The oleonitrile peaks are shown with a vertical line through them, and the background spectra were taken from the left where no other significant peaks could be found. Much greater background noise was observed for m/z 123 in the conditioned media (Figure 5-15, panel A) than in the cell extract, making this subtraction necessary. Along with the 122, 123 and 124 set, two other isotopic sets were examined and are shown in Table 5-2. The relative intensity of each m/z peak for each unlabeled (122, 136 and 164), singly labeled (123, 137 and 165) and doubly labeled (124, 138 and 166) were used in the ratios shown in Equation 5-2. A graphical representation of those ratios is shown in Figure 5-16.
Table 5-2: Fragments Used for Isotopic Ratio Assignment

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Oleonitrile is a direct reaction product of derivitization of oleamide with BSTFA. The intensities ($i$) of each isotopic peak were added to obtain a relative intensity of each isotopic contributor.
Figure 5-15: Background Levels in N_{18}TG_{2} Conditioned Media and Cell Samples

GC of N_{18}TG_{2} media (A) and cells (B) after incubation with 3-^{13}C,^{15}N-oleoylethanolamine. The TIC is shown in black and m/z’s overlaid in different colors. 122 is a characteristic peak for unlabeled oleonitrile, 123 for a singly-labeled oleonitrile, and 124 for the doubly-labeled oleonitrile. The background is much higher for m/z 123 in the media sample, necessitating a subtraction of the background spectra from the oleonitrile spectra before assignment of isotopic ratios.
The fact that there is much greater labeled product in the samples incubated with labeled NOE (turquoise and orange bars in Figure 5-16) indicates that the labeling comes from conversion of the labeled NOE to oleamide and not co-eluting contamination. In addition, the incorporation of the label seems to reach a saturation point between 0-12 hours, as the ratio of labeled to unlabeled compound stays steady from 12-48 hours.

The ratio of doubly to singly labeled oleamide is approximately 0.99 ± 0.04 in the cells and 1.09 ± 0.09 in the media, indicating that there is an equal amount of singly- and doubly-labeled oleamide present. This implies that the ethanolamine has an equal likelihood of being oxidized to the NAG and being hydrolyzed to the FFA before glycination; that there is more than one biosynthetic mechanism for NAGs in the N18TG2 cells and that they are likely to be acting at roughly equal rates.
5.7 Conclusions

A PFAM, oleamide, has been isolated from human aortic endothelial cells. Incubation with a FAAH inhibitor results in a great increase in the amount of oleamide found after incubation with OA (Figure 5-1). Therefore, FAAH is likely at work in the cardiovascular system, or at least in endothelial cells. Although FAAH has been found in endothelial cells of other organs (kidney\textsuperscript{20} and umbilical vein\textsuperscript{21}), there is no previous evidence for its action in the heart\textsuperscript{15,22}. The finding of PFAMs in these cells is the first report of its kind and suggests an important role in vasoregulation and angiogenesis is played by these molecules in the cardiovascular system.

An overview of the findings detailed in this thesis regarding NAG and PFAM metabolism is summarized in Figure 5-17. The quantitative NAG assay described in Chapter 2 was successfully demonstrated here in N\textsubscript{18}TG\textsubscript{2} cells to measure endogenous levels of NOG and labeled \textsuperscript{13}C\textsubscript{18}-NOG after incubation with \textsuperscript{13}C\textsubscript{18}-OA and PAM siRNA. This is explicit proof of the involvement of PAM in PFAM biosynthesis \textit{in cellulo}, as the siRNA targets PAM specifically and its knockdown leads to large intercellular levels of NOG and very small levels of oleamide even when incubating with OA. Maintaining PAM levels at normal concentrations results in the finding of very low cellular NOG levels (Figure 5-5) In addition, incubation with PAM siRNA results in very low cellular oleamide levels (Figure 5-6). The amount of NOG produced upon PAM knockdown was lower than the amount of oleamide found with normal PAM levels. This could mean that NOG has other metabolic fates, or simply that it is a poor FAAH substrate relative to oleamide.
Figure 5-17: NAG and PFAM Biosynthesis in N18TG2 Cells
Overview of what is now known about biosynthesis of NAGs and PFAMs in N18TG2 cells. Double arrows represent metabolic flow from one metabolite to another. Single arrows are known enzymatic reactions. The NAE to NAG double arrow is dashed because the NAG was not isolated itself but the corresponding PFAM was isolated. Isotopic labeling implies oxidation to the NAG before oxidative cleavage to the PFAM.
Abbreviations: ACS, acyl-CoA synthetase; ADH, alcohol dehydrogenase; AIDH, aldehyde dehydrogenase; CYP4F, cytochrome P450; EA, elaidic acid; FAAH, fatty acid amide hydrolase; LOA, linoleic acid; NAE, N-acylethanolamine; NAG, N-acylglycine; NOE, N-oleoylethanolamine, OA, oleic acid; PA, palmitic acid; PAM, peptidylglycine α-amidating monooxygenase; PFAM, primary fatty acid amide; POA, palmitoleic acid; TDA, tridecanoic acid; TDEA, N-tridecanoylethanolamine.
After incubation with $^{13}$C, $^{15}$N-oleoylthanolamine, both $^{13}$C, $^{15}$N-oleamide and $^{13}$C-oleamide were found in N$_{18}$TG$_2$ cells (Figure 5-13) in roughly equal proportion (Figure 5-16). This indicates that not only are both pathways at work to synthesize NOG (oxidation of NOE and glycination of OA), but that both pathways may be functioning with equal discrimination. This is in agreement with recent work by Bradshaw et al. who show that N-arachidonoylglycine is formed by two distinct pathways in C6 glimoa cells.$^{41}$ In their case, however, hydrolysis of the NAE followed by glycination was the preferred mode of NAG biosynthesis as the amounts of unlabeled NAG produced were much higher (see Chapter 3 for more details of the Bradshaw experiments). Because oxidation of NOE is occurring and ADH3, ADH4, AlDH3A1 and AlDH3A2 were not found to be expressed in N$_{18}$TG$_2$ cells (Chapter 4), other ADH isoform(s) are likely responsible for NAE oxidation in vivo. Indeed, ADH3 and ADH4 did not have particularly favorable kinetics for formation of the acylaldehyde$^{42}$ or NAG,$^{43}$ although the in vitro nature of the studies did not allow for analysis of long chain NAEs in the aqueous solutions used. Further studies of ADH5 and ADH6 are required, as these two ADHs are poorly investigated to date.

The levels of oleamide found after incubation with cyt c siRNA and OA were not significantly lower than that found in the absence of cyt siRNA (Figure 5-9). Although it is possible that other cells show a different mechanism for oleamide biosynthesis via cyt c, cyt c is not likely to play a role in direct PFAM biosynthesis in N$_{18}$TG$_2$ cells, either by glycination or amidation of oleoyl-CoA. Because there is no other candidate for NAG biosynthesis, as they have been ruled out by the expression studies in Chapter 4 and other enzymologic studies, and because the isotopic labeling studies indicate that both pathways are at work in the cells, it may be concluded that an unknown enzyme is responsible for in vivo NAG biosynthesis: an undiscovered long chain-specific glycine N-acyltransferase. These findings do not rule out the activation of the FFAs to another intermediate, such as an acyl-adenylate similar to the amino acid adenylates involved in activating tRNAs, or a fatty acid thioester linked to a cysteine in a carrier protein, similar to intermediates observed in fatty acid biosynthesis.
Although there is now a much better understanding of how PFAMs are synthesized and in what amounts, there is much still to be explored. The activities of ADH5 and ADH6 need to be investigated for their ability to convert long-chain NAEs to aldehydes and potentially NAGs, as ADH4 is capable of doing for shorter chain NAEs. If these are incapable of the dismutation, aldehyde dehydrogenases (AlDHs) should be investigated. Although the long-chain specific AlDHs were not found in N\textsubscript{18}TG\textsubscript{2} cells (Chapter 4), other AlDHs could be candidates. Rabbit ADH was shown to oxidize dodecanal ($K_M = 20 \mu\text{M}$) in the presence of NAD$^+$ and demonstrated a preference for longer-chain aldehydes. Rat liver mitochondrial AlDH also oxidized dodecanal. Longer-chain aldehydes have not been reported as AlDH substrates, either because they were not found to be substrates or, more likely, because of solubility issues preventing their analyses.

The existence of an unknown glycination enzyme poses the greatest challenge to elucidating this pathway. Initial attempts to locate this enzyme using bioinformatic tools to mine the mouse genome for ACGNAT, NMT and BAAT-like regions of DNA have yielded no novel expressed mRNA (data not shown). Several studies are underway in the Merkler laboratory to identify this enzyme, including a CoA-ylomics approach in which a biotinylated CoA was developed to probe for novel CoA-binding proteins.

These long-chain amide signaling molecules show much potential for therapeutic targeting, as discussed in detail in Chapter 1. The diverse set of receptors which they target provide a broad spectrum for pharmacological treatment, and elucidating PFAM and NAG biosynthesis will provide excellent targets for a huge range of disorders, including anxiety, depression, sleep disorders, and many motor and neurodegenerative diseases. If a long chain-specific $N$-acyltransferase is identified, as the work in this dissertation would suggest, it could provide a specific target for regulating the metabolism of these molecules while avoiding broader effects seen by targeting enzymes such as FAAH or ACS’s. Finally, the revelation that NAGs and PFAMs are made by more than one biosynthetic scheme adds another dimension to the potential regulation and biosynthesis of these molecules. The field of lipidomics is a growing one that has broadened our view of the molecular world, and although the pharmacologic actions of oleamide have been appreciated for 15 years, most of these lipid signaling molecules
were unknown even 3-5 years ago. Evidence demonstrated in this dissertation represents a significant contribution to understanding the biosynthetic mechanisms of these unique and difficult molecules to which aqueous chemistry has been aspiring for 15 years.

5.8 **References**


Appendices
Appendix A: Synthesis of a Novel N-Acylglycine Derivative

Silylation is the most widely used derivitization for GC analysis, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) one of the more common silylating agents. Derivitization with BSTFA results in trimethylsilylation of reactive groups of compounds in the following preference: alcohol > phenol > carboxylic acid > amine > amide. Each active hydrogen is replaced with a trimethylsilyl (TMS) group (Equation A, top), where “active” hydrogens constitute those connected to an electronegative atom. In addition, the formation of a nitrile is observed when the compound being derivitized is a primary fatty acid amide (PFAM) (Equation A, bottom).

Upon derivitization of an N-acylglycine (NAG), one would expect to see three products: NOG-TMS$_2$, NOG (N-TMS), and NOG (O-TMS), the last two of which would
Appendix A: (Continued)

co-elute. However, a fourth compound is observed under normal derivitization conditions, as shown in Figure A. From the MS, a structure was hypothesized to be an “enyliminoacetate.” Figure B Figure C show the fragmentation scheme for mono-and di-TMS NAGs. Figure D shows the structure of the proposed N-oleoylglycine enyliminoacetate derivative and its fragmentation scheme.
Figure A: GC-MS of N-Oleoylglycine Derivitized with BSTFA
Products of NOG derivitization with BSTFA. The “enyliminoacetate” is an unexpected product. Panel A shows the whole GC chromatogram (top) and closeup of NOG peaks (bottom). Panel B shows the MS spectrum for the enyliminoacetate derivative. Panel B shows the MS of NOG with two TMS groups attached. Panel D shows mono-derivitized NOG.
Figure B: Fragmentation Scheme for Di-TMS NAGs
Figure C: Fragmentation Scheme for Mono-TMS NAGs
In order to verify the structure of this enyliminoacetate, a series of NAGs were synthesized and derivitized with BSTFA, including the linoleoyl-, palmitoyl-, palmitoleoyl- and deuterated heptadecanoyl (D₃₃)- glycines. A detailed description of NAG synthesis can be found in Chapter 2, Section 2.2.2.3. GC-MS conditions can be found in Section 2.2.10.
Appendix A: (Continued)

If the predicted structure is correct, the fragmentation of each additional NAG derivative could be predicted, as shown in Figure E. Figure F through Figure J show the resulting GC-MS of the synthesized NAGs, the MS of each enyliminoacetate derivative, and the fragmentation pattern that matches the predicted fragmentation pattern. (For MS of NAG-TMS and NAG-(TMS)$_2$ derivatives, see Appendix B.)
Figure E: Fragmentation Patterns for Enyliminoacetate Derivatives of N-Acylglycines
Fragmentation patterns predicted for enyliminoacetate derivatives of each NAG synthesized.
Figure F: GC-MS of Enyliminoacetate Derivative of NOG and Fragmentation
GC of the enyliminoacetate derivative of N-Oleoylglycine (top), it MS (middle), and fragmentation (bottom)
Figure G: GC-MS of Enyliminoacetate Derivative of NLG and Fragmentation
GC of the enyliminoacetate derivative of N-linoleoylglycine (top), it MS (middle), and fragmentation (bottom).
Figure H: GC-MS of Enyliminoacetate Derivative of NPOG and Fragmentation
GC of the enyliminoacetate derivative of N-palmitoleoylglycine (top), it MS (middle), and fragmentation (bottom).
Figure I: GC-MS of Enyliminoacetate Derivative of NPG and Fragmentation
GC of the enyliminoacetate derivative of N-palmitoylglycine (top), its MS (middle), and fragmentation (bottom).
Appendix A: (Continued)

A predicted reaction mechanism is given in Figure K and is based on the formation of a nitrile-like intermediate as has been seen for primary amides (Equation A). Of the reactive groups, the amide is the least reactive, and the additional steric hindrance of the glycine makes this reaction even slower than for a primary amide. The reaction
proceeds with a nucleophilic attack upon the silicon atom of the TMS donor, followed by the protonation of the derivitized oxygen from its neighboring amine or HCl available from the reaction mixture. The electrons are then drawn up towards the positively charged oxygen, forming a transient nitrile and causing the TMS group to leave. This mechanism is similar to one reported by Ichiyama involving a primary amide and L-chloropropionic acid as the electrophile,¹ and would mark the end of the reaction for a primary amide to form a nitrile. In this case, the nitrile formed is less stable because the nitrogen is also connected to glycine, leaving a positive charge on the nitrogen. The glycine α-hydrogen is highly acidic in this transition state and is readily abstracted, leaving behind electrons that then form a di-imino intermediate. The iminoacetate structure is finally formed with the abstraction of the hydrogen β to the nitrogen, forming the energetically favorable structure with resonance stabilization. The resonance structures are shown in Figure L.
Appendix A: (Continued)

Figure K: Proposed Reaction Mechanism for the Formation of an Enyliminoacetate Derivative of NAGs
Appendix A: (Continued)

Reference

Appendix B: GC-MS of Synthesized Standards

Throughout these experiments standards had to be synthesized where not commercially available both for the formation of standard curves and for entry into the library database for compound verification in cell and media extracts. The GC-MS of these standards are shown below.

**GC-MS of PFAMs**

Primary fatty acid amides (PFAMs) were synthesized as described in Chapter 2, Section 2.2.2.2. Where commercially available, acyl chlorides were used. Where they were not available, acyl chlorides were synthesized from fatty acids as described in Chapter 2, Section 2.2.2.1. The primary product of derivitization with BSTFA was an acyl nitrile, although the amide-TMS was also observed.
Figure M: GC-MS of $^{13}\text{C}_{18}$-Oleamide
Panel A shows the GC of U-$^{13}\text{C}_{18}$-oleamide. The nitrile spans from 14.5-15.3 minutes. The large peak from 16.1-17 is the fatty acid. The PFAM-TMS is around 18 minutes. Panel B shows the annotated MS of U-$^{13}\text{C}_{18}$-oleamide-TMS. Panel C shows the annotated MS of U-$^{13}\text{C}_{18}$-oleonitrile.
Figure N: GC-MS of Oleamide

Panel A shows the GC of oleamide (top) with a close-up of oleonitrile and oleamide-TMS peaks (bottom). The unlabeled peak is underivitized oleamide. Panel B shows the annotated oleonitrile MS. Panel C shows the annotated oleamide-TMS MS.
Figure O: GC-MS of Tridecanamide
Panel A shows the GC of tridecanamide. Overlaid on the tridecanamide-TMS close-up is the SIM for m/z 131, a characteristic m/z for PFAM-TMS resulting from a McLafferty rearrangement. The peak had been split into two but both had MS for tridecanamide-TMS. Contamination at the source caused the GC spectrum to show increased contaminants as the temperature increased (top right). Panel B shows the MS of tridecanamide-TMS
Figure P: GC-MS of Palmitamide

GC-MS of synthesized palmitamide. Panel A shows the GC chromatogram over entire time period (top) and a close-up of the palmitonitrile peak (bottom). Palmitamide-TMS is at retention time 16.25 minutes. Panel B shows the MS of palmitonitrile. Panel C shows the MS of palmitamide-TMS.
Figure Q: GC-MS of Palmitoleamide
GC-MS of synthesized palmitoleamide. Panel A shows the GC chromatogram over entire time period (top) and a close-up of the palmitoleonitrile peak (bottom). Palmitoleamide-TMS is at retention time 16.1 minutes. Panel B shows the MS of palmitoleonitrile. Panel C shows the MS of palmitoleamide-TMS.
Appendix B: (Continued)

Figure R: GC-MS of Elaidamide
GC-MS of synthesized elaidamide. Panel A shows GC chromatogram over entire time period (top) and a close-up of the elaidonitrile peak (bottom). Elaidamide-TMS is at retention time 17.8 minutes. Panel B shows the MS of elaidonitrile. Panel C shows the MS of elaidamide-TMS.
Appendix B: (Continued)

Figure S: GC-MS of Linoleamide
GC-MS of synthesized linoleamide. Panel A shows GC chromatogram over entire time period (top) and a close-up of the linoleonitrile peak (bottom). Linoleamide-TMS is at retention time 17.7 minutes. Panel B shows the MS of linoleonitrile. Panel C shows the MS of linoleamide-TMS.
**GC-MS of NAGs**

*N*-acylglycines (NAGs) were synthesized as described in Chapter 2, Section 2.2.2.3. Where commercially available, acyl chlorides were used. Where they were not available, acyl chlorides were synthesized from fatty acids as described in Chapter 2, Section 2.2.2.1. The primary product of derivitization with BSTFA under normal derivitization conditions (60 min 60°C) was the enyliminoacetate derivative, followed by NAG-TMS. If abundant enough NAG was present, the NAG-(TMS)_2 was also observed.
Appendix B: (Continued)

Figure T: GC-MS of $^{13}$C$_{18}$-N-Oleoylglycine
Panel A shows the GC spectrum of $^{13}$C$_{18}$-N-oleoylglycine. Left peak at 18.8 min is the enyliminoacetate. Peak from 20-21 min is $^{13}$C$_{18}$-N-oleoylglycine-TMS. There is less enyliminoacetate in this reaction mixture because the reaction was only allowed to proceed 15 minutes instead of the normal 60. Panels B-D show MS from the large peak from 20-21 min with fragments annotated.
Figure U: GC-MS of N-Palmitoylglycine
Panel A shows the GC of N-palmitoylglycine-TMS. The mono-derivitized NPG has a retention time of 20.6 minutes and its MS is shown in panel B. Panel C shows the MS for di-TMS NPG (retention time 20.2 minutes). Panel D shows the MS for the enyyliminoacetate derivative of NPG (retention time 17.3 minutes).
Appendix B: (Continued)

Figure V: GC-MS of N-Palmitoleoylglycine
Panel A shows the GC of N-palmitoleoylglycine-TMS. The mono-derivitized NPOG has a retention time of 19.2 minutes and its MS is shown in panel B. Panel C shows the MS for di-TMS NPOG (retention time 18.85 minutes). Panel D shows the MS for the enyliminoacetate derivative of NPOG (retention time 17.2 minutes).
Figure W: GC-MS of N-Linoleoylglycine
Panel A shows the GC of N-linoleoylglycine-TMS. The mono-derivitized NLG has a retention time of 19.3 minutes and its MS is shown in panel B. Panel C shows the MS for di-TMS NLG (retention time 19.05 minutes). Panel D shows the MS for the enyliminoacetate derivative of NLG (retention time 17.4 minutes).
Appendix B: (Continued)

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GC-MS of NAEs

*N*-acylethanolamines (NAEs) were synthesized as described in Chapter 3, Section 3.3.2.3. The primary product of derivitization with BSTFA was a mono-TMS derivative, but di-TMS product was also observed.

Figure X: GC-MS of *N*-Elaidoylglycine
Panel A shows the GC of *N*-elaidoylglycine-TMS. The mono-derivitized NEG has a retention time of 20.6 minutes and its MS is shown in panel B. Panel C shows the MS for the enyliminoacetate derivative of NEG (retention time 18.8 minutes).
Figure Y: GC-MS of N-Tridecanylethanolamine
Panel A shows the GC of N-tridecanylethanolamine (TDEA). The peak indicated with a vertical line is for mono-derivitized TDEA and its MS is shown in panel B. The peak at 15.9 minutes is for bi-derivitized TDEA and its MS is shown in panel C.
Appendix C: GC-MS of Mammalian Cells

The results of incubations of each fatty acid and N-tridecanoylethanolamine in SCP and N_{18}TG_{2} cells and media are shown below. For brevity, only one timepoint example from each cell and media incubated with each fatty acid or TDEA is shown. Many of the MS shown below are the TICs (full range of m/z), but for integration purposes only the select ions specific to each acyl nitrile and/or PFAM-TMS were used (see Chapter 3, Section 3.3.10 for more information). Both nitriles and PFAM-TMS were observed products, and both samples showing nitriles and PFAM-TMSs are shown below.
Appendix C: (Continued)

**GC-MS of Palmitoleic Acid-Incubated Cells Over Time**

Figure Z: GC-MS of N_{18}TG_{2} Cell Extract after Incubation with POA for 12h, Showing Palmitoleamide-TMS

GC-MS of N_{18}TG_{2} cell extract after incubation with POA for 12h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitoleamide-TMS). Panel C shows the library database MS for palmitoleamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM POA in 0.25mM BSA for 12 hours in this case before extraction.
Appendix C: (Continued)

Figure AA: GC-MS of N<sub>18</sub>TG<sub>2</sub> Media Extract after Incubation with POA for 12h, Showing Palmitoleamide-TMS and Interference from OA

GC-MS of N<sub>18</sub>TG<sub>2</sub> media extract after incubation with POA for 12h. Panel A shows the whole GC and zoom in of the peak of interest. The palmitoleamide-TMS peak is buried within the OA peak. Panel B shows the MS of the indicated GC peak (palmitoleamide-TMS). Panel C shows the library database MS for palmitoleamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM OA in 0.25mM BSA for 12 hours in this case before extraction.
Figure BB: GC-MS of SCP Cell Extract after Incubation with POA for 48h, Showing Palmitoleamide-TMS

GC-MS of SCP cell extract after incubation with POA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitoleamide-TMS). Panel C shows the library database MS for palmitoleamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM POA in 0.25mM BSA for 48 hours in this case before extraction.
Figure CC: GC-MS of SCP Media Extract after Incubation with POA for 48h, Showing Palmitoleonitrile

GC-MS of SCP media extract after incubation with POA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitoleonitrile). Panel C shows the library database MS for palmitoleonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM POA in 0.25mM BSA for 48 hours in this case before extraction.
Appendix C: (Continued)

**GC-MS of Palmitic Acid-Incubated Cells Over Time**

For data analysis, only the palmitonitrile was integrated and plotted against a corresponding standard curve in which only the palmitonitrile was integrated. This was due to the interference of oleic acid and octadecanoic acid -TMS esters co-eluting with palmitamide-TMS in both cell and media samples. One sample is shown with palmitamide-TMS where resolution was slightly better (Figure ).
Appendix C: (Continued)

Figure DD: GC-MS of N18 TG2 Cell Extract after Incubation with PA for 24h, Showing Palmitonitrile
GC-MS of N18TG2 cell extract after incubation with PA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitonitrile). Panel C shows the library database MS for palmitonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM PA in 0.25mM BSA for 24 hours in this case before extraction.
Figure EE: GC-MS of N_{18}TG\textsubscript{2} Media Extract after Incubation with PA for 48h, Showing Palmitoleamide-TMS

GC-MS of N_{18}TG\textsubscript{2} media extract after incubation with PA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. The single ion trace m/z 131, characteristic for derivitized amides, is shown overlaid in red. Panel B shows the MS of the indicated GC peak (palmitamide-TMS). Panel C shows the library database MS for palmitamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM PA in 0.25mM BSA for 48 hours in this case before extraction.
Figure FF: GC-MS of SCP Cell Extract after Incubation with PA for 48h, Showing Palmitonitrile

GC-MS of SCP cell extract after incubation with PA for 48h. Panel A shows the whole GC and
zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitonitrile). Panel C shows the library database MS for palmitonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM PA in 0.25mM BSA for 48 hours in this case before extraction.
Appendix C: (Continued)

Figure GG: GC-MS of SCP Media Extract after Incubation with PA for 24h, Showing Palmitonitrile
GC-MS of SCP media extract after incubation with PA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (palmitonitrile). Panel C shows the library database MS for palmitonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM PA in 0.25mM BSA for 24 hours in this case before extraction.

**GC-MS of Elaidic Acid-Incubated Cells Over Time**

Unambiguous assignment of unsaturated acyl chains can pose a problem in some samples. Separation of cis/trans isomers is not ideal on a nonpolar matrix such as the
dimethylpolysiloxane GC column that is commonly used for lipid analysis. A highly polar bis(cyanopropyl) polysiloxane phase has been shown to provide this separation but was not available for these analyses to determine whether the complex lipid extract would be separable on such a column. The assumption is made that any 18:1 fatty acid amide found after incubation with elaidic acid is elaidamide.
Figure HH: GC-MS of N\textsubscript{18}TG\textsubscript{2} Cell Extract after Incubation with EA for 48h, Showing Elaidamide-TMS

GC-MS of N\textsubscript{18}TG\textsubscript{2} cell extract after incubation with EA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (elaidamide-TMS). Panel C shows the library database MS for elaidamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM EA in 0.25mM BSA for 48 hours in this case before extraction.
Figure II: GC-MS of N18TG2 Media Extract after Incubation with EA for 48h, Showing Elaidonitrile

GC-MS of N18TG2 media extract after incubation with EA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (elaidonitrile). Panel C shows the library database MS for elaidonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM EA in 0.25mM BSA for 48 hours in this case before extraction.
Figure JJ: GC-MS of SCP Cell Extract after Incubation with EA for 24h, Showing Elaidamide-TMS

GC-MS of SCP cell extract after incubation with EA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (elaidamide-TMS). Panel C shows the library database MS for elaidamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM EA in 0.25mM BSA for 48 hours in this case before extraction.
Figure KK: GC-MS of SCP Media Extract after Incubation with EA for 24h, Showing Elaidonitrile

GC-MS of SCP media extract after incubation with EA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (elaidonitrile). Panel C shows the library database MS for elaidonitrile. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM EA in 0.25mM BSA for 24 hours in this case before extraction.
Appendix C: (Continued)

**GC-MS of Tidecanoic Acid-Incubated Cells Over Time**

Figure LL: GC-MS of N_{18}TG_{2} Cell Extract after Incubation with TDA for 48h, Showing Tridecanamide-TMS

GC-MS of N_{18}TG_{2} cell extract after incubation with TDA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDA in 0.25mM BSA for 48 hours in this case before extraction.
Appendix C: (Continued)

Figure MM: GC-MS of N\textsubscript{18}TG\textsubscript{2} Media Extract after Incubation with TDA for 48h, Showing Tridecanamide-TMS
GC-MS of N\textsubscript{18}TG\textsubscript{2} media extract after incubation with TDA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDA in 0.25mM BSA for 48 hours in this case before extraction.
Figure NN: GC-MS of SCP Cell Extract after Incubation with TDA for 12h, Showing Tridecanamide-TMS

GC-MS of SCP cell extract after incubation with TDA for 12h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDA in 0.25mM BSA for 12 hours in this case before extraction.
Figure OO: GC-MS of SCP Media Extract after Incubation with TDA for 24h, Showing Tridecanamide-TMS

GC-MS of SCP media extract after incubation with TDA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDA in 0.25mM BSA for 24 hours in this case before extraction.
Appendix C: (Continued)

**GC-MS of Tidecanoylethanolamine-Incubated Cells Over Time**

Figure PP: GC-MS of N\textsubscript{18}TG\textsubscript{2} Cell Extract after Incubation with TDEA for 48h, Showing Tridecanamide-TMS

GC-MS of N\textsubscript{18}TG\textsubscript{2} cell extract after incubation with TDEA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDEA in 0.25mM BSA for 48 hours in this case before extraction.
Figure QQ: GC-MS of N$_{18}$TG$_2$ Media Extract after Incubation with TDEA for 12h, Showing Tridecanamide-TMS

GC-MS of N$_{18}$TG$_2$ media extract after incubation with TDEA for 12h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDEA in 0.25mM BSA for 12 hours in this case before extraction.
Figure RR: GC-MS of SCP Cell Extract after Incubation with TDEA for 24h, Showing Tridecanamide-TMS

GC-MS of SCP cell extract after incubation with TDEA for 24h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDEA in 0.25mM BSA for 24 hours in this case before extraction.
Figure SS: GC-MS of SCP Media Extract after Incubation with TDEA for 48h, Showing Tridecanamide-TMS
GC-MS of SCP media extract after incubation with TDEA for 48h. Panel A shows the whole GC and zoom in of the peak of interest. Panel B shows the MS of the indicated GC peak (tridecanamide-TMS). Panel C shows the library database MS for tridecanamide-TMS. Panel D shows the difference between panels B and C. The cells were extracted as described in the methods section in Chapter 3. The cells had been incubated with 2.5mM TDEA in 0.25mM BSA for 48 hours in this case before extraction.

Reference

Appendix D: Primer Sequences

The primers used in Chapter 4 for RT-PCR expression studies are shown in Table A. Not all sequences were known for sheep and so those primers are based on multiple sequence alignments of those from closely related species. The FAAH isoforms probed were all FAAH-1, as that is the only isoform expressed in mice and sheep.¹

Table A: Primer List, Predicted Fragment Size, and Successful PCR Experiments

<table>
<thead>
<tr>
<th>Primer target</th>
<th>sequence</th>
<th>Expected fragment size (bp)</th>
<th>Target DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACGNAT: sense</td>
<td>GTGGACAAGTGCCCTGATTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACGNAT: antisense</td>
<td>CAGTTTTGGGGATCTTTGG</td>
<td>119</td>
<td>human kidney, liver</td>
</tr>
<tr>
<td>acyl-CoA synthetase 4:</td>
<td>CACCATGACCATTTTCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acyl-CoA synthetase 4:</td>
<td>GCCCTCAGTTTGCTTTCCAG</td>
<td>268</td>
<td>N₁₈TG₂, SCP</td>
</tr>
<tr>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acyl CoA synthetase 3:</td>
<td>TGTGCGACAGCTTTGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h acyl CoA synthetase 3:</td>
<td>CTGGGAGTGCTGCTTTATCAG</td>
<td>292</td>
<td>human brain, kidney, liver</td>
</tr>
<tr>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acyl CoA synthetase 5 and 6:</td>
<td>TTCGAAGAGGCTGCAAGAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h acyl CoA synthetase 5 and 6:</td>
<td>AGAAATCGAGCCACACTGTC</td>
<td>205</td>
<td>human brain, kidney</td>
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<tr>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAAT: sense</td>
<td>TGGCCTTGCCCTACCATAAC</td>
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</tr>
<tr>
<td>BAAT: antisense</td>
<td>CGTGCTGCTGACTGCTTT</td>
<td>197</td>
<td>human liver</td>
</tr>
<tr>
<td>FAAH: sense</td>
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<td></td>
</tr>
<tr>
<td>FAAH: antisense</td>
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<td>230</td>
<td>N₁₈TG₂</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>FAAH: antisense</td>
<td>GTGGAGGTGCTGCTTTATCAG</td>
<td>276</td>
<td>human brain, kidney</td>
</tr>
<tr>
<td>FAAH: sense</td>
<td>GCACCGCTTCCGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAH: antisense</td>
<td>TAGAGCAAGGCGCTGCC</td>
<td>228</td>
<td>human brain, kidney, liver</td>
</tr>
<tr>
<td>PAM1: sense</td>
<td>CACTGGATATTCGCACTG</td>
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<td></td>
</tr>
<tr>
<td>PAM1: antisense</td>
<td>ACTAGATGTGCCGCGGAT</td>
<td>(nested)</td>
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<tr>
<td>PAM2: sense</td>
<td>GACACTGTCCACATATG</td>
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<td></td>
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<tr>
<td>PAM2: antisense</td>
<td>CCTAAATGTTGAGAT</td>
<td>431</td>
<td>N₁₈TG₂, SCP</td>
</tr>
<tr>
<td>PAM: sense</td>
<td>TTGCCTTTCACGTGAAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAM: antisense</td>
<td>CACACCTGTTGTGATG</td>
<td>194</td>
<td>human brain, kidney, liver</td>
</tr>
<tr>
<td>Cytochrome c: sense</td>
<td>GTCAGGCACCCCTGGAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c: antisense</td>
<td>TCTGGCCCTTTCCCTGCT</td>
<td>157</td>
<td>Human brain, kidney, liver</td>
</tr>
<tr>
<td>Cytochrome c: sense</td>
<td>CCAATCTCCACGCTGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c: antisense</td>
<td>GTCAGGCACCCCTGGAAT</td>
<td>192</td>
<td>N₁₈TG₂</td>
</tr>
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<td>Cytochrome c: sense 2</td>
<td>CCAACCTCCATGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c: antisense</td>
<td>TTTCCTCTTAATGCC</td>
<td>177</td>
<td>SCP</td>
</tr>
<tr>
<td>Gene</td>
<td>Sense Sequence</td>
<td>Antisense Sequence</td>
<td>Position</td>
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<td>---------------------------------</td>
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<tr>
<td>ADH3: sense</td>
<td>TGATGGGCACCACCAGATTTA</td>
<td>ACAGCCCATGATGACTGACA</td>
<td>262</td>
</tr>
<tr>
<td>ADH3: antisense</td>
<td>AGTTGGGGTTGTGGAGAGTG</td>
<td>AAAATCCACAGCCCGATCAAG</td>
<td>320</td>
</tr>
<tr>
<td>ADH3: sense 2</td>
<td>GCTGCTGTAAAACTG</td>
<td>CTGGTCCCATAGTTTCAT</td>
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<tr>
<td>ADH3: antisense 2</td>
<td>GGGAGGGAAAGTCCATGTT</td>
<td>ALDH 3A2: sense</td>
<td>GGTTGGGGCTATGTAGCGTGT</td>
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<tr>
<td>CYP4F12: sense</td>
<td>GGAGCTCAGTGCCCCCTTGTAG</td>
<td>CYP4F12: antisense</td>
<td>GCCTCCAAACATGAAGGTGT</td>
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<td>CYP4F17: sense</td>
<td>ACCAGCCCTCCTGTACCTT</td>
<td>CYP4F17: antisense</td>
<td>GCCTCCAAACATGAAGGTGT</td>
</tr>
</tbody>
</table>

**Reference**

About the Author

Emma Katherine Farrell received her B.A. in Biology with a minor in Chemistry from the University of Missouri – St. Louis, where she also taught as an undergraduate teaching assistant and received an award for her work. She made the transition from biology to biochemistry for her graduate studies at the University of South Florida. Emma wrote and was awarded two grants for her work on fatty acid amide biosynthesis through the USF Thrust Life Sciences Program administered by the Florida Center of Excellence for Biomolecular Identification and Targeted Therapeutics. In addition to six other travel awards, Emma received full support to attend the 2009 Education without Borders conference in Dubai, UAE as a delegate. Emma presented her work at 8 meetings, co-chaired the student-led Castle Conference, and mentored four undergraduate students during her time at USF.