Antidepressants Accumulate in Lipid Rafts and Modify the Acylation State of G alpha S

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A DISSERTATION

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biopharmaceutical Sciences
in the Graduate College of the
University of Illinois at Chicago, 2015

Medical Center
Chicago, Illinois

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To my parents, Roger and Becky, and the memory of my grandmother,

Thank you for everything.

ACKNOWLEDGEMENTS

My pursuit of a Ph.D. and this dissertation research would not have been possible without the help and support of so many people in my life. I must first thank my advisor, Dr. Mark M. Rasenick, for his hard work, invaluable training, guidance throughout the research contained within this thesis, and for being there for me when others were not. I cannot truly express how much having you accept me into your laboratory means. I have learned so much, and without the vast amount of knowledge you have passed on to me, this would not have been possible. The knowledge that I have gleaned from my time working with you is immeasurable and I will forever view any piece of data differently. Just imagine what we could have accomplished if I was in your lab for five years instead of one and a half! Thank you so much for making me a better scientist and for making this process a great experience.

I must sincerely thank all of the members of my dissertation committee. This includes my research advisor Dr. Mark Rasenick and my thesis advisor Dr. William Beck, as well as Dr. John Nitiss, Dr. Amynah Pradhan, and Dr. Gerd Prehna. I would like to especially thank Dr. Prehna for your assistance in improving this dissertation and for your friendship. I feel so fortunate to have received guidance from such a talented and knowledgeable group of professors. My doctoral research has benefitted greatly from all of your valued advice and criticisms over the past couple of years.

I would also like to thank Dr. William T. Beck for your invaluable insights and for always reminding me to, "Never assume anything." Your ability to see the

ACKNOWLEDGEMENTS (CONTINUED)

unseen has helped greatly with constructing and writing this dissertation. I will always remember your unique ability to see a result from more points of view than seemingly possible. The knowledge that I have gleaned from my time working with you is immeasurable and I will forever view any piece of data differently.

I would also like to thank the Department of Biopharmaceutical Sciences, including my professors, staff, and students for all of the assistance that each of you provided. Specifically, Dr. Karl Larsen our director of graduate studies for graciously allowing me to use the GC-MS in the forensics laboratory for studying the accumulation of antidepressants in membrane fraction microdomains.

Lastly, I must thank everyone in my laboratory for challenging me, for your advice regarding my experiments, and your friendship; thank you Andy Czysz, Bob Donati, Harinder Grewal, Sia Koutsouris, Jeff Schappi, and Nate Wray. When each of you read this, your names are in alphabetical order and not according to order of importance. You all have been good friends in my time at UIC. I hope that we will keep in contact in the future.

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ABBREVIATIONS

ANOVA	Analysis of variance
A.U.	Absorbance units
AC	Adenylyl Cyclase
APCI	Atmospheric pressure chemical ionization
BDNF	Brain derived neurotrophic factor
cAMP	cyclic Adenosine mono-phosphate
cDNA	Complementary DNA
co-IP	Co-immunoprecipitation
CREB	cAMP response element binding protein
C.I.	Confidence Interval
Cys-NEM	NEM conjugated Cysteine
Da	Dalton
DHHC	Asp-His-His-Cys motif
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DSP	3,3'-dithiobis-succinimidyl propionate
DUIS	Dual ionization mode detection
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
ECD	Electron Capture Dissociation
ECS	Electroconvulsive shock
ESI	Electrospray ionization

ESI MS/MS	Electrospray ionization tandem mass spectrometry
FT	Fourier Transform
G418	Geneticin
Gα	G protein alpha subunit
GAP	GTPase activating protein
$Gα_s$	G alpha S
$Gα_s^{C3S}$	Acylation deficient $Gα_s$-GFP
Gβγ	G protein beta and gamma subunit
GC-MS	Gas Chromatography Mass Spectrometry
GDNF	Glial cell derived neurotrophic factor
GEF	Guanine exchange factors
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GRB2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GTP	Guanine nucleotide triphosphate
Gγ	G protein gamma subunit
HEK293	Human embryonic kidney clone 293 cells
$HEK-5-HT_{3A}$	HEK293 cells expressing the serotonin 3A receptor
HRP	Horse radish peroxidase
ICR	Ion cyclotron resonance
IPTG	Isopropyl β-D-1-thiogalactopyranoside

kDa	Kilo Dalton
$LiAlH_4$	Lithium aluminum hydrate
LC-ESI-FTICR-MS	LTQ-FT hybrid linear ion trap - Fourier Transform ICR mass spectrometer
LTQ	Linear Trap Quadrapole
m/z	mass to charge ratio
mAb	Monoclonal antibody
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDD	Major Depressive Disorder
MSD	Mass selective detection
MIP	Molecular ion profile
M_X	Measurement
NaSSA	Noradrenergic and specific serotonergic antidepressant
NB35	Nanobody 35
NB37	Nanobody 37
NEM	N-ethylmaleimide
NEM-$G\alpha_s$	NEM conjugated $G\alpha_s$
NET	Norepinephrine reuptake transporter
NETN	NaCl, EDTA, Tris-HCl, Nonidet P-40
NHS	N-hydroxyl succinimide
NIST	National Institute of Standards and Technology
nM	Nanomolar

PBS   Phosphate buffered saline

PMSF   Phenylmethylsulfonyl fluoride

PPM   Parts per million

PTM   Post-translational modifications

PVDF   Polyvinylidene fluoride

PKA   cAMP dependent protein kinase

PET   Positron emission tomography

PDE4   Phosphodiesterase 4

RGS   Regulators of G-protein signaling

RNA   Ribonucleic acid

RT-PCR   Reverse transcription polymerase chain reaction

SEM   Standard error of the mean

Ser   Serine

SERT   Serotonin reuptake transporter

SNRI   Selective norepinephrine reuptake inhibitor

SOS   Son of Sevenless

SSRI   Selective serotonin reuptake inhibitor

STAR*D   Sequenced Treatment Alternatives to Relieve Depression

Sw II   Switch II segment of $G\alpha_s$

TCA   Tricyclic antidepressant

Thr   Threonine

TX-100   Triton X-100 (non-raft)

ABBREVIATIONS (CONTINUED)

TX-114   Triton X-114 (raft)

Tyr   Tyrosine

UHPLC-MS/MS   Ultra High Performance Liquid Chromatography Tandem
                Mass Spectrometry

µM   Micromolar

wt   Wild type

SUMMARY

Depression is a complex and significant public health problem for which currently available medications are often ineffective and their therapeutic effects routinely delayed by 1-2 months after initial administration. Due to the relative lack of understanding of the biochemical etiology of depression and for the mechanistic activities of available antidepressant medications, establishing an appropriate system to model a depressed state and evaluate the mechanisms by which antidepressants act is difficult. Establishing a model that adequately presents all of the intricacies and complexity of such a biological system is likely not possible. Nevertheless, previous studies from our laboratory have shown that: 1) $G\alpha_s$, the protein that activates adenylyl cyclase, localizes to lipid rafts in depressed subjects and 2) that chronic antidepressant treatment mediates translocation of $G\alpha_s$ out of lipid rafts. Translocation of $G\alpha_s$ presents a potential mechanistic explanation for the delayed onset of therapeutic action, but the precise molecular mechanisms orchestrating $G\alpha_s$ translocation remain.

Published data suggests that localization of $G\alpha_s$ to the plasma membrane results from N-terminal palmitoylation, and it appears that the localization of $G\alpha_s$ to lipid rafts requires palmitoylation (3). Based on this, I proposed that the gradual accumulation of antidepressants in lipid rafts resulted in an antidepressant-induced depalmitoylation of $G\alpha_s$. Moreover, I proposed that the translocation of $G\alpha_s$ to non-raft regions of the plasma membrane is a mechanistic factor describing antidepressant hysteresis.

SUMMARY (CONTINUED)

We have generated and established stably transfected C6 glioma cells with (i) Gα$_s$-GFP N-terminal acylation mutants that prevent Gα$_s$ N-terminal palmitoylation (Cys3Ser) and (ii) mutant Gα$_s$ that is both myristoylated and palmitoylated (Asn6Ser), which modifies Gα$_s$ similar to Gα$_i$. Analysis by cellular fractionation of both mutant Gα$_s$-GFP constructs displayed an antidepressant insensitive Gα$_s$. Furthermore, immunoprecipitation of Gα$_s$ with conformation specific nanobodies revealed that chronic antidepressant treatment and acylation state of Gα$_s$ directly influence the molecular partners to which Gα$_s$ associates. These results may provide new molecular insights and targets that allow for the eventual discovery of novel therapies for depression.

I evaluated those molecular associations of Gα$_s$ that direct Gα$_s$ to the plasma membrane using a conformation specific nanobody (NB35). I then compared the effects of different antidepressant treatments (drug, time, and concentration) and analyzed the associations of all immunoprecipitations using electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis.

In Aim 1, I correlated the accumulation of antidepressants in plasma membrane microdomains with the localization of Gα$_s$. Since C6 cells do not express reuptake transport proteins, while antidepressants still mediate translocation of Gα$_s$ from lipid raft microdomains, there is likely another target(s) that is/are mediating chronic antidepressant treatment effects. The principle hypothesis behind experiments in these aims is that modulation of the cytoskeletal components in association with Gα$_s$, function to maintain Gα$_s$ in lipid raft

SUMMARY (CONTINUED)

microdomains and that chronic antidepressant treatment destabilizes the

aforementioned interactions. Upon destabilization, $G\alpha_s$ is able to translocate from

the lipid raft and interact with Adenylyl Cyclase (AC).

In aim 2, I evaluated the effects of acylation on the subcellular localization of

$G\alpha_s$ with stably transfected C6 cells expressing wt GFP-$G\alpha_s$,

palmitoylated/myristoylated ($G\alpha_i$-like) GFP-$G\alpha_s$, and non-acylated GFP-$G\alpha_s$

recombinant constructs. Acylation of $G\alpha_s$ is important for plasma membrane,

whereas deacylation suggests activation and altered subcellular localization of $G\alpha_s$.

As such, I evaluated the effects of chronic antidepressant treatment on changes to

the association partners and the palmitoylation state of $G\alpha_s$. Immunoprecipitations

of $G\alpha_s$, with NB35, suggest that gradual antidepressant accumulation in lipid raft

microdomains correlates with a depalmitoylation of $G\alpha_s$ and an acylation state

directed panel of molecular association partners.

The results obtained and presented in this dissertation suggest a mechanism

of action for antidepressants that is apart from the inhibition of monoamine

reuptake. These observations are likely not the only accompanying mechanistic

action that chronic antidepressant treatments mediate, nor do they discount the

importance of the accumulation of monoamines (serotonin and norepinephrine) in

response to antidepressant therapy. These results have the potential to provide

new molecular targets in the antidepressant signaling cascade(s) and may allow for

the discovery of novel therapies that reduce the therapeutic latency characteristic of

antidepressant treatments. In the future, I would accomplish this through packaging

SUMMARY (CONTINUED)

a selected antidepressant with adjuvant therapies designed to modulate the

palmitoylation of $G\alpha_s$ in the acute phase or through peptides designed to disrupt the

lipid or protein anchors holding $G\alpha_s$ in the lipid raft of target cells.

Chapter 1 – Literature Review

1.1 Introduction

"It is an outrage that a person with a biochemical problem in their liver is treated with compassion, whereas a person with a biochemical problem in their brain is scorned!"
~Edward M. Kennedy

Mental health disorders are serious, costly, and debilitating illnesses for which the social stigma associated is often sufficient to prevent a person suffering from one or more to avoid seeking treatment. As recently reported in the New York Times, the overall cost of mental illness in the United States, which is the sum of reduced wages, usage of poverty services, direct medical costs, and lost productivity are costing the U.S. economy roughly $500 billion dollars per annum (4). Some of this expense however, is hard to quantify, as depression leads to reduced motivation and consequently a reduction in potential productivity. Even so, depression is one of the most commonly diagnosed psychiatric disorders (5). Moreover, while effective in some patients, therapeutic options have improved little beyond the available antidepressant medications that have been on the market for years. This is due to a lack of understanding of the biochemical etiology of depression as well as an incomplete understanding of the biochemistry by which antidepressants mediate their full therapeutic action.

In the treatment of depression, antidepressants have revealed that their mechanisms of action are more pharmacologically complex than a simple monoamine-mediated process. Specifically, the fact that therapeutic efficacy, if achieved at all, depends upon sustained antidepressant administration over weeks to months (hysteresis), all while the presence of monoamines increases within days of beginning a

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treatment regimen, suggests that alternative mechanisms of action exist and that unexploited targets that may provide better treatment options exist as well. Therefore, fully understanding the biochemical mechanisms that account for antidepressant pharmacological action is necessary to more precisely characterize and tailor effective treatments for patients suffering from depression.

1.2 Depression

Depression is a chronic illness that affects the way sufferers feel, think, and behave. The persistent feelings of sadness can result in a loss of interest in previously enjoyable activities (anhedonia) and may lead to an array of emotional and physical problems as well. Major Depressive Disorder (MDD) is the leading cause of long term disability in the industrialized world(6) and it is estimated that ~15% of the world's population is affected at some point during their lifetime (7). In diagnosing depression, at least five of the nine diagnostic criteria for MDD, outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM), must be present nearly every day. According to the DSM, MDD is diagnosed based on the presence of long-lasting key symptoms that include: low mood or irritability most of the day, anhedonia, feelings of worthlessness, guilt, or despair, significant appetite or weight change (>5%), sleep disturbances (insomnia or hypersomnia), psychomotor issues (fatigue and anergia), diminished ability to think and concentrate, and suicidal ideation. However, before diagnosing depression, physical illness, medication, substance abuse, dysthymia (a mild chronic mood disturbance that persists for at least two years), adjustment disorder, or bipolar disorder (depressive phases alternate between periods of mania and hypomania) must be ruled out as potential causes.

1.3 Biology of Depression

Since the observations of Schildkraut in the 1950s and '60s, depression has been associated with a global reduction in monoamine content in the brain (8). However, there is a significant hysteresis between the increase in monoamines and therapeutic efficacy with antidepressant therapies. Alternatively, depression may result from a reduction in the overall size and volume of specific brain regions most affected by depression: hippocampus, prefrontal cortex, amygdala, cingulate gyrus, and nucleus accumbens (9-13) and that chronic antidepressant therapy induces neurogenesis in afflicted regions (14-16). Supporting evidence for this theory derives from the similarity in lengths of time in the maturation cycle of newly incorporated granule cells with the hysteresis in antidepressant therapeutic efficacy (17). Moreover, neuronal atrophy in the depressed state is due to a decrease in the expression of brain derived neurotrophic factor (BDNF) that chronic antidepressant treatment appears to restore (18-24). However, the observed decrease in hippocampal volume in depressed patients is less than the variability that occurs naturally (25) and brain regions not normally associated with depression, such as the cerebellum, also shrink in volume. Therefore, the importance of neurogenesis in response to chronic antidepressant treatment as a potential mechanism of action in treating depression remains doubtful.

There is a growing consensus however that depression is caused by altered synaptic plasticity (synaptogenesis) affecting cognitive and behavioral functions (6). Synaptogenesis is a process by which new connections between neurons occur without producing a new cell (neurogenesis). Though they appear related, synaptogenesis and neurogenesis are independent processes. Glial cells are known to support neuronal

maintenance, but conduct a rather strange task of preventing neuronal cell differentiation (26). Almost half of the human brain is composed of glial cells (27) that play an important role in synapse formation. Recent evidence suggests that astroglial cells control the number of synapses formed, are integral to synaptic stability, necessarily exert influence over postsynaptic function, and mediate structural and functional synaptic changes throughout the nervous system (28, 29). Furthermore, chronic antidepressant treatment increases the expression and release of glial cell derived neurotrophic factor (GDNF) in glial cell populations (30-33), which further implicates synaptogenesis in depression and the antidepressant response. Regardless, they are likely two sides of the same coin, as chronic antidepressant treatment results in an increased accumulation of cellular cyclic adenosine monophosphate (cAMP) (34), which necessarily mediates phosphorylation and activation of the cAMP response element-binding protein (CREB) (30, 35-37), and subsequently activation of BDNF (or GDNF) in a CREB-mediated process (22, 38).

Recent positron emission tomography (PET) evidence showed that cAMP is diminished (throughout the brain) in depressed patients, but rebounds in subjects responding to antidepressants (39). Currently the prevailing theory of how antidepressants function, this could be termed the cAMP theory of depression. However, CREB deficient mice, which effectively could be achieved from reduced expression of CREB or from reduced cAMP accumulation, display increased neurogenesis and experience a rapid onset of action with chronic desipramine (34); serotonin (5-HT) depletion reverses the effects of CREB deficiency. The serotonin receptor family is composed of members coupled to both $G\alpha_i$ and to $G\alpha_s$; $5\text{-HT}_{1/5}$ are

$G\alpha_i$ coupled and 5-HT$_{4/6/7}$ are $G\alpha_s$ coupled; 5-HT$_2$ is $G\alpha_q$ coupled and 5-HT$_3$ is an ion channel. Thus, serotonin appears to be signaling through 5-HT$_{1/5}$ in the system they are observing as these receptors are $G\alpha_i$ coupled, which will result in an increase in cellular cAMP accumulation and activated CREB. This suggests that the monoamine serotonin is not terribly important for antidepressant action, but also that cAMP and neurogenesis are opposing forces. Moreover, if serotonin were a significant mediator of the chronic antidepressant response, there would not be a hysteresis to therapeutic efficacy.

Regardless, many still consider depression a result of a deficiency in monoamine neurotransmitters in the synaptic cleft (monoamine hypothesis). However, antidepressant hysteresis suggests that mechanism(s) apart from signaling through the serotonin, dopamine, and/or adrenergic receptors exist (40-43), which is in contrast to the prevailing dogma that antidepressants work via a presynaptic mechanism. The monoamine and cAMP (neurogenesis/synaptogenesis and genomic) theories of depression together appear to only begin to explain the complexity of depression and the complex pharmacology of antidepressants. Thus, an alternative molecular target(s) for each antidepressant rather than the monoamine transporters or monoamine oxidase (MAO) appears to exist.

1.4 Antidepressants

Psychoactive agents and drugs have been used for thousands of years for therapeutic, hallucinogenic, and various other purposes. However, until the relatively recent rapid advances in medical science, the mechanism(s) by which many of these drugs acted were largely unknown. While the scientific community is now better able to address these gaps in knowledge and characterize the mechanism(s) by which many of these drugs act, for some very commonly prescribed psychoactive drugs (e.g. antidepressants) there still remains a relative lack of understanding of precisely how they work. Much of the hindrance to the discovery of new antidepressant therapies and the precise mechanism antidepressants engage results from the social stigma associated with depression and the complex pharmacology that antidepressants exhibit (hysteresis, etc.).

Among others, Schildkraut first proposed in 1965 the most widely known theory of antidepressant action, "the catecholamine hypothesis of affective disorders," more commonly referred to as the monoamine hypothesis. In which, he asserts that, "some, if not all, depressions are associated with an absolute or relative decrease in catecholamines, particularly norepinephrine, available at central adrenergic receptor sites," and that, "Elation, conversely, may be associated with an excess of such amines" (8). This assessment was rooted in observations made in the 1950s that hydrazine agents, used to treat tuberculosis, also exhibited antidepressant effects and the fact that the same compounds were later found to inhibit monoamine oxidase (44). Therefore, the reasoning was that it must be true that a deficiency in signaling associated with the monoamine neurotransmitters was the root cause. Even though depression has long

been thought of as an imbalance (i.e. deficiency) in monoamine neurotransmitters, the monoamine hypothesis fails to address the fact that antidepressants exhibit delayed onsets of action, of at least a week and often longer, that cannot be accounted for by a simple increase in monoamine neurotransmitter density (40-43). This increase occurs relatively soon after treatment begins, but the hysteresis to therapeutic action cannot fully account for the assumption that monoamines alone are responsible.

Owing to the monoamine hypothesis, the current treatment options for depression are pharmacological agents designed to enhance the density of serotonin, norepinephrine, or a combination of the two in the synaptic cleft. While beneficial in many patients, many more do not respond to conventional therapies. For instance, the racemic mixture drug citalopram (Celexa) is associated with a remission rate of only 36.8 %, and 40% of patients on antidepressants relapse within a year (45). It is not surprising that treatment efficacies are not improving, as each new drug is essentially a derivative of the preceding one. As the low hanging fruit has essentially all been picked, it is necessary to understand on a more fundamental level the mechanism(s) by which current antidepressants act, so that novel or newly devised adjunct therapies for the treatment of MDD be designed.

Antidepressants are particularly unusual in that their effects take weeks to manifest (46, 47). Moreover, patients routinely cease taking a prescribed antidepressant, often citing improved health or that they do not work. Often the symptoms relapse, but about one third of individuals do not respond to a first line antidepressant regimen. The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial taught us that nearly 70% of sufferers of MDD fail to respond with a first

line antidepressant regimen (48, 49). This assessment may be due to an actual lack of response or it may occur before realizing the therapeutic effects. However, the former is most likely as all participants were treated for two months. For these reasons, the mechanisms by which antidepressants act and the reasons for their hysteresis have been under investigation for some time. A listing of the currently available antidepressants, their class, and characterized function is summarized in TABLE I. However, a conclusive explanation accounting for the delay between beginning a treatment regimen and achieving a desired physiological response remains.

A mechanistic explanation accounting for the delayed onset in therapeutic action (hysteresis) remains elusive and presents a significant gap in our understanding of the complex pharmacology antidepressants display. Much of the currently available antidepressants are designed with the monoamine hypothesis in mind, but the increase in monoamine neurotransmitters occurs in hours to days, whereas the therapeutic effects in patients that respond manifest over weeks of treatment. Apart from increasing monoamine density in the synaptic cleft, the hysteresis to therapeutic efficacy coupled with long-term (chronic) antidepressant treatment suggests the engagement of other signaling pathways. This dissertation addresses the salient features of a key mechanism that accounts for the manifestation of depression as well as antidepressant hysteresis. Experiments test the hypothesis that: different antidepressant drugs display distinct patterns of action, which may be through direct interaction with a protein or lipid component of lipid rafts, through interference with associations anchoring $G\alpha_s$ in lipid rafts coupled with mediating depalmitoylation of $G\alpha_s$.

TABLE I. Antidepressant Classes, Drug Structures, and Designed Mechanistic Function.
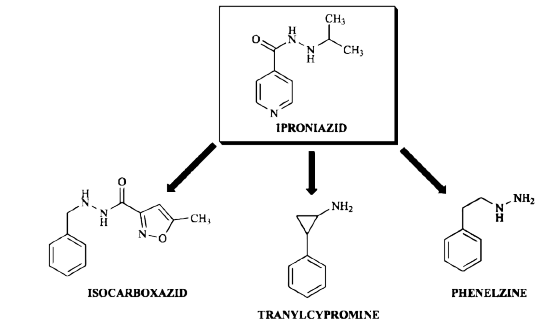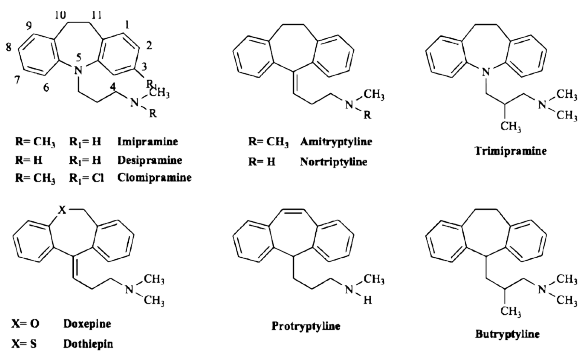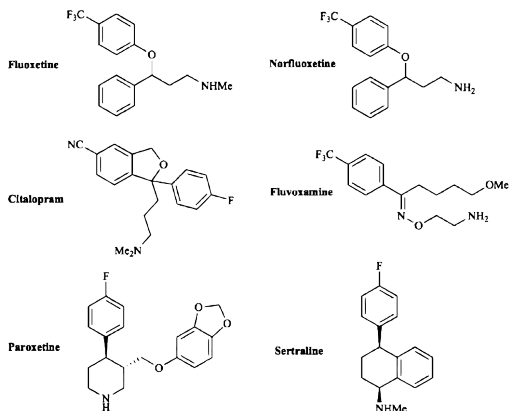
Class	Antidepressants	Activity
Monoamine Oxidase Inhibitor (MAOI) **1960s**		**Inhibit Monoamine Catabolism**
Tricyclic Antidepressant (TCA) **1960s - 80s**		**Inhibit Serotonin/ Norepinephrine Reuptake Transporters**
Selective Serotonin Reuptake Inhibitor (SSRI) **1980s -**		**Inhibit Serotonin/ Norepinephrine Reuptake Transporters**

Table 1: Antidepressant drug classes, associated drugs, and their canonical mechanistic function. Representatives from each class of antidepressant are assayed in the following experiments: Phenelzine (MAOI), Desipramine (TCA), Fluoxetine (SSRI), and Escitalopram (SSRI).

1.5 G-protein coupled receptors

G protein-mediated signaling pathways are highly conserved throughout the evolutionary spectrum and transmit signals regulating numerous cellular processes. G protein-coupled receptors (GPCRs) are responsible for most of the signaling of hormones and neurotransmitters via activation of heterotrimeric guanine nucleotide binding proteins (G protein) (50). GPCRs are a diverse class of receptors, most of which are integral membrane proteins that contain seven transmembrane domains. Many of the structural characteristics of the GPCR super family are based upon sequence homology with the first GPCR to be crystallized, Bacteriorhodopsin (51) and later the first mammalian GPCR crystal structure for Rhodopsin (52).

GPCRs transmit their associated signals via significant ligand mediated conformational rearrangements of the receptor, most notably through forming a cavity due to outward movement of the transmembrane domains 5 and 6 (50, 53), coupled with conformational rearrangement and activation of the coupled G protein (Figure 1) (50, 53-55). These conformational rearrangements promote association with guanine nucleotide exchange factors (GEFs) that exchange the bound GDP to GTP on the Gα subunit. GTP bound Gα subunits dissociate from Gβγ and are free to engage signaling pathways specific to the particular Gα subunit (56). Effectively, a GPCR is a GEF for heterotrimeric G proteins.

1.6 G-proteins and signaling

There are two classes of G-proteins, monomeric and heterotrimeric, which are activated via signals external to the cell transmitted inward through a receptor. Both classes are regulated via GDP/GTP exchange to mediate a variety of functional activities; hence G (guanine nucleotide binding) protein. However, the types of receptors that activate each are markedly different.

Monomeric G proteins, more commonly referred to as small GTPases or the Ras superfamily of GTPases, is quite large with over 100 members (57), but essentially consists of five subfamilies (Rab, Ran, Ras, Rho, and ARF) (58). One of the ways in which small GTPases are activated is through receptor tyrosine kinase (RTK) activation. RTKs are comprised of two subunits that unite and cross phosphorylate each other, which facilitates the docking of adaptor protein growth factor receptor-bound protein 2 (Grb2) through its Src homology 2 (SH2) domain as well as the Son of Sevenless homolog (SOS) through Src homology 3 (SH3) domains of Grb2 (59). SOS in turn binds the monomeric G protein and recruits a guanine nucleotide exchange factor (GEF) to the membrane that induces GDP to GTP exchange (active state) (60).

The active G protein dissociates and affects kinases or cytoskeletal and vesicle trafficking targets downstream. The Rab family principally targets vesicle trafficking machinery (61), the Ras family primarily affects growth and differentiation involved kinase cascades (62), the Rho family regulates actin filament polymerization (63-65), and ARF plays a role in the formation of vesicle formation (66). Association with GTPase activating proteins (GAPs) results in termination of signaling through small monomeric G proteins.

Heterotrimeric G proteins by contrast consist of α, β, and γ subunits. There are 27 Gα, 5 Gβ, and 14 Gγ subunits currently known to exist, which presents a possible diversity of 27×5×14=1890 combinations of heterotrimers (67). However, the actual number of potential combinations is likely much lower due to tissue specific expression patterns. The fact that there are 27 different Gα subunits suggests that it, rather than β and γ subunits, is the principal mediator of the diverse signaling pathways GPCRs transmit. The Gα subunits consist of four families: $G\alpha_i$ ($G\alpha_{i0/1/2/3}$ $G\alpha_z$ $G\alpha_t$ $G\alpha_{gust}$), $G\alpha_{q/11}$ ($G\alpha_q$ $G\alpha_{11}$ $G\alpha_{14}$ $G\alpha_{15/16}$), $G\alpha_s$ ($G\alpha_s$ $G\alpha_{olf}$), and $G\alpha_{12/13}$ ($G\alpha_{12}$ $G\alpha_{13}$). Upon activation, G protein coupled receptor (GPCR) conformational rearrangements promote exchange of GDP for GTP, resulting in Gα activation, and functional dissociation of Gα from Gβγ (56). Moreover, there is significant conformational rearrangement of the Gα subunit when GTP bound, as opposed to GDP bound, which promotes this dissolution (1, 2, 50, 53-55) (Figure 1). GPCR mediated signaling pathways are turned off via the intrinsic GTPase activity of the respective Gα subunits, which can be accelerated by regulators of G-protein signaling (RGS) (68-70).

Figure 1. Heterotrimeric Gα proteins dissociated from Gβγ and undergo significant conformational rearrangement upon activation.

Inactive GDP bound Heterotrimeric G protein

Active GTP bound Gα

PDB: 1GG2

PDB: 1GIA
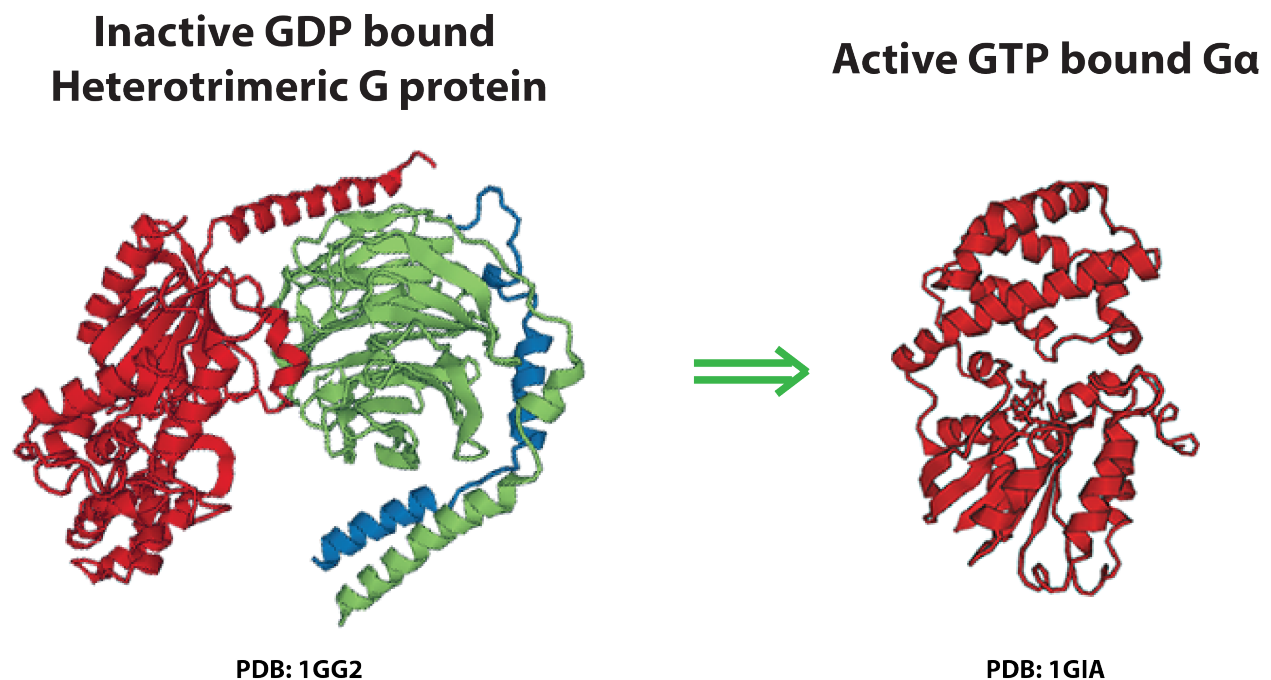
Exchange of GDP for GTP on the Gα subunit of heterotrimeric G-proteins results in activation through significant conformational rearrangement of the Gα subunit, and dissolution of the complex between Gα and Gβγ. GDP bound G protein heterotrimer. Gα$_i$ (G203A) complexed with Gβγ (PDB entry 1GG2); Gα - red, Gβ - green, and Gγ – blue (1). GTP bound and conformationally active Gα$_i$ subunit (PDB entry 1GIA); Gα – red (2).

1.7 Acylation

Proteins that interact with the hydrophobic plasma membrane lipid bilayer require hydrophobic surfaces for insertion, such as *α*-helices or *β*-sheets, or be modified with lipid anchors (acylated). The latter serves the purpose of inserting into the hydrophobic hydrocarbon core of the lipid bilayer. Examples of acylated proteins include the lipidated forms of receptors, monomeric and heterotrimeric G-proteins (71-73), and protein tyrosine kinases (74, 75).

Acylation is a common protein modification that enables and directs membrane-associated proteins to the inner leaflet and regulates their signaling capacity. Acyl modifications essentially belong to two categories: I) Glyocophosphatidylinositol (GPI) linked (76, 77) and cholesteroylation (78-80) modifications that orient the protein extracellularly. GPI linkage aids the trafficking of proteins through the secretory pathway from the endoplasmic reticulum. II) Those that mediate association with the cytosolic face of the plasma membrane. Cytosolic lipidations are further divided into: N-myristoylation (81, 82), prenylation (71, 83-86), and palmitoylation (87-94).

The two most common acyl modifications are myristoylation (14 carbon) and palmitoylation (16 carbon) (95). Both modifications may be dynamically regulated, but the linkage of myristate to the N-terminus of glycine, as opposed to the S-linkage with cysteine in the case of palmitoylation, makes this a more stable bond and less readily turned over (95). N-myristoylation occurs via an amide linkage between the 14-carbon saturated fatty acid myristate and the N-terminal amino group of a target protein with the sequence Met-Gly-protein. The N-terminal methionine is cleaved by methionine aminopeptidase and N-myristoyltransferase catalyzes the amide linkage of myristoyl-

CoA to the N-terminal glycine (81). Prenylation, by contrast, occurs on a C-terminal cysteine thiol on which farnesyl (15 carbon) or geranylgeranyl (20-carbon) are thioester linked by farnesyltransferase (96, 97) or geranylgeranyltransferase (98-100) respectively. Finally, the comparatively more exciting from a signaling perspective, palmitoylation occurs through thioester linkage of the saturated 16-carbon fatty acid to a cysteine thiol side chain of a target protein (87-91, 94, 101-109).

Palmitoylation is catalyzed by protein palmitoyltransferases (PATs), but the target motifs that many PATs recognize are poorly characterized (94, 110). The primary function of palmitoylation is to direct palmitoylated proteins to the membrane (94). Palmitoylation also targets proteins to lipid rafts, as when palmitoylation is blocked by mutagenesis or PAT inhibition, proteins no longer localize to lipid rafts (111-114). However, the unique feature of this modification is that it is reversible and that rapid palmitoylation turnover allows modified proteins to shuttle between the plasma membrane and other subcellular regions (69, 87, 115-123). Therefore, the dynamic reversibility of palmitoylation differentiates it from the other types of acyl modifications, and makes it the comparatively more interesting and important acyl modification because it serves a dual purpose as a lipid anchor, but also allows proteins to shuttle between cellular regions via a cycle of palmitoylation and depalmitoylation. The structure and catalyzing enzyme families for each lipid modification are summarized in TABLE II.

There are a considerable number of proteins that are only palmitoylated. In fact, a single, reversible palmitoylation catalyzed by a DHHC motif containing protein (DHHC3/7) (124) anchors $G\alpha_s$ to the plasma membrane, which enhances its interaction

with Gβγ, an essential step in cell signaling cycles (68). Moreover, regulators of G-protein signaling (RGS) are also singly palmitoylated, which regulates membrane localization and inactivation of G proteins by turning off GPCR mediated signaling pathways (68, 69). Acylation of many G protein alpha subunits and small GTPases is what directs their association with the inner leaflet of the plasma membrane and may control their association with lipid rafts (3, 124, 125), in part because it regulates the association between Gα and Gβγ, the latter associating with the membrane via prenylation (126, 127). Moreover, $G\alpha_s$ is the only Gα subunit that exhibits activation-induced translocation coupled with depalmitoylation (88, 107, 128-132).

Dual acylation with a palmitoyl and a prenyl or myristoyl group is also possible. For example, the Ras proteins, H-Ras and N-Ras, are palmitoylated and farnesylated (96, 133) and the G protein $G\alpha_i$ is palmitoylated and myristoylated (128, 134-136). In each case, the first modification (e.g. prenylation and myristoylation) provides a weak membrane interaction and the subsequent palmitoylation generates sufficient hydrophobicity for a strong membrane affinity (3, 107, 137, 138). $G\alpha_s$ is the most difficult Gα to extract from the membrane with detergent (139), likely owing to the fact that palmitate, in contrast with myristate (137), is more than capable of mediating strong association with the plasma membrane even though it is reversible (104). Taken together, it may be possible that one, or a companion, of the potential mechanism(s) of antidepressant-induced translocation of $G\alpha_s$ from lipid rafts is via attenuating its N-terminal palmitoylation.

Although antidepressants mediate translocation of $G\alpha_s$ from lipid rafts, until the results presented herein it was unknown if they mediated depalmitoylation of $G\alpha_s$, or

possibly even activated $G\alpha_s$. Where DHHC3/7 are responsible for palmitoylating $G\alpha_s$ (124), acyl-protein thioesterase 1 (APT1) is the enzyme responsible for depalmitoylating $G\alpha_s$ (140). Knockdown of DHHC3 and DHHC7 appear to have little effect apart from impairing membrane localization of their protein targets (124, 141), whereas knockdown of APT1 reduces synaptic spine volume (142). Thus, knockdown of either palmitoylating or depalmitoylating enzymes does not appear to be toxic to cells and likely only affects localization of palmitoylated proteins (122, 124, 141). Interestingly, inactivation of the closely related palmitoyl protein thioesterase 1 (PPT1) results in infantile neuronal ceroid lipofuscinosis, which is characterized by degradation target accumulation in the lysosome, neurodegeneration, and ultimately death (143). Regardless, an acute activator of APT1, or acute inhibitor of DHHC3/7 could be therapeutically useful in combination with a chronic antidepressant treatment in treating MDD.

TABLE II. Structures and Catalyzing Enzymes for the Principle types of Protein Lipid Modification.
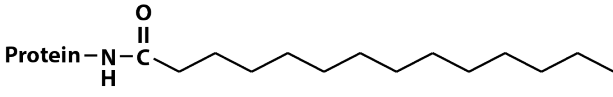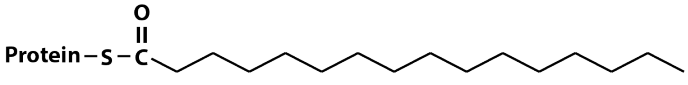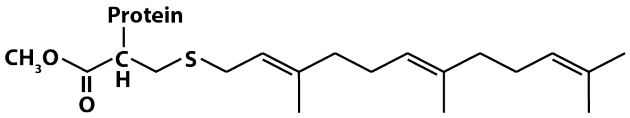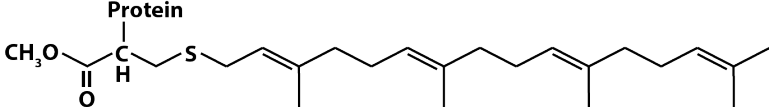
Lipid Modification		Enzyme
	Myristoylation	N-myristoyl transferase
	Palmitoylation	Palmitoyl transferase
	Farnesylation	Farnesyl transferase/ Geranylgeranyl transferase I
	Geranylgeranylation	Geranylgeranyl transferase II

Table 2: The hydrophobic chains allow for insertion into the hydrophobic plasma membrane lipid bilayer, which mediates the association of the accompanying protein with the membrane. In the case of acylation by either myristoylation or palmitoylation, the chemistry of attachment determines the lability of turnover in that covalent linkage with the cysteine thiol is inherently less stable than covalent linkage to the N-terminal amine. Prenylation by contrast is sometimes referred to as iso-lipidation due to the way in which bonding occurs intrachain as opposed to the terminus.

1.8 Plasma membrane and Lipid Raft microdomains

The fluid mosaic model proposes that the plasma membrane is a fluid lipid bilayer in which integral and associated proteins are able to freely diffuse laterally. In some cases, this is true, but the compartmentalization of the membrane into microdomains via interactions between different lipids, proteins, and the cytoskeleton greatly restrict much of this lateral mobility. In particular, regions of the plasma membrane rich in Caveolin, cholesterol, sphingolipids, and GPI-anchored proteins known as lipid rafts, contain many of the anchoring cytoskeletal-associated membrane structures (144-146). Lipid rafts bring together and facilitate molecular association(s) of a vast array of different membrane imbedded and membrane-associated proteins to theoretically initiate intracellular signaling. However, lipid rafts are enriched with sphingomyelin and low in phosphatidylcholine, presumably to maintain similar choline content between the raft and non-raft regions of the plasma membrane. Moreover, due to the rigid nature of cholesterol, it preferentially partitions into the lipid rafts where acyl chains of the sphingolipids and others are more saturated and less fluid; thus maximizing van der Waals interactions. Thus, the rigidity and tight packing of lipid rafts is sufficient to restrict lateral diffusion of integrated as well as membrane-associated proteins.

1.9 G proteins and Lipid Raft microdomains

While lipid rafts can facilitate the clustering of signaling molecules (147, 148), the rigid structure afforded by increased cholesterol content appears to actually have a globally dampening effect on G protein signaling, as many GPCRs are lipid raft localized (149). For example, $G\alpha_s$ is a membrane-associated protein whose signaling is impaired by lipid raft microdomains, presumably through inhibiting association(s) between raft and non-raft based molecules (132, 150). Dampened signaling, through $G\alpha_s$ and/or $G\alpha_s$ coupled receptors, is consistent with the observed increase in $G\alpha_s$ association with rafts as well as damped cAMP signaling seen in MDD (151). Accordingly, $G\alpha_s$ content within lipid rafts is diminished after chronic treatment with fluoxetine, desipramine, and escitalopram (152, 153), cAMP is increased (154), and enhanced neurite outgrowth ensues (155, 156); presumably through induction of GDNF expression (30-33). Moreover, lipid raft disruption through cholesterol depletion or cytoskeletal disruption displaces many raft proteins, but activation or antidepressant treatment displaces only $G\alpha_s$, as there was not any change in raft localization of $G\alpha_i$ or $G\alpha_q$ (139, 153).

Displacement of $G\alpha_s$ from lipid rafts could mean intracellular translocation. However, increased $G\alpha_s$ and Adenylyl Cyclase (AC) physical coupling was observed by co-immunoprecipitation (co-IP) after chronic, but not acute antidepressant treatment, which resulted in enhanced activation of AC; chronic amphetamine did not show similar effects (157). This suggests rather that translocation is into non-raft regions of the plasma membrane as AC is quite large and less likely to readily internalize. The overall amount of $G\alpha_s$ was unchanged and intrinsic GTP binding nor intrinsic AC activity was

altered; $G\alpha_i$ and AC inhibition were not altered (154). Furthermore, chronic (3 week) but not acute (1 week) treatment of rats with amitriptyline, desipramine, imipramine, iprindole, or electroconvulsive shock (ECS) increased activation of AC in the cortex and hypothalamus, but not in the liver or kidney (158). Lastly and importantly, escitalopram increases $G\alpha_s$/AC coupling and lipid raft translocation to non-raft regions of the plasma membrane of C6 glioma cells in a time and concentration dependent manner in which the inactive enantiomer R-citalopram had no effects (152). Taken together, this suggests that antidepressant effects are mediated through induction of the cAMP generating system: G alpha S ($G\alpha_s$)–Adenylyl Cyclase (AC)–cAMP dependent protein kinase (PKA) in mediating the antidepressant response. However, the known targets of currently available antidepressants are the reuptake transporters or monoamine oxidase (MAO), neither of which couples with $G\alpha_s$. Together, these findings suggest a significant role for $G\alpha_s$ in depression and in mediating the physiological effects of antidepressants.

1.10 Aim of the Dissertation

Although antidepressants are the most widely prescribed class of drugs in the United States, the precise mechanisms by which they function are not well defined. The goal of this study is to better understand the molecular mechanisms accounting for the delayed onset of their therapeutic action. Depression is a significant public health problem and the hysteresis of antidepressant action complicates this problem. The goal of any drug treatment is a rapid, sustained, and complete remission of symptoms. However, antidepressant drugs exhibit a hysteresis to their physiological effects that may last several weeks to months. Previous studies from our laboratory have shown that chronic antidepressant treatment mediates movement of $G\alpha_s$ out of lipid rafts (132, 139, 151-153, 157, 159-163), which presents a potential mechanistic explanation for the delayed onset of therapeutic action, but the molecular mechanisms mediating movement of $G\alpha_s$ into and out from lipid rafts presents a significant knowledge gap. Therefore, in response to chronic antidepressant treatments, I directly evaluated the effects that antidepressants have on $G\alpha_s$ and its localization.

1.11 Innovation and Impact

       The suggestion that one action of antidepressants is to move $G\alpha_s$ out of lipid rafts is a unique and novel biochemical mechanism. We propose that this finding and the results contained within this dissertation will establish $G\alpha_s$ as a new diagnostic marker of depression. Moreover, that the biochemical alterations that antidepressants mediate upon $G\alpha_s$ are exploitable in creating novel targeted pharmacological therapies. Successful completion of the proposed experiments have furthered our understanding of a possible site of action of a variety of disparate drugs that act as antidepressants and may lead to the rational design of new antidepressant therapies. Data derived from this project might also lead to new screening methodologies for newly developed antidepressant drugs by analyzing the modification status of the biomarker $G\alpha_s$. We suggest that the localization of $G\alpha_s$ in lipid rafts represents a biological signature of depression and that antidepressant-induced translocation of $G\alpha_s$ through accumulation in lipid rafts is a useful indicator of antidepressant responsiveness. This provides the overriding rationale for the studies in this dissertation:

1.12 Hypothesis of Antidepressant Action

In response to chronic, but not acute antidepressant treatment, $G\alpha_s$ moves out of lipid raft microdomains and increasingly interacts with AC. The molecular anchors localizing $G\alpha_s$ to the lipid raft regions of plasma membrane and changes to them consequent to chronic antidepressant treatment remain poorly characterized. Therefore, I tested a novel hypothesis that **chronic treatment with antidepressants leads to their gradually accumulation in lipid rafts, which mediates the depalmitoylation of $G\alpha_s$, the remodeling of the molecular associations in which $G\alpha_s$ participates, and ultimately the membrane localization of $G\alpha_s$.**

1.13 Specific Aims

Previous studies from our laboratory have shown that **chronic antidepressant treatment mediates movement of Gα$_s$ out from under the inhibitory effects of lipid rafts** (132, 139, 151-153, 157, 159-163). The precise molecular mechanisms of this phenomenon are not well defined and present a significant knowledge gap. Specifically, I employed the following aims to test the hypothesis (Figure 2):

AIM 1: Determine the molecular associations/mechanisms that modulate Gα$_S$ translocation from lipid rafts under chronic antidepressant treatment *in vitro*.

I directly evaluated the molecular mechanisms acting on Gα$_s$ in response to acute (1 hr) and chronic (72 hrs) antidepressant treatments. I analyzed molecular associations of Gα$_s$ with ESI-MS/MS and correlated Gα$_s$ localization with the expression of cytoskeletal factors (e.g. Tubulin isoforms) in C6 glioma cells that lack any monoamine transporter system **(antidepressants also exert post-synaptic effects).** Further, I determined the accumulation of antidepressants in lipid rafts by extraction and analysis with absorbance and confirmed with GC-MS. Antidepressant presence was determined by screening obtained mass spectra against available libraries. I expect that **antidepressants alter cytoskeletal architecture; enabling the key molecular event of Gα$_s$ movement out from lipid rafts, before achieving a therapeutic response.**

    1A. Determine the extent of gradual accumulation of different antidepressant drugs in lipid raft and non-raft fractions of the plasma membrane and correlate this with Gα$_s$ subcellular localization.

1B. Evaluate changes to the molecular associations in which Gα$_s$ participates

mediated by chronic antidepressant treatment, specifically filament proteins.

AIM 2: Determine the effects of acylation state of Gα$_S$ on its subcellular localization and molecular associations that maintain lipid raft localization.

Acylation of Gα$_s$ appears to direct its subcellular localization and likely the molecular associations in which it is involved. Wild type (wt) Gα$_s$ is palmitoylated in the N-terminus (125), localizing it to the plasma membrane. I will stably transfect C6 cells with wt Gα$_s$-GFP, as well as mutant variants that are palmitoylated/myristoylated (Gα$_i$ like) Gα$_s$-GFP mutants, and acylated deficient Gα$_s$-GFP for examining changes in association partners. I compared acute (1 hr) and chronic (72 hrs) antidepressant treatments for alterations in Gα$_s$ complexes, precipitated using conformationally specific (GTP bound) camelid nanobodies (164). Molecular associations were analyzed by ESI-MS/MS, confirmed via western blot analysis, and acyl modification(s) determined by GC-MS. Taken together, I examined the effects that acylation has on the antidepressant mediated **molecular event of translocation of Gα$_s$ out of lipid rafts, before achieving a therapeutic response.**

2A. Evaluate the effects of acylation state on the subcellular localization of Gα$_s$ and

how this affects changes to the nascent molecular associations in which Gα$_s$

participates in response to chronic antidepressant treatment.

2B. Determine whether chronic treatment with antidepressants mediates

depalmitoylation of Gα$_s$ and if depalmitoylation affects lipid raft anchoring of Gα$_s$.

Figure 2. Proposed model of chronic antidepressant mediated effects upon Gα$_s$ plasma membrane localization.
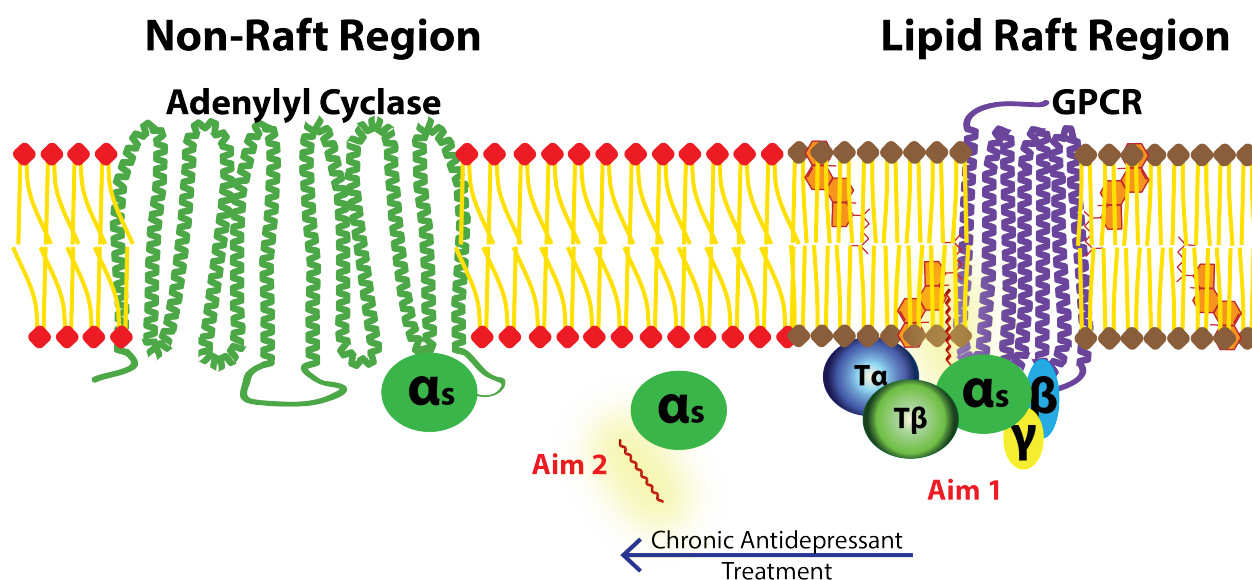
N-terminal palmitoylation directs Gα$_s$ to the plasma membrane, preferentially to lipid raft regions. I hypothesize that Gα$_s$ is enriched in lipid rafts during depression. Subsequent to chronic treatment with various antidepressant compounds, Gα$_s$ is translocated from lipid raft regions of the plasma membrane to non-raft membrane regions that allow greater interaction with/activation of Adenylyl Cyclase. I further hypothesize that translocation of Gα$_s$ is accompanied by dissociation of Gα$_s$ from Tubulin (Tα/Tβ) or additional raft anchors; which is accompanied by depalmitoylation of Gα$_s$.

Chapter 2 – Research Design and Methods

2.1 Model Systems

Any system will be fraught with some level of complexity. In this dissertation, we will be using C6 astroglial cells because of their relative ease of biochemical and pharmacological manipulation, but most importantly because of their lack of expression of monoamine transport proteins. The latter is of paramount importance for the study of the post-synaptic effects attributed to the chronic presence of antidepressant drugs, which is suggests by the hysteresis of antidepressant action that will be described in detail throughout the rest of this document.

I evaluated the mechanism(s) of antidepressant action and the consequent movement of $G\alpha_s$ out of lipid rafts in rat C6 glioma cells, which is a system that others and we have used for years. While C6 cells have transporters for glutamate, they lack any monoamine transporter system, which does not discount the relevance of monoamine reuptake inhibition, but rather does suggest that antidepressants also exert post-synaptic effects independent of reuptake. I suggest that this "transmitter/transporter-independent" effect is due to some reordering of membrane components and that it is a process requiring days (in cells) to weeks (in rodents or humans).

2.2 Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, and penicillin/streptomycin were purchased from Sigma-Aldrich, St Louis, MO. Cell culture flasks were from NUNC (VWR International, West Chester, PA). Escitalopram and *R*-citalopram were kindly provided from H. Lundbeck A/S, Copenhagen, Denmark. Desipramine hydrochloride and olanzapine were purchased from Tocris Bioscience, Ellisville, MO. Phenelzine sulfate, fluoxetine hydrochloride, N-ethylmaleimide, and Hydroxylamine were purchased from Sigma-Aldrich, St Louis, MO.

2.3 Western blotting

Westerns were conducted according to standard protocols with a mouse mono-clonal anti-Gα_s (1:1,000), rabbit mono-clonal anti-Cav1 (1:10,000), and mouse mono-clonal anti-β-actin (1:5,000). Membranes were blocked in 5% milk for 30 min at RT, primary antibody incubations conducted in 5% BSA, 0.2% NaN$_3$, and 1X protease inhibitors for 4 hrs at 4 ^{o}C. Secondary antibody incubations were conducted in 5% milk for 2 hrs at RT.

2.4 Drug Treatments

To date, most drugs investigated relate in some way to monoaminergic transmission. The STAR*D study suggests that a subset of patients started on citalopram that fail to respond see some improvement with the addition of a second drug (48). We are in a position to test such "combination therapies" in our simple model system.

C6 cells were cultured in DMEM, 4.5 g of glucose/L, 10% newborn calf serum (Hyclone Laboratories, Logan, UT), 100 mg/mL bacteriostatic penicillin-streptomycin at 37 °C in humidified 5% CO_2 atmosphere to a confluence of ~40% before chronic treatments were begun. Treatment with 10 μM antidepressant for 72 hrs is a standard assay condition (152) and parallels doses used in rat studies (139, 165), even though these drugs are effective at concentrations as low as 50 nM over the same period (160). This is ~ 2x the plasma concentration seen after a 20 mg/day dose, but closer to a biologically consistent level. Culture media and drug were changed daily and no apparent change in cell morphology occurred during treatment.

Intact cells were rinsed twice with pre-warmed 1X phosphate buffered saline (PBS) to remove debris and wash away unbound drugs. C6 cells were stimulated with 0, 0.01, 0.1, 1, and 10 μM escitalopram for dose response curve generation and 10 μM escitalopram for temporal stimulation at 0 hr, 3 hr, 12 hr, 24 hr, 48 hr, 72 hr, and 120 hr; 100 nM escitalopram for 120 hrs was also tested; R-citalopram served as the control (152, 166, 167).

2.5 GFP tagging

Fluorescent tagging of proteins, particularly with GFP, has proven to be a powerful tool in analyzing the localization and trafficking of different proteins in live cell imaging. We have generated a GFP tagged $G\alpha_s$ construct that behaves much the same as untagged wild type $G\alpha_s$ when transfected into C6 cells and treated with antidepressants (moves out of lipid rafts) (168). Importantly, $G\alpha_s$-GFP however accumulates in detergent resistant membrane fractions, whereas endogenous $G\alpha_s$ predominates in the buoyant fraction with lipid rafts. We have thus modified the GFP tag to be monomeric, according to published methods (169). We have verified the oligomeric state of $G\alpha_s$-GFP through decreased accumulation in the detergent resistant membrane fractions, similar to endogenous $G\alpha_s$, and observing enhanced membrane localization of $G\alpha_s$-GFP. Our lab has constructed acylation mutants in the N-terminus of $G\alpha_s$-GFP at Cys3Ser and Asn6Ser residues to affect the palmitoylation and myristoylation state respectively. Modification of Cys3 impairs palmitoylation of $G\alpha_s$ and mutation of Asn6Ser provides the recognition sequence necessary for myristoylation (170) of the nascent Gly2 residue of $G\alpha_s$. These acylation mutant $G\alpha_s$ constructs are invaluable tools for evaluating the effects of antidepressants on the localization and associations of $G\alpha_s$. When kept to a moderate level of expression (2 to 3 fold that of endogenous $G\alpha_s$), the expression of GFP-$G\alpha_s$ is transparent to cellular physiology while allowing a window on the movements of $G\alpha_s$ (159).

2.6 Lipid Raft Isolation

Cells were washed and harvested in ice-cold 1X PBS. Lipid raft fractions were prepared as previously described with minor modification (171). C6 cells were scraped into 0.75 mL of HEPES buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, and protease inhibitors) containing 1% TX-100. Samples were homogenized and mixed 1:1 v/v with an 80% sucrose HEPES buffer, and loaded into an ultracentrifuge tube. A sucrose gradient was sequentially layering 30, 15, and 5% over the homogenate and centrifuged at 200,000xG for 20 hrs in an SW55 rotor (Beckman, Palo Alto, CA). Lipid raft bands exist between 15 and 30% sucrose layers (171). 500 μL fractions were collected from the top down into 1.5 mL ultrafuge tubes, diluted 3:1 in HEPES buffer, and pelleted at 20,000xG. Pellets were resuspended in HEPES buffer and analyzed by western blot.

Alternatively, treated C6 cells were separated by Triton-X100/114 (Tx100/114). The ratio of $G\alpha_s$ in Tx-100 (non-raft) vs. Tx-114 (raft) extracts is comparable to sucrose gradient preparation, but offers a much higher throughput (139). Cells were pelleted, washed with 1X PBS, lysed in TME buffer (10 mM Tris-HCl, 1 mM MgCl2, 1 mM EDTA, pH 7.5, protease inhibitors), and centrifuged at 100,000xG for 30 min. at 4°C to pellet membranes. Cytosolic supernatant was aspirated and saved. Membrane pellets were resuspended and rotated in TME containing 1% Tx-100 and 150 mM NaCl, for 30 min. at 4°C. Membranes were pelleted at 100,000xG for 30 min. at 4°C (Tx-100 non-raft fraction). Pellets were resuspended and homogenized in TME containing 1% Tx-114 and 150mM NaCl at 4°C (Tx-114 lipid raft fraction). All procedures are carried out under ice-cold conditions.

2.7 Accumulation of Antidepressants measured by UV-Vis

Similar to protocols for determining tableting efficiency (172), the ratio of escitalopram absorbance at 238 nm (S- and R-citalopram) was normalized to protein absorbance at 280 nm. Eisensamer and colleagues observed the accumulation of antidepressants and other psychoactive drugs in membranes by spiking membrane fractions with known concentrations of drug and subjecting them to HPLC (173); detection is through their characteristic absorbance. Although minimized by column chromatography, biomolecular absorbance may obscure any "drug" readings detected. In the case of citalopram, peptide bond absorbance (190-220 nm) likely obscures its absorbance maximum at 238 nm. This is why I have normalized my measurements to protein content (280 nm) and point out that the reported values are only useful in qualitatively suggesting drug presence.

The UV absorbance of antidepressants was used to determine their association with membrane fractions as before (173), with modifications. C6 cells chronically treated (72 hrs) with 10 µM escitalopram, R-citalopram, fluoxetine, desipramine, phenelzine, or olanzapine were extracted by Tx100/114 and the cytosolic, non-raft membrane, and lipid raft fractions analyzed by UV absorbance and normalized to protein content (λ = 280 nm). Furthermore, 500 µL sucrose density gradient fractions were spiked with a final concentration of 10 µM escitalopram or R-citalopram. S- and R-citalopram absorbance (λ = 238 nm) in each fraction was assessed before and after spiking, measurements normalized to protein and blanked. The drug absorbance units per mg protein (238/280 ratio) suggests antidepressant accumulation in lipid rafts.

2.8 Antidepressant drug hydrophobicity

Partition coefficients of drugs were determined as previously described (174) in a 1:1 v/v octanol to ddH$_2$O and the UV-Vis spectrum recorded for each phase. If the distribution of a drug in this system predominates in the octanol phase, it is more hydrophobic and if the drug predominates in the water phase, it is more hydrophilic. The mixtures were prepared with 100 nmol of drug in a total volume of 210 μL (0.48 mM), vortexed 3 X 20s, and centrifuged at 2000 x G to separate the phases; octanol has a density of 824 kg/m^3 and water is 999.97 kg/m^3.

The UV-Vis spectrum was then taken for each phase (n=3). Absorbances: phenelzine (256 nm), desipramine (252 nm), fluoxetine (226 nm), citalopram (238 nm), or olanzapine (270 nm). The partition coefficients were calculated using:

$$\log P_{\text{oct/wat}} = \log \left(\frac{[\text{solute}]_{\text{octanol}}^{\text{un-ionized}}}{[\text{solute}]_{\text{water}}^{\text{un-ionized}}} \right)$$

2.9 Accumulation of Antidepressants measured by Gas Chromatography Mass Spectrometry (GC-MS)

The accumulation of antidepressants in lipid rafts and non-raft membranes of C6 glioma cells was measured via GC/MS to accompany results obtained via increases in the UV absorbance spectrum for escitalopram as opposed to R-citalopram. C6 cells were chronically treated (72 hrs) with 10 μM escitalopram, R-citalopram, fluoxetine, desipramine, phenelzine, or olanzapine. More elaborate concentration and temporal measurements were restricted to escitalopram. The accumulation of increasing concentrations, 10 nM to 10 μM, of escitalopram over 72 hrs, as well as temporally from 3-120 hrs with 10 μM escitalopram was measured in lipid raft and non-raft membrane; R-citalopram served as the control.

1) Cells were trypsinized and pelleted at 1500 rpm. Membranes were fractionated into Tx-100 soluble and Tx-114 soluble fractions. The ratio of $G\alpha_s$ in Triton X-100 (non-raft) vs. Triton X-114 (raft) extracts is comparable to sucrose gradient preparation (30, 15, and 5%), where rafts exist between the 15 and 30% sucrose layers (171), but offers a much higher throughput (139, 151). Sucrose gradients, however, are more sensitive and more likely to detect subtle changes in raft localization. I measured the accumulation of antidepressants in lipid rafts and non-raft membranes of C6 glioma cells. I further measured the accumulation of increasing concentrations, from 10 nM to 10 μM, of escitalopram for 3 days in order to be consistent with plasma concentrations and studies done in cells and animals; R-citalopram served as the control.

2) Extraction of accumulated antidepressant drugs in lipid rafts (Tx-114 fraction) may be assessed on large volume samples as previously described (175), but is not appropriate for small volumes here. Extraction of antidepressants from membrane structures, in order, with ammonium hydroxide, n-butyl chloride/ethyl ether, 2N sulfuric acid, hexane, ammonium hydroxide, and butyl acetate failed as the elimination of leftover lipid and/or cholesterol emulsions by the addition of excess ethyl ether following the n-butyl chloride/ethyl ether extraction step, likely removed the drugs as well. Membrane fractions were chloroform-methanol precipitated as previously described (176) and the water, chloroform, and methanol phases vacuum centrifuged to recover accumulated drug. Desiccant was dissolved into 1 mL of methanol for direct injection onto an Agilent capillary column.

3) GC-MS analyses were performed using an Agilent HP-6890 gas chromatograph, equipped with an Agilent 19091S-602 HP-1MS capillary column (25 m, 0.20 mm, 0.33 µm, 7 inch cage), and interfaced with an Agilent HP-5973 mass selective detection (MSD) spectrometer equipped with a Single Flame Ionization Detector, Single 100 psi EPC Split/Splitless Injection Ports, 7673C-6890 Auto sampler: 6890 Control Electronics, 6890 Injector, 100 Position Tray and 6890 Mounting Bracket. Helium was used as the carrier gas at 1.0 mL/min in corrected constant flow mode. Primary oven temperature was programmed at 70 °C for 2 min. and increased at 20 °C/min to 230 °C where it was held for 10 min. The front inlet thermal modulator was set to 20 °C higher relative to the primary oven and 18.91 psi. Constant flow injection of 1 µL was used and inject split mode to splitless. The injector, transfer line, and ion source temperatures were

maintained at 250, 280, and 230 °C, respectively, throughout each analysis. Data

acquisition was performed in the full scan mode from m/z 50 to 550 with an acquisition

rate of 20 Hz. Molecular ion profiles (MIP) were matched against the standard mass

spectral database of the National Institute of Standards and Technology (NIST).

2.10 Nanobody expression and purification

Nanobodies are single chain antibodies derived from the variable domains of various species of the *Camelidae* family, such as Camels, Llamas, and Alpacas (camelid antibodies). Their relatively small size and high affinity make them ideal for use in experiments where a larger antibody might sterically restrict an interaction(s). Moreover, the lack of an Fc portion in their structure prevents activation of compliment and possibly detection of proteins not actually in complex with the protein of interest. These characteristics make them ideal for protein-protein interaction study.

We have received expression vectors for camelid nanobodies that recognize different regions of $G\alpha_s$ from Dr. Brian Kobilka at Stanford University. NB35 specifically recognizes the GTP binding domain of $G\alpha_s$, and NB37 the alpha helical domain of $G\alpha_s$ (164). I expressed nanobody constructs in BL21 DE3 *E. coli* cells and purified them to relative homogeneity (Figure 3). Incubation of transformed BL21 DE3 *E. coli* cells, containing NB35, NB37, or His-$G\alpha_s$ expression constructs, were grown with 100 µg/mL ampicillin at 37°C and 220 rpm until an OD_{600} of 0.8. I cooled cultures on ice and induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C for 16 additional hours. I then harvested cells by centrifugation at 4°C, for 10 min at 7000 rpm (15min., 6000rpm for 1 L bottles). Supernatants were discarded and 15 mL TES buffer (0.2 M Tris pH 8, 0.5 mM EDTA, 0.5 M sucrose) was added/pellet of 1L culture and shook for 1 hour on ice. Add 30 mL of TES/4 / pellet of 1L culture and shaken for another 45 min on ice. Cell debris was removed by centrifugation for 30 min at 8000 x G, discarded, and the lysate was added to a 1 mL bed of 50% Ni^{+} NTA slurry in 1X PBS and rotated at 25°C for 1 hour. I next transferred bound resin to a column and washed

with one column volume (10 mL) of phosphate buffer1 (50 mM Na2HPO4, 1M NaCl, pH 7), 3 column volumes (30 mL) of phosphate buffer 2 (50 mM NaH_2PO_4 1M NaCl, pH 6), and eluted with 200 mM imidazole in phosphate buffer 2. Recombinant proteins were dialyzed overnight into 1X PBS, the OD_{280} measured, and stored at 4^oC.

Figure 3. General Nanobody structure and NB35 precipitates Gα$_s$.

Nb35:
MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLVQPGGSLRLSCAASGFTFSNY**K**MNWVRQAPG**K**GL
EWVSDISQSGASISYTGSV**K**GRFTISRDNA**K**NTLYLQMNSL**K**PEDTAVYYCARCPAPFTRDCFDVTSTTYA
YRGQGTQVTVSSHHHHHH
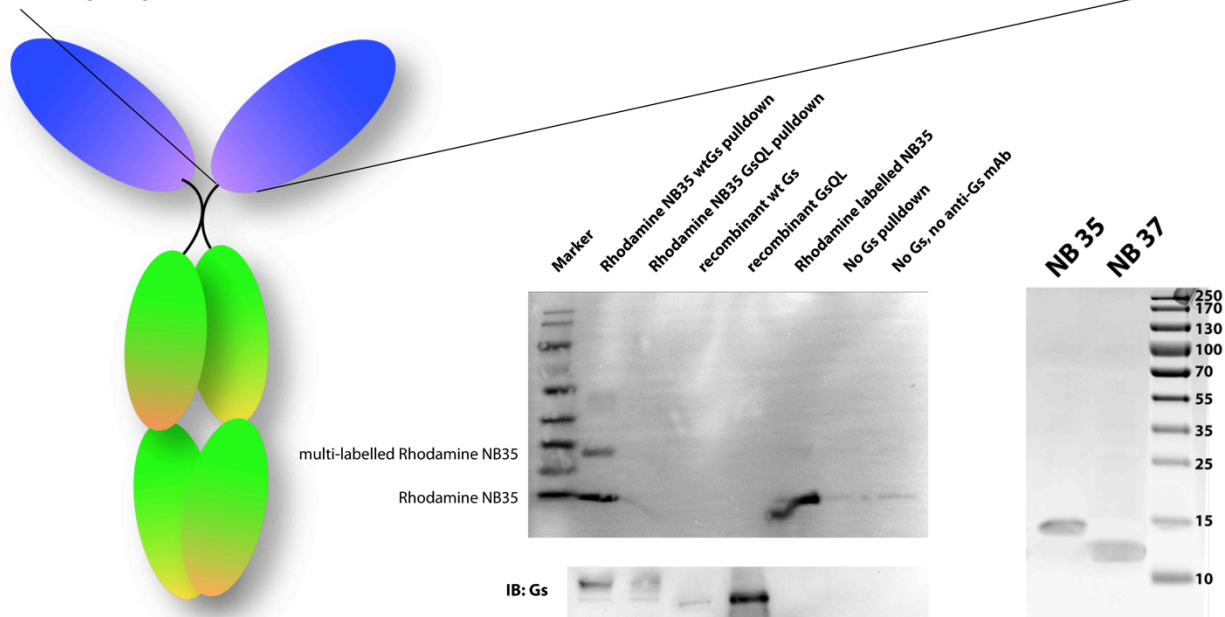
NB35 was purified to apparent homogeneity and binds purified Gα$_s$. NB35 was labeled with NHS-rhodamine, potential sites are indicated in red in the above sequence, and *in vitro* binding (1:1 density ratio) with recombinant purified Gα$_s$, both native and constitutively active Gα$_s$QL, occurred in 1X PBS. Immunoprecipitation occurred via anti-Gα$_s$ mAb and binding was confirmed through visible red band for Rhodamine-NB35 (presented in gray scale) and Gα$_s$ by immunoblotting.

2.11 Gα$_s$ immunoprecipitation and Binding Partner Identification

Immunoprecipitation of Gα$_s$ complexes with 5 µg total NB35 that specifically detects conformationally active (GTP bound) states of Gα$_s$ (164) was conducted on 1 mg total membrane protein from lipid rafts derived from parent, Gα$_s$-GFP, palmitoylation deficient Gα$_s$-GFP, and myristoylated/palmitoylated Gα$_s$-GFP C6 cells that were treatment naïve, chronically treated with 10 µM escitalopram, or 10 µM desipramine. C6 cells were treated with the water-soluble and membrane permeable reversible crosslinking agent 3,3'-dithiobis-succinimidyl propionate (DSP); crosslinking was terminated with Tris-HCl. Gα$_s$ immunoprecipitates from lipid rafts of parent as well as GFP acylation mutants were digested with 1 µg Trypsin and subjected to strong cation exchange and reverse phase liquid chromatography followed by electrospray ionization on a Thermo Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer with peptide mass tolerance of ± 10 ppm and fragment mass tolerance of ± 0.6 Da.

2.11.1 Database Searching

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped. I analyzed all MS/MS samples using Mascot (Matrix Science, London, UK; version 2.2.07). I conducted Mascot searches of the SwissProt_57.15 database (selected for Rattus, 7497 entries) with the digestion enzyme set to trypsin, a fragment ion mass tolerance of 0.60 Da, and a parent ion tolerance of 10.0 ppm. I specified carbamidomethyl cysteine as a fixed modification and deamination of asparagine and glutamine as a variable modification in Mascot.

2.11.2 Criteria for Protein Identification

I used Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Peptide Prophet algorithm (177) with Scaffold delta-mass correction. I accepted protein identifications if established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (178). I grouped proteins that contained similar peptides and not differentiated based on MS/MS analysis alone to satisfy the principles of parsimony.

2.12 Gα$_s$ Acylation Determination

Lipid rafts were isolated from C6 cells chronically treated (72h) with 10 μM phenelzine, desipramine, fluoxetine, S-citalopram, R-citalopram, or olanzapine via sucrose density gradient. Gα$_s$ was immunoprecipitated with an anti-Gα$_s$ monoclonal antibody and the extent of palmitoylation determined by LC-MS. All immunoprecipitations were digested with 0.5 μg Trypsin and prepared as previously described (179), without radiolabelled N-ethylmaleimide (NEM). Thus, peptide identification must be accomplished via peak identification in the LC-MS spectrum (93). Briefly, protein samples were treated as follows:

1) Free sulfhydryls were covalently blocked with 100 mM NEM and unreacted NEM removed with a 10K molecular weight cutoff (MWCO) spin filter to prevent undesired side reactions; the resulting S-C linkage is very stable (180, 181).

2) Samples were reduced with 1M hydroxylamine; Cys-palmitoyl is reduced whereas Cys-NEM is not reducible with hydroxylamine (180). Importantly, a sample omitting hydroxylamine was kept to control for false positive detection. Unreacted hydroxylamine was removed with a 10K MWCO spin filter to prevent undesired effects on precipitating antibodies.

3) Samples were precleared with sepharose A resin and immunoprecipitated with 2 μg of an anti-Gα$_s$ monoclonal antibody (UC Davis/NIH NeuroMab Facility clone N192/12). It is important to immunoprecipitate Gα$_s$ only after treatment of samples with NEM and hydroxylamine as immunoprecipitation may remove palmitoylation.

4) Immunoprecipitations were washed and digested with 1 μg Trypsin. Peptides were acidified with 0.1% Formic acid, column bound and desalted with C18 zip-tips (Millipore®) in 2% CH_3CN, and eluted with 40% CH_3CN.

5) Palmitoylation of $G\alpha_s$ (free sulfhydryl present at Cys3) in the plasma membrane fraction was analyzed on a Thermo Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer.

2.12.1 Liquid Chromatography Mass Spectrometry (LC-MS)

Chromatographic separation of peptides was accomplished by gradient elution on an Agilent 1200 binary HPLC coupled to a Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was conducted using a ZORBAX 300SB-C18 microbore column (5 × 0.3 mm ID, 5 μm; Agilent Technologies, Santa Clara, CA) at 4 °C. Peptides were detected by full-scan mass analysis from *m/z* 400 to 1800 at a resolving power of 30,000 (at m/z 400, full width at half maximum [FWHM]) and followed by data-dependent multiple stage mass analysis at a resolving power of 7500, which was triggered by the most abundant ions from a parent list of triply-, doubly-, and singly-charged peptides at a flow rate of 250 nL min^{-1} into the ESI source. CID was conducted with an isolation width of 3 Da, normalized collision energy of 30%, and an activation time of 10 millisec. Data acquisition and reduction was carried out using Xcalibur version 2.1 (Thermo Fisher Scientific, Bremen, Germany).

2.13 Antidepressant mediated Conformational Change of $G\alpha_s$

Binding of Fluoride to Gα-GDP mimics the GTP bound conformational state (182, 183). The resulting changes to the emitted fluorescence of tryptophan residues in the switch domains of $G\alpha_s$ indicate the conformational viability of a recombinant Gα construct. I incubated 200 nM $G\alpha_s$ at room temperature in 1x PBS alone, with 10 mM NaF and 30 µM $AlCl_3$, or with 10 µM antidepressant. I measured fluorescence in a 96 well plate with excitation at 280 nm and emission at 340 nm. Fluorescence increase is expressed as a percent change of the initial fluorescence (F_o): $\Delta F\ (\%) = (F - F_o)/F_o \times 100\%$.

2.14 Statistical Analysis

I present all measurements as the mean (n=3) ± standard error of the mean (SEM) and propagate calculation error throughout each calculation $\sqrt{[(SEM_1)^2+(SEM_2)^2]}$ = SEM_N for addition/subtraction calculations and $\sqrt{[(SEM_1/M_1)^2+(SEM_2/M_2)^2]} * |M_N|$ = SEM_N for multiplication/division calculations (M_X: measurement). I further subjected each data set to statistical analyses using GraphPad Prism (version 5.0), using a one-way analysis of variance (ANOVA) followed by a post-hoc Student's t-test (two groups) or Dunnett's t-test (multiple groups) (95% C.I.).

Chapter 3 – Results

AIM 1: Determine the molecular associations/mechanisms that modulate Gα$_S$ translocation from lipid rafts under chronic antidepressant treatment *in vitro*.

Aim 1A. Gradual accumulation of antidepressant drugs in plasma membrane microdomains correlates with Gα$_s$ subcellular localization.

3.1.1 Background

  Chronic treatment with antidepressant drugs mediates Gα$_s$ translocation from

lipid rafts to non-raft regions of the plasma membrane, which is likely through drug

specific mechanisms. Initially, I hypothesized that the activation by chronic

antidepressant treatment of GPCRs coupled to Gα$_s$ in the lipid raft mediated the

translocation of Gα$_s$. Since the only known modes of action of currently available

antidepressants are either through inhibiting the catabolism of serotonin and

norepinephrine (MAOIs) or through inhibiting their reuptake in the presynaptic bulb

(TCAs, SSRIs, SNRIs, and NaSSAs). It necessarily follows then that monoamine

oxidase (MAO) or the serotonin (SERT) or norepinephrine (NET) reuptake transporter is

present and somehow coupled with Gα$_s$. However, the hysteresis in their action

suggests that an alternative target exists. Treatment of HEK293 cells, transfected with

the serotonin 3A receptor (HEK-5-HT$_{3A}$), with a number of antidepressant and

psychoactive compounds (fluoxetine, fluphenazine, clozapine, and haloperidol) reveals

a concentration of those compounds in lipid rafts (173). Thus, the gradual accumulation

of antidepressants in lipid rafts may be a possible causative occurrence mediating Gα$_s$

translocation out of lipid rafts. However, this is not a perfect correlation, as

phenothiazine antipsychotics, which do not alter Gα$_s$ raft association or coupling to

adenylyl cyclase, also appear to concentrate in lipid rafts. Moreover, the only

antidepressant assayed that did accumulate in lipid rafts was fluoxetine (SSRI); reboxetine (NET), mirtazapine (NaSSA), and moclobemide (MAOI) did not. The lack of accumulation in lipid rafts though is likely due to a deficiency in the method of detection rather than a lack of accumulation.

I hypothesized that antidepressants preferentially associate with rafts, which correlates with the degree to which they mediate the mobility of $G\alpha_s$ out of lipid rafts. It is possible that the active sites for some antidepressants are downstream from their membrane binding sites. However, I still expected to see an accumulation in either the lipid raft or the non-raft regions of the membrane unless the drug is able to translocate and bind an intracellular target. Moreover, different/multiple mechanisms are likely to exist for the actions of different antidepressants. For example, tricyclics may require Arrestin and Spinophilin for certain actions whereas SSRIs do not (184). Regardless, chronic treatment with each antidepressant examined thus far has the effect of moving $G\alpha_s$ from lipid rafts, but this does not imply a single mechanism of action. Rather, it suggests that antidepressants have a similar molecular footprint that to exploit for the purposes of diagnostics. For these reasons, I assessed the accumulation of representative drugs from each antidepressant class MAOI (phenelzine), SSRI (escitalopram/inactive stereoisomer R-citalopram and fluoxetine), and TCA (desipramine), as well as the antipsychotic (olanzapine).

3.1.2 Results

I used C6 glioma cells for accumulation of antidepressant drug experiments because they do not express reuptake transport proteins, yet still respond to antidepressant treatments. Based upon a previous report by Eisensamer et. al. (173), I expected that antidepressants would gradually accumulate in raft fractions of C6 cells over time. Repeating this experiment without HPLC purification, using an analogous detection method (UV absorbance), but treating cells instead of spiking membranes, I assessed the accumulation of different antidepressants. Treatment of C6 cells with different antidepressants and subsequently measuring the protein normalized and blanked absorbance specific to each drug, it was observed that all drugs, with varying degrees of efficiency, might accumulate over time in the membrane (Figure 4), but that escitalopram and possibly phenelzine and fluoxetine are able to accumulate in the lipid raft fraction (Figure 5). In this case, only the method of detection is similar, as I treated cells over 72 hrs, whereas Eisensamer and colleagues spiked prepared membranes with known concentrations of drug.

To parallel the experiments by Eisensamer and colleagues, I chose escitalopram for spiking prepared membranes because it accumulated to a significantly larger degree compared with other antidepressants and R-citalopram is available as a negative control. Escitalopram is the active isomer and R-citalopram the inactive and our lab has previously demonstrated the difference between escitalopram and R-citalopram as well as establish the most effective treatment conditions of C6 cells: 10 uM of drug for three days (152). Spiking of membrane fractions from C6 cells showed escitalopram, but not R-citalopram associates with lipid raft fractions of the plasma membrane in fractions

corresponding to Caveolin rich lipid rafts (Figure 6); the values are presented in TABLE III. In both cases, spiking of an isolated membrane fraction is less of a physiological observation than assaying the gradual accumulation over time in treated cells. Moreover, detection of drug via its characteristic absorbance is fraught with inconsistency as other biological absorbance might obscure readings and the absorbance efficiency of the drugs might be different. To minimize background measurements as much as possible for this method of detection, I normalized the readings to protein (280 nm) and subtracted the control absorbance. For these reasons, it is necessary to directly measure the presence of drug through mass spectrometry.

Figure 4. Chronic treatment of C6 cells with Antidepressants results in the accumulation of drug in the plasma membrane.
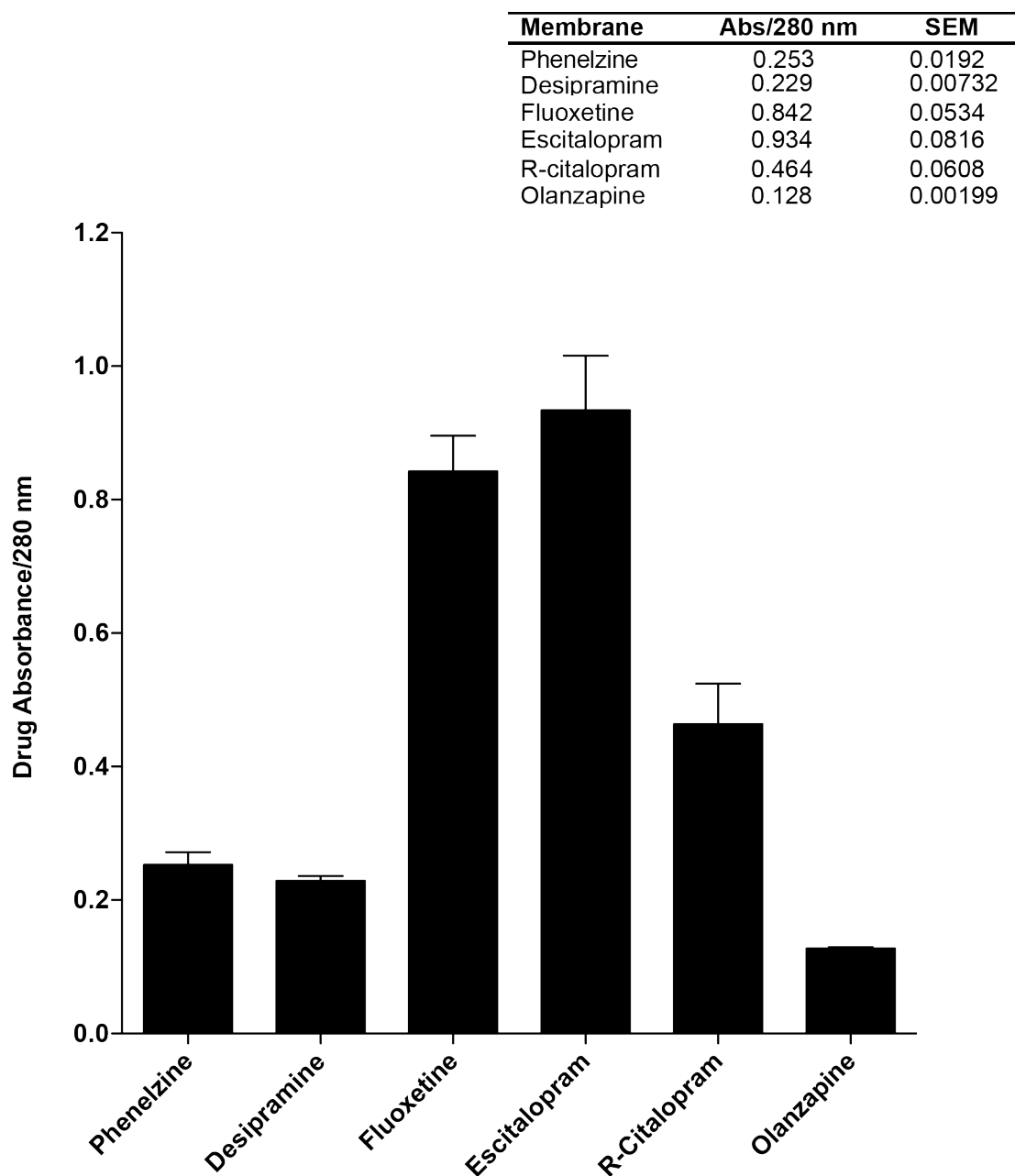
Membrane	Abs/280 nm	SEM
Phenelzine	0.253	0.0192
Desipramine	0.229	0.00732
Fluoxetine	0.842	0.0534
Escitalopram	0.934	0.0816
R-citalopram	0.464	0.0608
Olanzapine	0.128	0.00199

C6 cells were culture according to standard protocols with media changes daily. Cells were treated with 10 μM of antidepressant each day for a total of 3 days (72 hrs). UV absorbance recordings for each drug (inset Table) in prepared membranes, normalized to protein content (abs 280 nm) and blanked with a no treatment control, result in accumulated dug content (n=3).

Figure 5. Chronic treatment of C6 cells with Antidepressants results in the accumulation of drug in Lipid rafts.

Raft	Abs/280 nm	SEM
Phenelzine	0.177	0.00434
Desipramine	0.0606	0.00303
Fluoxetine	0.188	0.00885
Escitalopram	0.501	0.0197
R-citalopram	0.000	0.0194
Olanzapine	0.0535	0.000729
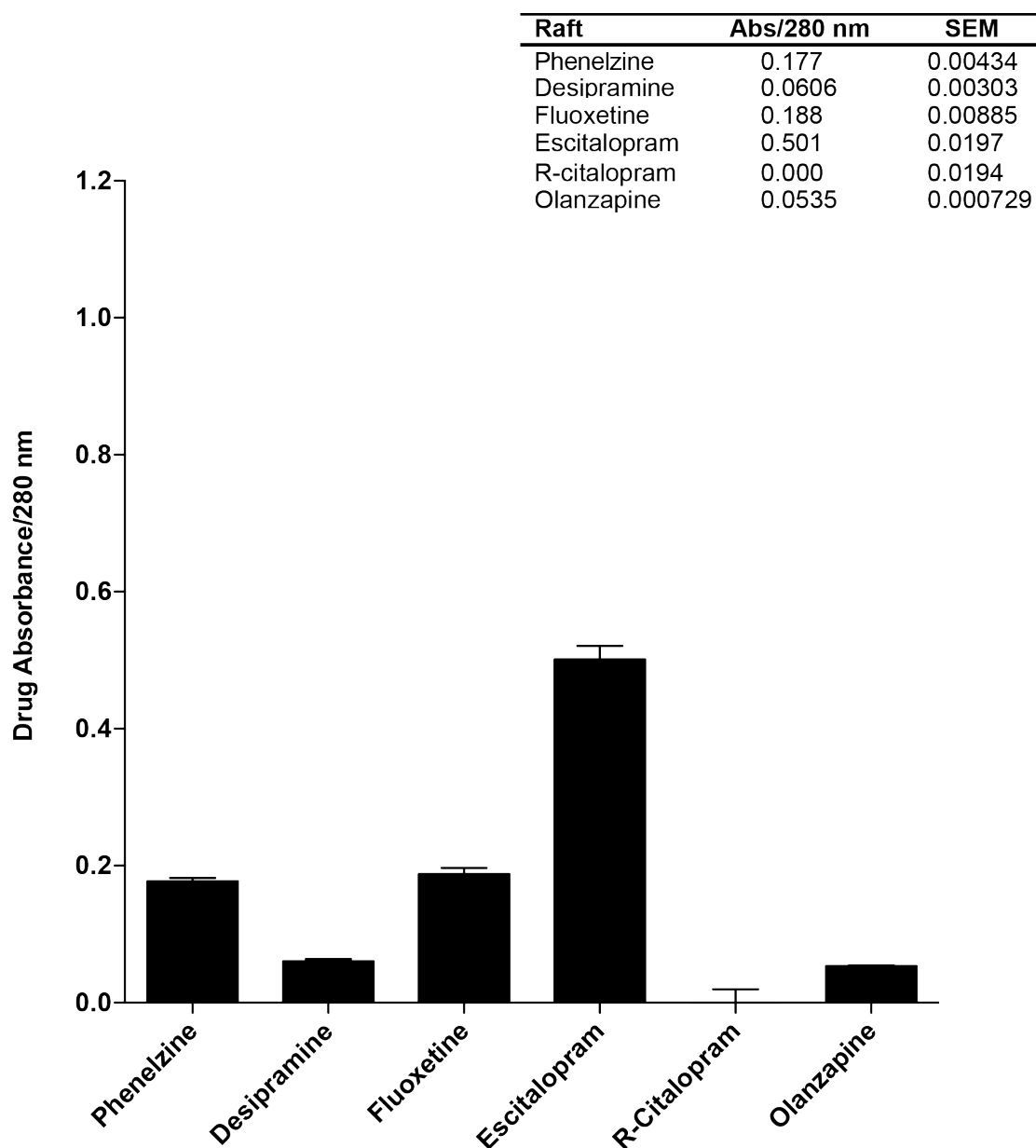
C6 cells were culture according to standard protocols with media changes daily. Cells were treated with 10 µM of antidepressant each day for a total of 3 days (72 hrs). Membranes were prepared, fractions separated by sucrose density gradient, the UV absorbance of each drug (inset Table) was recorded, and normalized to protein content (abs 280 nm). Protein normalized values were blanked with a no treatment control to result in accumulated dug content absorbance (n=3).

TABLE III. Membranes spiked with S-, but not R-citalopram, display association with Lipid Raft Fractions.

Fraction (Abs 238 nm):	Pre-Spike	R-citalopram Post-spike	SEM	Fold Change	escitalopram Post-Spike	Fold Change	SEM
1	1.66	0.76	0.14	0.46	3.22	1.94	0.49
2	1.83	0.77	0.15	0.42	8.45	4.61	1.71
3	0.67	1.47	0.69	2.20	5.80	8.70	1.76
4	0.87	1.52	0.70	1.75	4.09	4.71	1.19
5	1.25	1.48	0.29	1.18	4.05	3.22	0.45
6	1.47	1.47	0.76	1.00	2.95	2.01	0.24
7	1.49	1.55	0.66	1.04	1.82	1.22	0.22
8	1.25	1.96	0.25	1.57	1.38	1.11	0.11
9	0.67	1.65	0.63	2.45	0.69	1.02	0.24
10	1.23	1.73	0.55	1.41	1.64	1.33	0.33

Table 3: Values for spiking of sucrose gradient fractions from C6 cells with 10 µM final concentration of either the antidepressant escitalopram or the inactive stereoisomer R-citalopram. The UV absorbance at 238 nm was recorded before and after spiking in 500 µL fractions (n=3) and the change relative to unspiked control reported.

Figure 6. Membranes spiked with S-, but not R-citalopram, display association with Lipid Raft Fractions of C6 cell membranes.
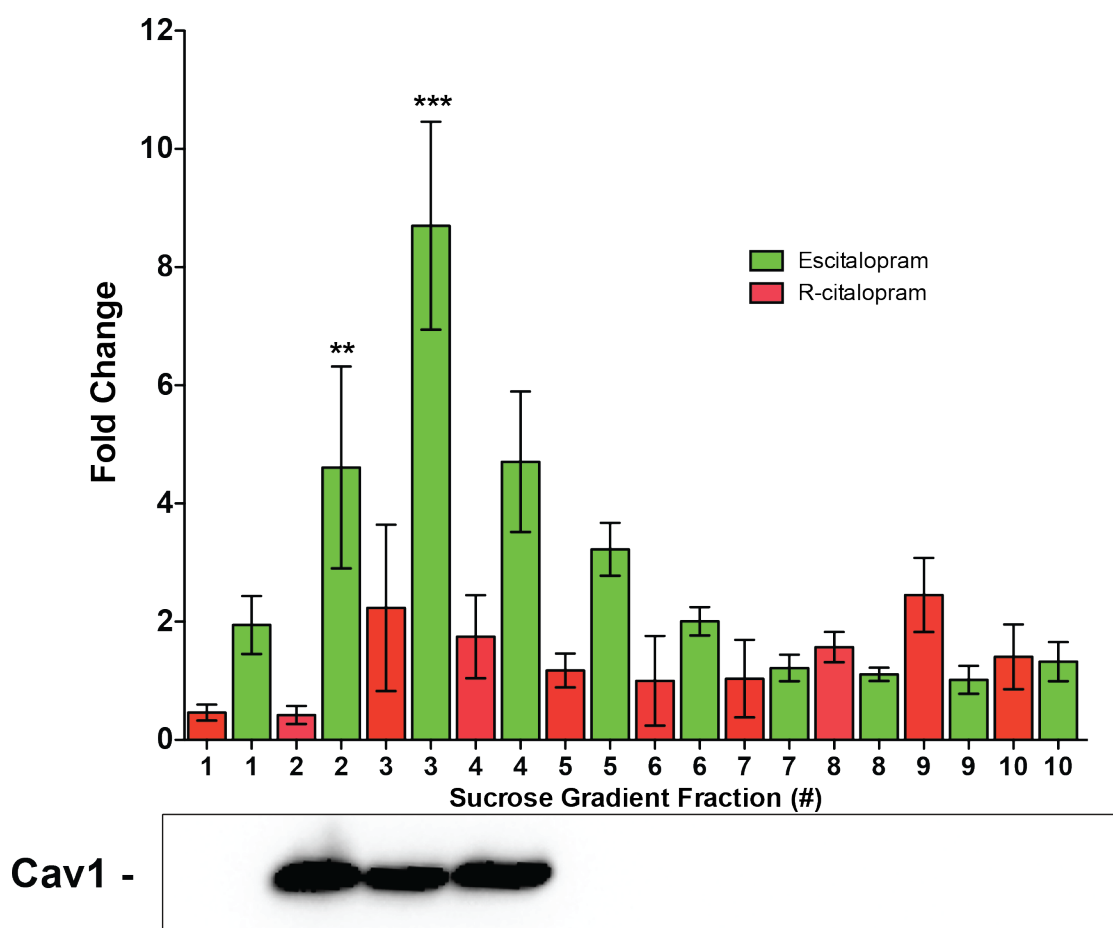
C6 cells were culture according to standard protocols with media changes daily. Membranes were prepared and fractions separated by sucrose density gradient. Five hundred µL fractions were collected and spiked with 10 µM final concentration of drug. Incubations were conducted overnight at 4°C and the membranes pelleted again. Pelleted membranes were solubilized to equivalent volumes before spiking. The UV absorbance at 238 nm was recorded before and after spiking in three separate samples and the change relative to unspiked control is reported for both Escitalopram (green) and R-citalopram (red). Fractions 2-4 where Caveolin-1 reactivity was observed also display accumulation of Escitalopram, but not R-citalopram.

GC-MS is sensitive and selective, due in large part to the separation efficiency achieved with capillary, as opposed to liquid, chromatography in the analysis of small molecules. Samples may also be analyzed by LC-MS, but the lack of ion suppression, as well as the comparatively higher separation power, makes GC-MS the preferable option for identification of small molecules. I again treated C6 cells for 72 hrs with 10 µM of antidepressant and extracted the lipid raft fraction for determination of drug presence. Somewhat surprisingly though, under chronic (72 hrs) treatment, accumulation of phenelzine (MAOI) (Figure 7) and fluoxetine (SSRI) (Figure 9) was observed in both the lipid raft and non-raft regions, whereas desipramine (TCA) (Figure 8) and olanzapine (antipsychotic) (Figure 10) did not accumulate over time. The lack of accumulation of desipramine was surprising, whereas the absence of olanzapine was less so as the former, but not the latter, mediates movement of $G\alpha_s$ out of rafts (161). However, the accumulation of drug in lipid rafts is based upon its detectable presence in the third through sixth 500 µL fractions of a sucrose gradient prepared from C6 cell homogenate and desipramine has been shown to mediate disruption of lipid raft microdomains (185). Moreover, there is a reduction in the presence of cholesterol in the desipramine treated cells (17.9 minute peak), consistent with a disruption of lipid raft integrity (Figure 8). The fact that olanzapine is so structurally similar to clozapine, which did accumulate for Eisensamer, makes this result surprising as well, but does support the assertion that **a distinct molecular target exists for each drug**. Regardless, the phenelzine (41.51 ± 4.52 µg/mg), fluoxetine (26.24 ± 1.41 µg/mg), and escitalopram (48.13 ± 5.35 µg/mg), but not desipramine, the inactive stereoisomer R-citalopram, nor the antipsychotic olanzapine accumulated in C6 lipid rafts (Figures 7-14).

Figure 7. Phenelzine accumulates in the lipid rafts of chronically treated C6 cells.
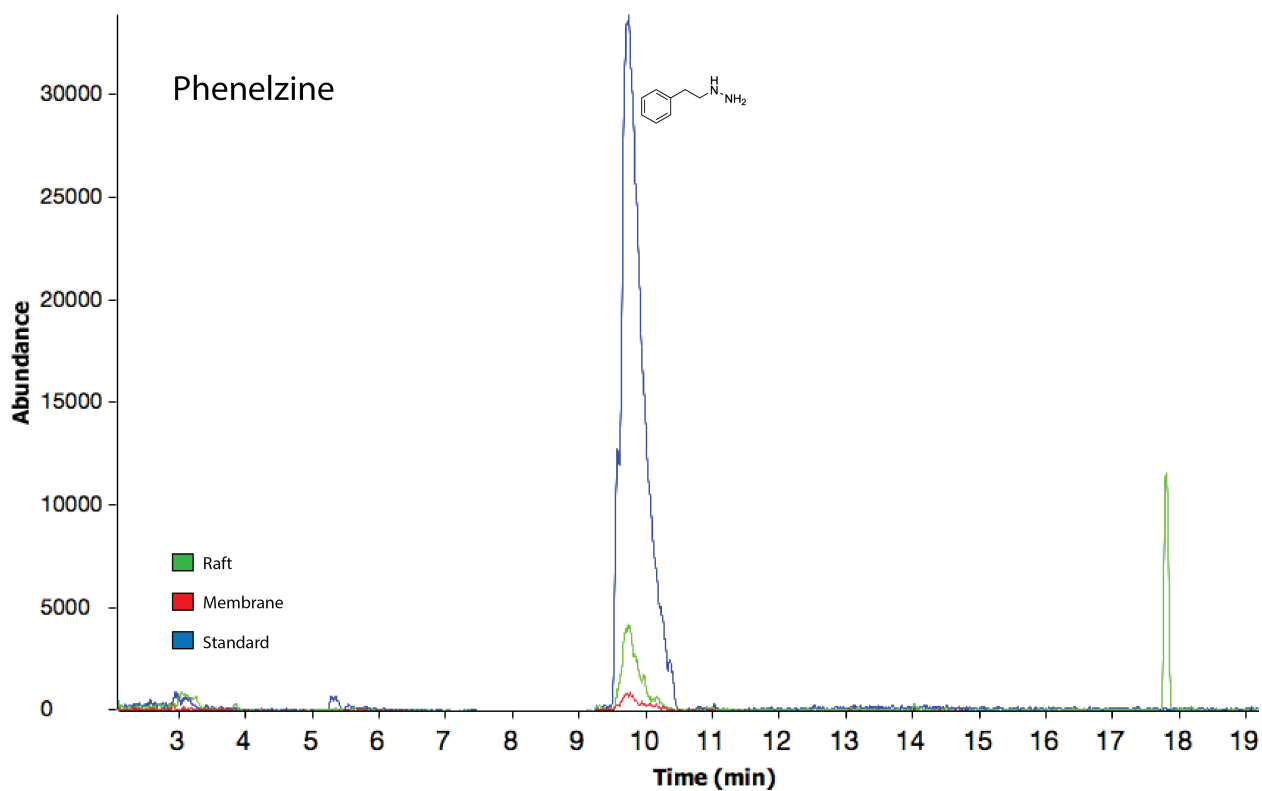
C6 cells were treated with 10 µM Phenelzine for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. Phenelzine elutes at 9.8 minutes and was detected in both the lipid raft as well as the non-raft membrane.

Figure 8. Desipramine does not accumulate in the membranes of chronically treated C6 cells.
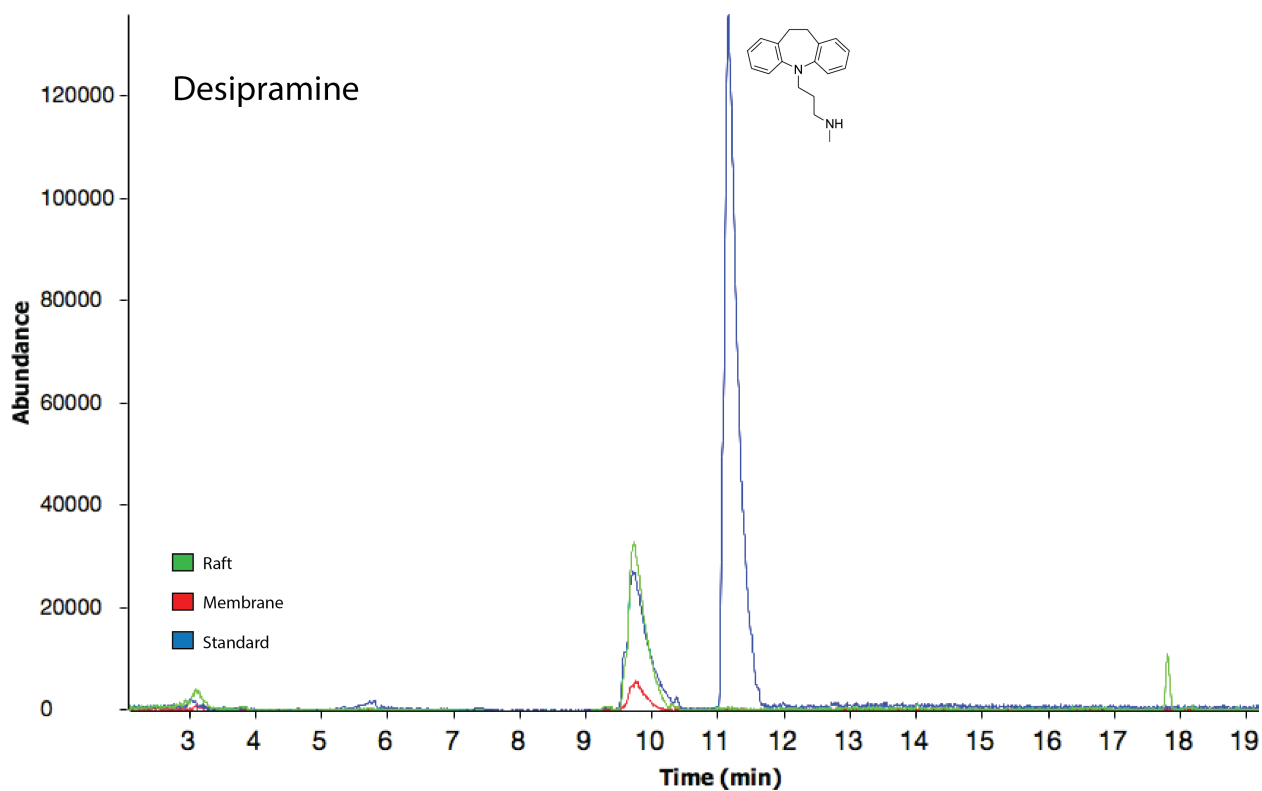
C6 cells were treated with 10 µM Desipramine for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. Desipramine elutes at 11.4 minutes, but was detected in neither the lipid raft nor the non-raft membrane.

Figure 9. Fluoxetine accumulates in the membranes of chronically treated C6 cells.
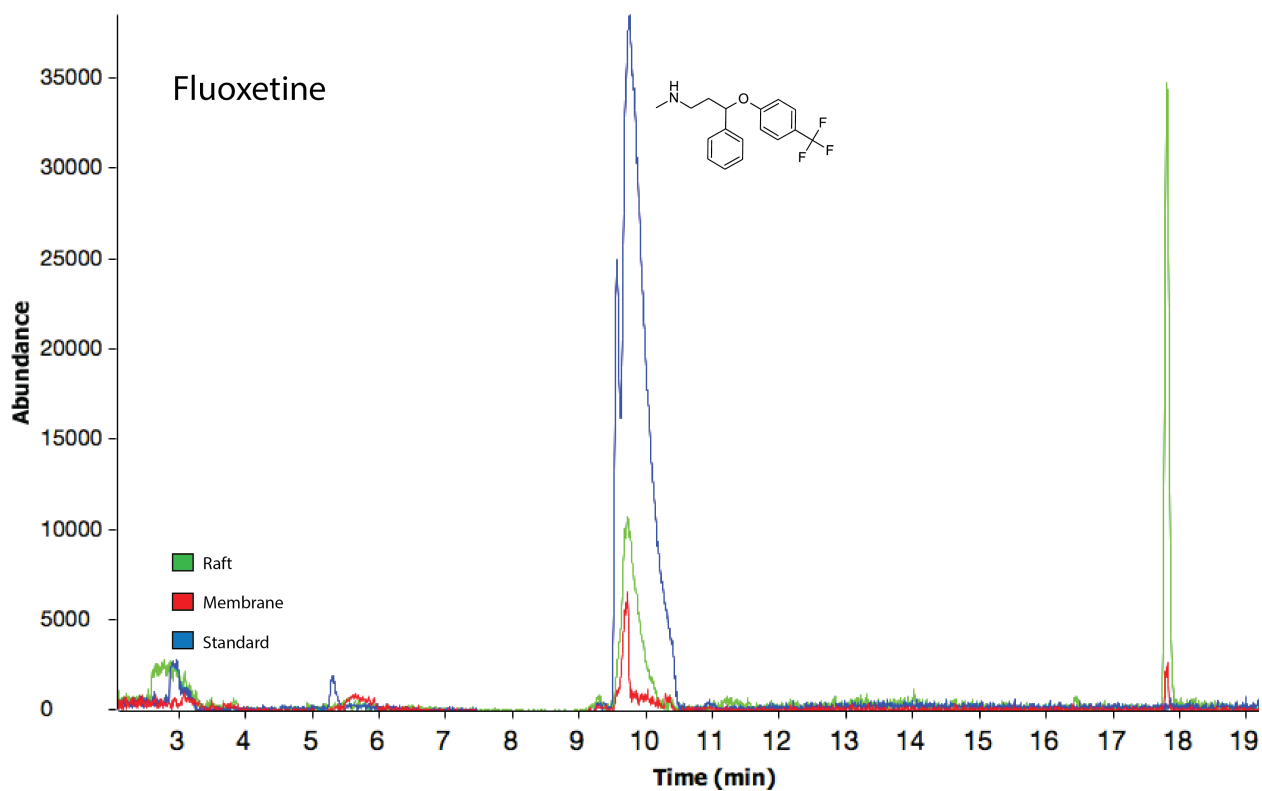
C6 cells were treated with 10 µM Fluoxetine for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. Fluoxetine elutes at 9.8 minutes and was detected in both the lipid raft as well as the non-raft membrane.

Figure 10. Olanzapine does not accumulate in the membranes of chronically treated C6 cells.
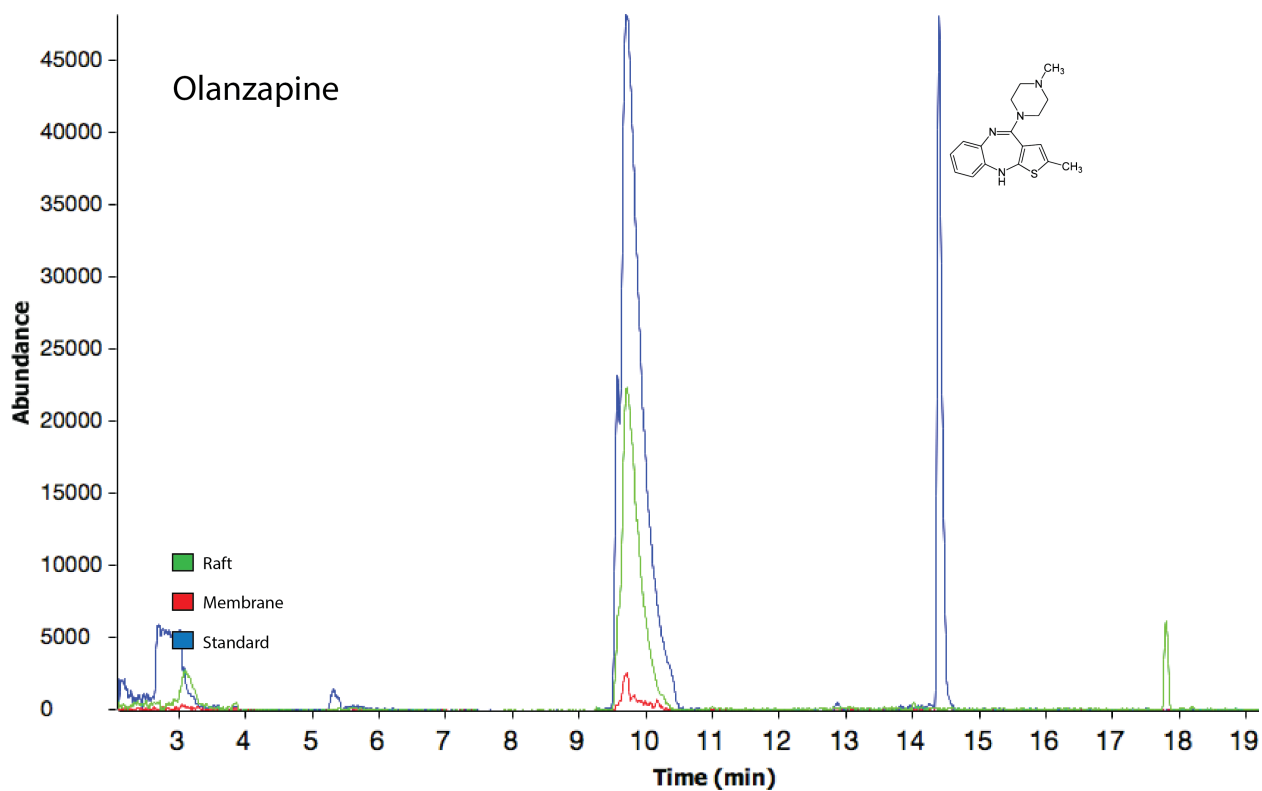
C6 cells were treated with 10 µM Olanzapine for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. Olanzapine elutes at 14.3 minutes and was detected in neither the lipid raft nor the non-raft membrane.

Figure 11. R-citalopram does not accumulate in the membranes of chronically treated C6 cells.
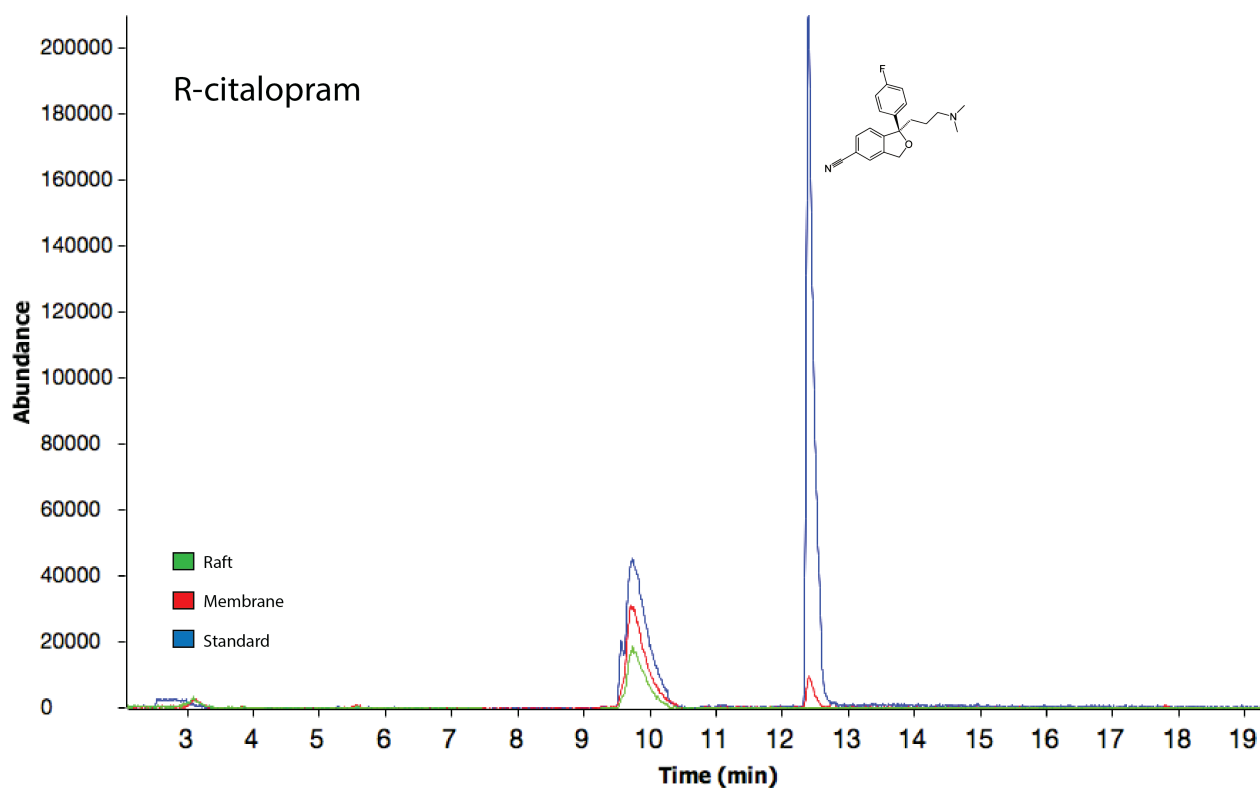
C6 cells were treated with 10 µM R-citalopram for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. R-citalopram elutes at 14.3 minutes and was detected in neither the lipid raft nor the non-raft membrane.

Figure 12. Escitalopram accumulates in the lipid rafts of chronically treated C6 cells.
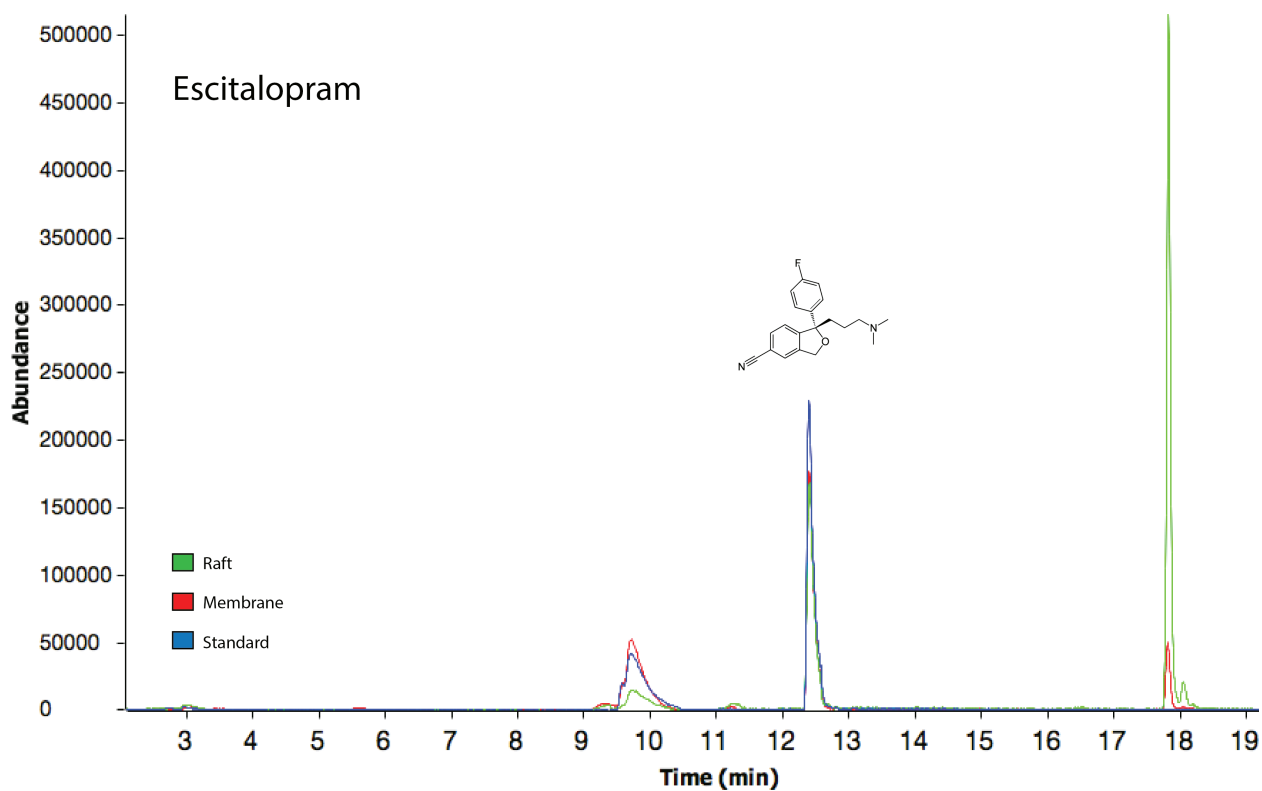
C6 cells were treated with 10 µM Escitalopram for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. Escitalopram elutes at 14.3 minutes and was detected in neither the lipid raft nor the non-raft membrane.

Figure 13. Phenelzine, fluoxetine, and escitalopram gradually accumulate in Lipid Rafts.
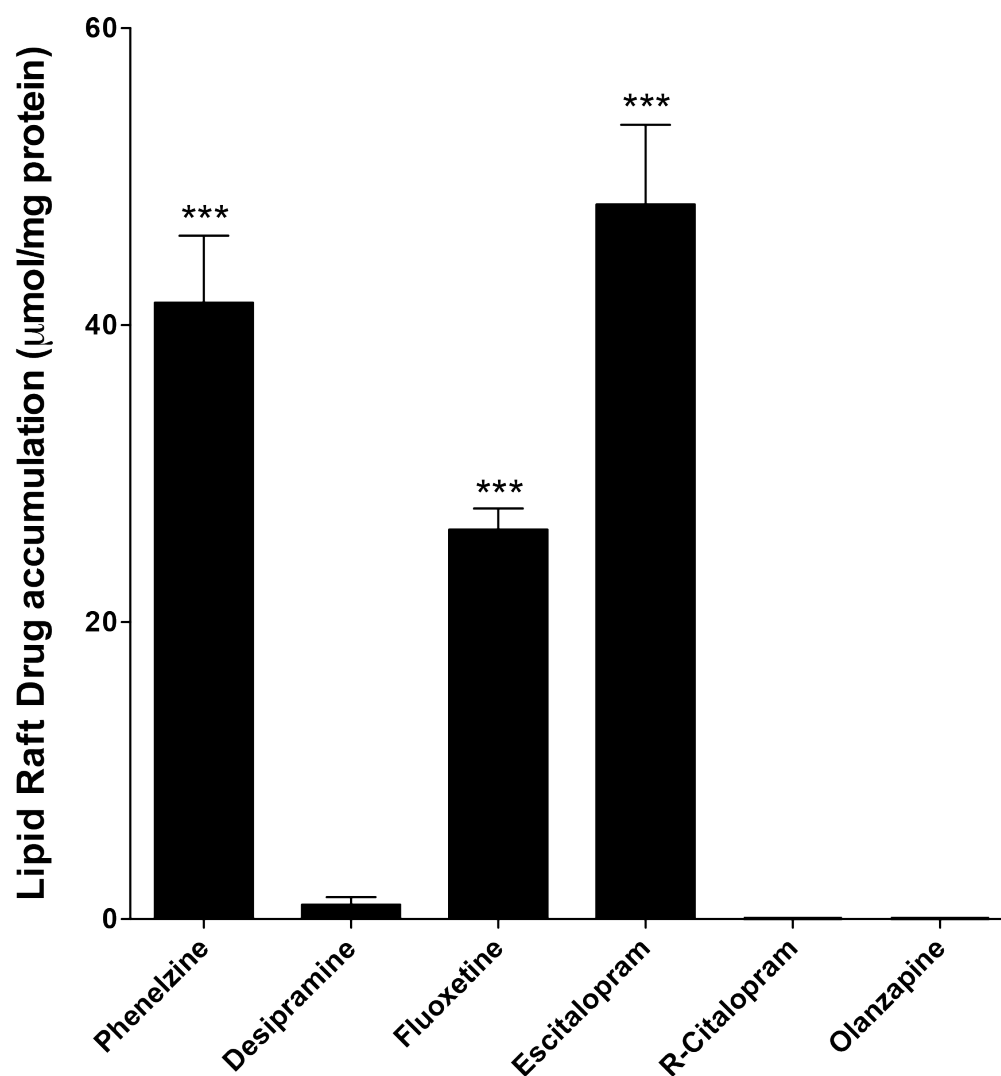
GC-MS elution peak intensities were compared with standards and values determined from a standard curve. The means peak intensities were normalized to protein content of each sample and error propagated between calculations. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc analysis of means for GC-MS quantification. Data are presented as mean ± SEM. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to vehicle).

Escitalopram, but not the inactive stereoisomer R-citalopram, accumulated in lipid raft fractions from C6 cells (Figure 11-14). As stereo-selectivity is a hallmark of proteins and no other macromolecules, the molecular target of at least escitalopram is a protein. Stereo-selective accumulation of escitalopram was further investigated due in part to the ability to compare it with the lack of accumulation in R-citalopram as well as its comparative importance, having been used as first line treatment in the STAR*D study. Escitalopram accumulates in a time and concentration dependent manner. Detectable accumulation occurred following 1 µM treatment for 72 hrs or 100 nM treatments for 120 hrs and at 24, 48, 72, and 120 hrs treatments with 10 µM escitalopram (Figure 15). There is a concern that treatment with escitalopram for 120 hrs is toxic to cells. However, images taken of C6 cells over the course of treatment with escitalopram revealed that escitalopram is in fact not toxic to cells (Figure 16). Regardless, the accumulation of escitalopram, fluoxetine, and phenelzine, but not R-citalopram and olanzapine parallels their capacity to mediate movement of $G\alpha_s$ from lipid rafts (161).

Figure 14. Escitalopram, but not its inactive stereoisomer R-citalopram, gradually accumulates in Lipid Rafts.
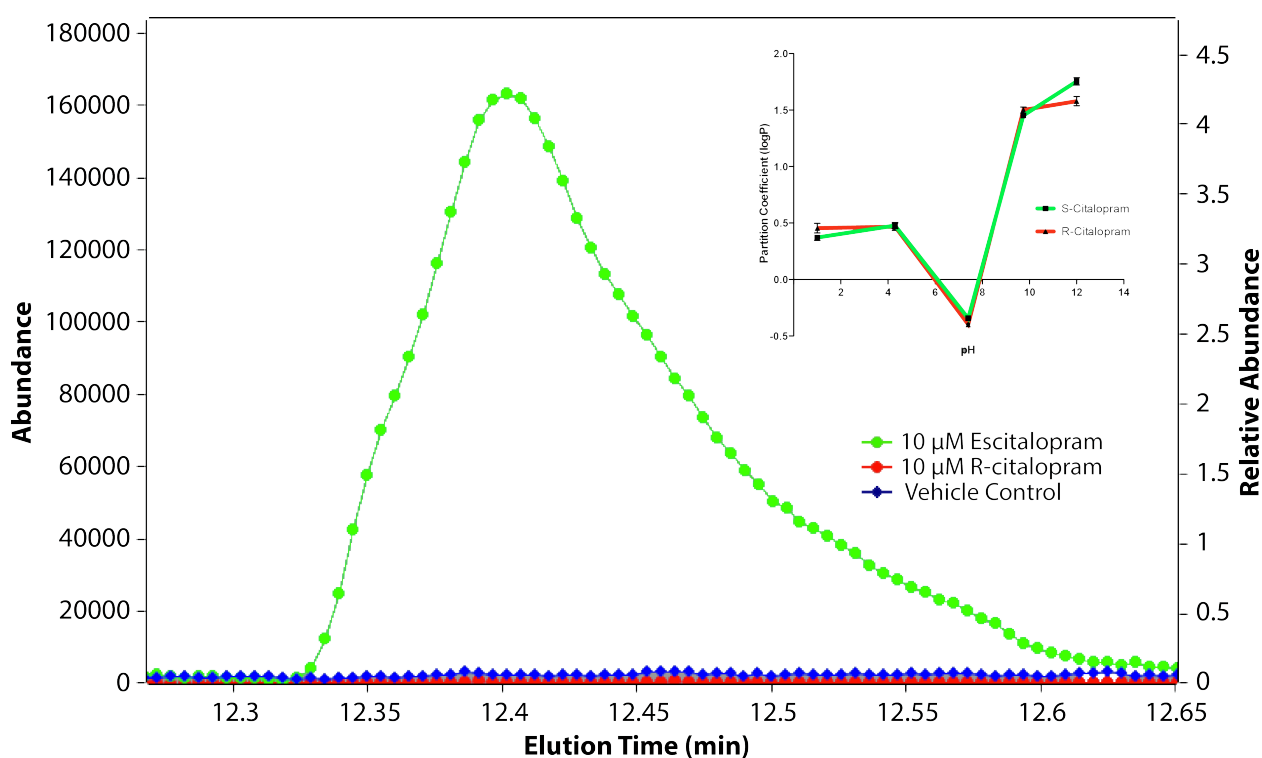

C6 cells were treated with either 10 μM S- or R-citalopram for 72 hrs with media changes every 24 hrs. Lipid Rafts were isolated into Tx-100/Tx-114 fractions (similar results were obtained by sucrose gradient fractionation), dissolved 1:100 in methanol to extract drug, and the supernatant subjected to GC-MS. While both S- and R-citalopram exhibit negative partition coefficients at neutral pH (inset), suggesting the presence of a protein target, escitalopram was observed to accumulate, but R-citalopram did not.

Figure 15. Accumulation of escitalopram is both temporal and concentration dependent.
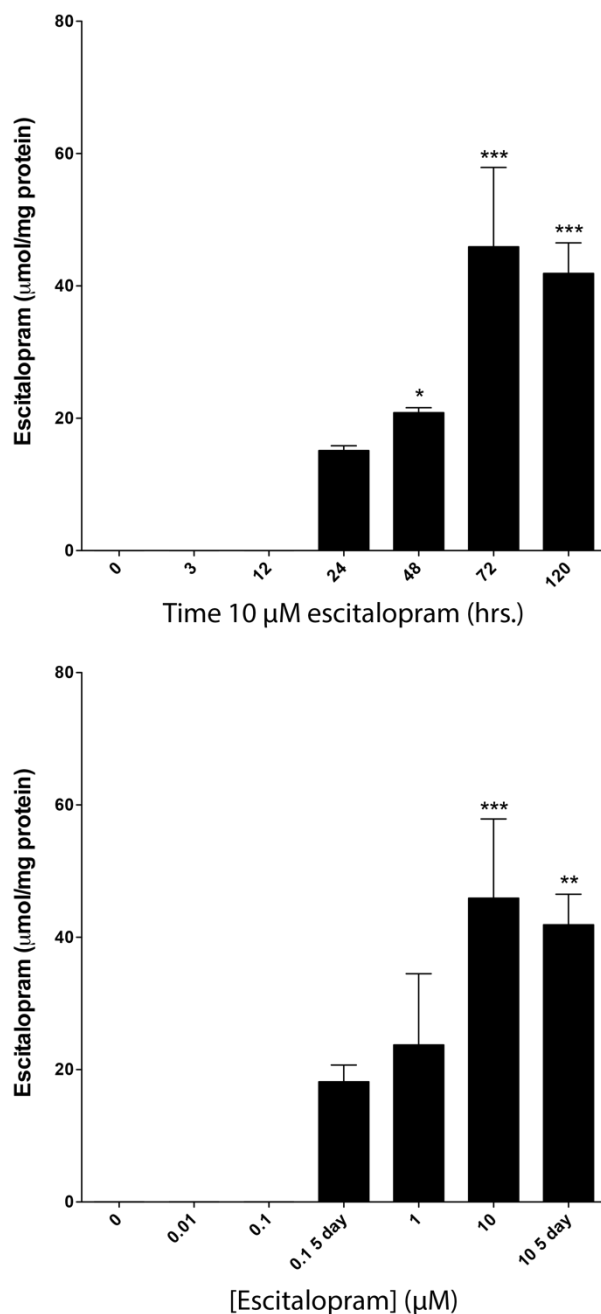
GC-MS elution peak intensities were compared with standards and values determined from a standard curve. The means peak intensities were normalized to protein content of each sample and error propagated between calculations. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc analysis of means for GC-MS quantification. Data are presented as mean ± SEM. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to vehicle).

Figure 16. Accumulation of escitalopram is not toxic to C6 cells.

**Region of Interest is the same for beginning and ending of treatment with Escitalopram.
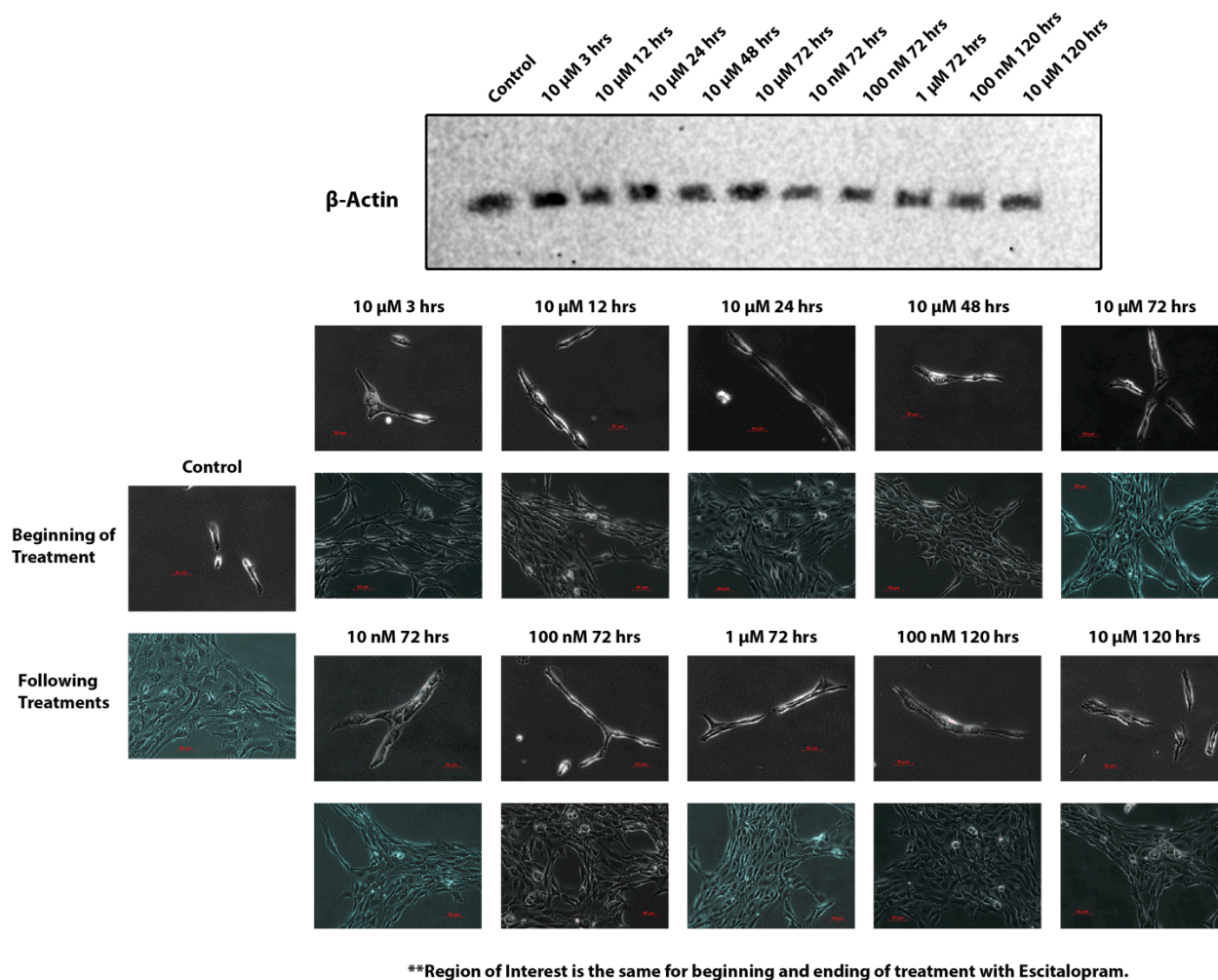
The lack of accumulation in C6 lipid rafts after 120 hrs of treatment with escitalopram suggests that escitalopram may be toxic to cells. However, imaging C6 cells for the dosing and time points at 20X resolution revealed the drug is in fact not toxic to cells. Each cell treatment was completed simultaneously and whole cell homogenates probed for β-actin.

3.1.3 Conclusions

Gradual accumulation of antidepressants in lipid rafts is potentially a causative mechanism accounting for the translocation of $G\alpha_s$ to non-raft regions of the plasma membrane. However, not all antidepressants assayed accumulated to a significant degree over time, but each antidepressant is still able to mediate translocation of $G\alpha_s$ from the lipid raft (161). Some might contend that this is merely a stochastic measure of lipophillicity of the drugs. If this were true, it is reasonable to expect that all assayed antidepressants would accumulate. The fact that only escitalopram, fluoxetine, and phenelzine accumulated supports the hypothesis that specific molecular targets exist for each drug. Moreover, the lack of accumulation of R-citalopram and the empirically determined partition coefficients for each drug, with the exception of olanzapine, suggests that the accumulation of drug in membrane fractions is specific (Figure 17). Therefore, the apparent enantio-selectivity with regard to citalopram, the structural selectivity between Clozapine and olanzapine, and the fact that C6 cells do not express transport proteins, suggests that **a protein target exists for each drug separate from the canonically accepted reuptake transport proteins**.

Figure 17. Partition Coefficients of Antidepressant Drugs reveal they are amphiphilic, whereas Olanzapine is very hydrophobic.
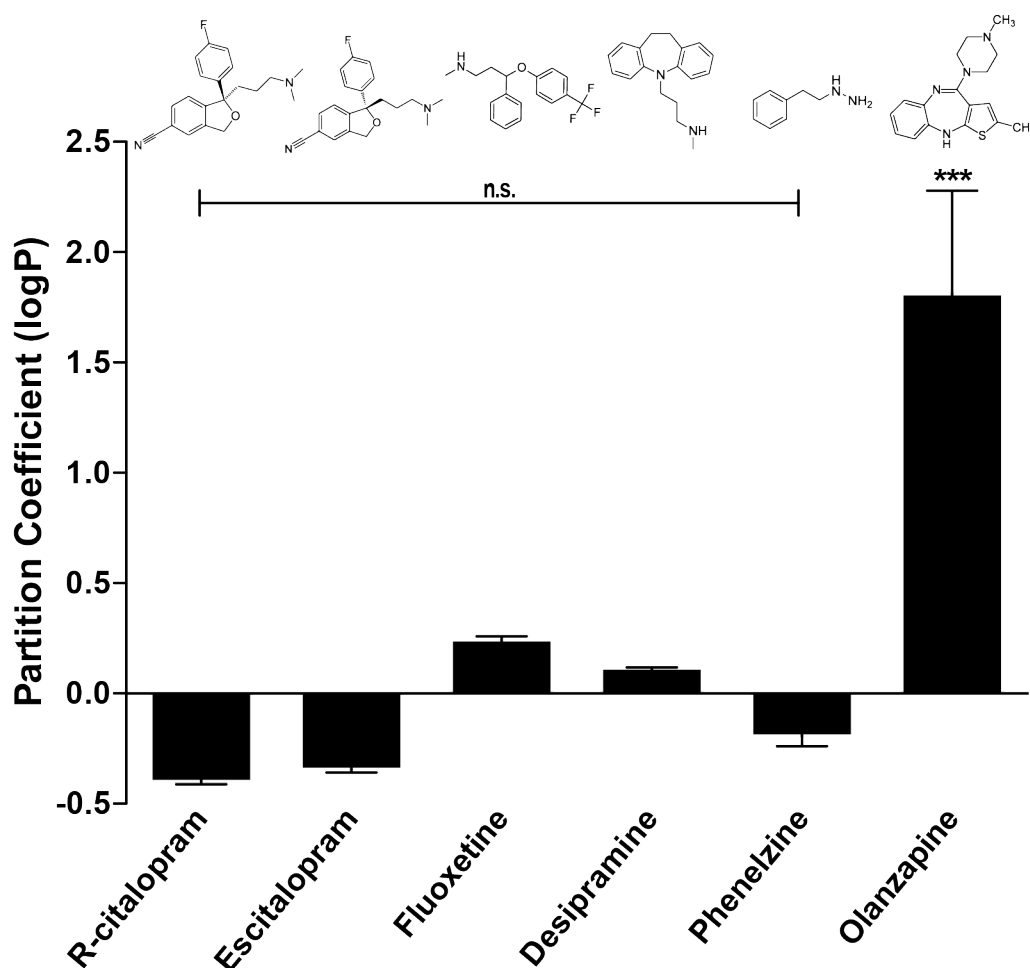
Oil-Water partition coefficients for all assayed drugs was conducted in a 1:1 octanol:water mix, with 10 mM drug added in a 1:2000 dilution. The characteristic UV absorbance for each drug was measured in three independent experiments at pH 7 or both the water and octanol phases. Partition coefficients were calculated per: **log P = log ([solute]$_{octanol}$/[solute]$_{water}$)**. Negative log P values suggest a hydrophilic compound, whereas positive log P values are hydrophobic. No drug is excessively hydrophilic, but olanzapine is extremely hydrophobic. This observation supports the contention that the antidepressant drugs found to accumulate in rafts are binding a protein(s) and not hydrophobically packing in the lipid bilayer. Data are presented as mean ± SEM. (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

3.1.4 Implications

Chronic treatment with antidepressant drugs mediates $G\alpha_s$ translocation from lipid rafts to non-raft regions of the plasma membrane, which is likely through drug specific mechanisms. As the currently established pharmacological actions of available antidepressants are through inhibiting monoamine oxidase (MAO) or inhibiting reuptake transporters (SERT or NET), it necessarily follows that MAO, SERT, or NET is present and somehow associated with $G\alpha_s$. However, the hysteresis in antidepressant therapeutic action suggests that alternative targets exist because the increase in monoamine density occurs within hours of administration.

A seemingly simplistic explanation for an antidepressant-mediated translocation of $G\alpha_s$ from the lipid raft is the accumulation of drugs in lipid raft regions of the plasma membrane. However, previous reports on the concentration of psychoactive drugs in the lipid raft (173) do not perfectly correlate with our findings, as drugs that do not alter $G\alpha_s$ raft association appear to concentrate in lipid rafts and antidepressants that do mediate $G\alpha_s$ translocation did not. The lack of accumulation in lipid rafts in this case is likely due to a deficiency in the method of detection rather than a lack of accumulation. Furthermore, these studies used HEK cells transfected with 5-HT$_3$; HEK cells are not a cell type in which antidepressants should have effects and 5-HT$_3$ is a ligand-gated Na+ and K+ cation channel. I observed that spiking of membranes with escitalopram and comparing with R-citalopram showed the former, but not the latter remains associated with raft fractions of the plasma membrane. Regardless, spiking of an isolated membrane fraction is less of a physiological observation than assaying the gradual accumulation over time in treated cells.

Although C6 cells do not contain any reuptake transport system, yet respond to chronic antidepressant treatment in the context of $G\alpha_s$ translocation from lipid rafts, which suggest the presence of another molecular target. It is possible that the active sites for some antidepressants are downstream from their membrane binding sites. However, I still predicted to see an accumulation in either the lipid raft or the non-raft regions of the membrane unless the drug translocates across the membrane to bind an intracellular target. This might be the case as tricyclics may require Arrestin and Spinophilin for certain actions whereas SSRIs do not (184). Regardless, we observe direct inhibition of binding between $G\alpha_s$ and Tubulin *in vitro* and that chronic treatment with all antidepressants examined thus far move $G\alpha_s$ from lipid rafts. This does not imply a single mechanism of action, but rather that antidepressants have a similar molecular footprint to exploit for the purposes of diagnostics.

Different/multiple mechanisms are likely to exist for the actions of different antidepressants. For example, I assessed the accumulation of representative drugs from each antidepressant class MAOI (phenelzine), SSRI (escitalopram/inactive stereoisomer R-citalopram and fluoxetine), and TCA (desipramine), as well as the antipsychotic (olanzapine). Somewhat surprisingly, I observed accumulation of phenelzine (MAOI) and fluoxetine (SSRI) in both the lipid raft and non-raft regions, whereas desipramine (TCA) and olanzapine (antipsychotic) did not accumulate over time. The lack of accumulation of desipramine is surprising, since Eisensamer observed it to moderately accumulate in low buoyant density fractions of the plasma membrane. However, the absence of olanzapine was less so as it does not move $G\alpha_s$ out of rafts (161). The fact that olanzapine is so structurally similar to clozapine, which

did accumulate for Eisensamer (173), makes this somewhat surprising. Yet again, this suggests that there are protein targets in the membrane apart from the canonical reuptake transporters that bind these drugs. Further support for a distinct molecular target of each drug, is observed with the enantio-selective accumulation of escitalopram, but not its inactive stereoisomer R-citalopram, and the empirically determined partition coefficients for each drug; stereo-selectivity is a hallmark of proteins.

Escitalopram accumulated in a time and concentration dependent manner, which potentially suggests an antidepressant mediated remodeling of the cytoskeletal architecture, but may also explain the relapse in patients that initially respond to antidepressants. Identification of escitalopram's receptor(s) in lipid rafts will provide insight into why ~70% of patients do not respond to first line antidepressant therapy.

Aim 1B. Changes in the molecular associations in which Gα_s participates directs the chronic antidepressant response.

3.2.1 Background

Cell fractionation and real time imaging of GFP tagged Gα$_s$ has revealed that chronic antidepressant treatment, but not acute, mediates its translocation into non-raft membrane domains (139, 152-154, 160, 161, 163, 168). Moreover, non-raft localized Gα$_s$ increasingly interacts with and activates AC, which results in an accumulation of cyclic adenosine monophosphate (cAMP) that we hypothesize to be the physiological event that ultimately mediates the antidepressant response. In support of this are the observations cAMP levels are low in depression (186) and those by Innis and colleagues that chronic administration of C[11]-rolipram, a phosphodiesterase 4 (PDE4) inhibitor, to depressed patients resulted in a global accumulation of cAMP that was coupled with an elevated mood in treated patients, consistent with an antidepressant effect (39, 187, 188). Schering AG originally developed rolipram as an antidepressant (189), but clinical trials showing its therapeutic window was too small to limit significant gastrointestinal inflammatory side effects led to its discontinuation (190), but these observations perfectly accompany our observations regarding translocation of Gα$_s$ from lipid rafts. However, the exact mechanisms by which antidepressants affect localization and resultant signaling may not be purely through engaging coupled signaling pathways. Although chronic antidepressant treatment mediates translocation of Gα$_s$ from the lipid raft, a fraction of Gα$_s$ remains, which is likely to have an entirely new panel of associated factors anchoring it to the membrane and trapped in lipid rafts.

Earlier studies from our lab found that association between Gα$_s$ and Tubulin diminishes subsequent to antidepressant treatment (152, 153) and that Tubulin may

actually exchange GTP with $G\alpha_s$ (29). Whether this association is specific to GTP bound Tubulin is unclear, as Tubulin may specifically interact with GTP bound $G\alpha_s$ and inhibit its intrinsic GTPase activity. However, the GTP-bound state of $G\alpha_s$ appears to preferentially bind Tubulin (156, 191). G-proteins cycle through inactive (GDP bound) and active (GTP bound) states that is dependent to a large degree upon the energy requirements of the cell. This is significant in that Tubulin thus appears to be a potential molecular anchor by which $G\alpha_s$ is retained in lipid rafts. We have established that lipid rafts are areas that dampen $G\alpha_s$ signaling, but may also form vesicles through which $G\alpha_s$ internalizes (132). At first glance, these appear to be incongruous findings, but $G\alpha_s$ exhibits very rapid GTPase activity, but the resulting loss of the "GTP-cap" allows for increased microtubule dynamics (192). Taken together, membrane-associated Tubulin may serve to activate $G\alpha_s$, but may also act as a molecular anchor to link a variety of membrane-associated signaling systems that lock $G\alpha_s$ in lipid rafts (29). In fact, the apparent importance of the association between $G\alpha_s$ and Tubulin may not be unique and other cytoskeletal components are likely to be involved.

The cytoskeleton is a filamentous network present in the cytoplasm of all cells that provides scaffolding and a structural support system that plays important roles in intracellular transport and cellular division. The cytoskeleton is composed of actin microfilaments, intermediate filaments, and microtubules as well as various associated proteins mediating attachments with the plasma membrane and structures throughout the cytoplasm. The cytoskeletal architecture plays an important role in many cellular functions. In light of the nascent and subsequent findings, it is reasonable to predict that the cytoskeletal architecture in depressed patients is contrary to a 'normal'

individual and that antidepressants mediate changes to these altered architectures to establish a 'normal' condition.

Since chronic antidepressant treatment mediates $G\alpha_s$ translocation to non-raft domains, these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades (193). For example, hippocampal neurofilament assembly rearranges in depressed animals (194). Moreover, the time required for such a rearrangement to occur might account for the observed hysteresis in therapeutic efficacy as well as the lack of response in ~70% of patients that take antidepressant drugs. Therefore, **I hypothesize that antidepressants mediate changes to the molecular associations holding $G\alpha_s$ in lipid rafts**.

3.2.2 Results

I have obtained two $G\alpha_s$ nanobodies (NB35 and NB37) from Brian Kobilka that recognizes two distinct, non-overlapping sites on $G\alpha_s$ with very high affinity (50). Initial immunoprecipitations suggest complete pull-down of $G\alpha_s$ in the presence of NB35 and nickel agarose beads. Moreover, even with complete immunoprecipitation of $G\alpha_s$, it is possible that sufficient material for analysis is not present. However, this should not present a major issue, as little as a couple picomole of sample is sufficient for LC-MS/MS analysis (195). If necessary, I will utilize the GFP tag in our $G\alpha_s$-GFP C6 cells, which express $G\alpha_s$-GFP about 3x normal $G\alpha_s$ expression. The GFP insertion is not in a position that interferes with binding of either nanobody. Another possible problem is that the conditions for immunoprecipitation and UHPLC-MS/MS analysis might remove palmitoyl groups. Covalent modification of free sulfhydryls with N-ethylmaleimide and reducing S-acylation sites with hydroxylamine before precipitation addresses this.

I used C6 cells, because of the lack of a reuptake transport system, for treatment with 10 µM escitalopram or desipramine for 72 hrs. Based upon previously reported results regarding the capacity of antidepressants, specifically escitalopram, this time and concentration of drug treatment is known to mediate translocation of $G\alpha_s$ out from the suppressive effects of the lipid raft (152, 161). Following chronic treatment with the aforementioned antidepressants, the lipid raft fractions of the plasma membrane were isolated by sucrose density gradient, $G\alpha_s$ localized to lipid rafts was activated with fluoride (196), and immunoprecipitated with NB35, a conformation specific nanobody recognizing active $G\alpha_s$ (50, 197); a representative peptide ion mass spectrum for precipitated $G\alpha_s$ from lipid rafts is given (Figure 18). Molecular associations were

identified by LC-MS/MS, grouped into functional categories, and bead proteome removed (198). The largest category of proteins found in association with $G\alpha_s$, in the no treatment control, as well as chronically treated with escitalopram or desipramine were cytoskeletal scaffolding (mediators of cytoskeletal attachment) and remodeling proteins (enzymes and factors recruiting cytoskeletal destabilizing proteins) (Figure 19). Both escitalopram and desipramine mediate the translocation of $G\alpha_s$ from the lipid raft and that only in the no treatment control does $G\alpha_s$ associate with a receptor.

Accumulation of certain antidepressant drugs (escitalopram, fluoxetine, and phenelzine) in (Figures 4-15), and the consequent translocation of $G\alpha_s$ out of the lipid raft suggests that antidepressants may be able to physically disrupt the $G\alpha_s$ and molecular anchors (*e.g.* β-Tubulin); the proteins found in association with $G\alpha_s$ from C6 lipid rafts can be found in TABLE IV. Incubation of escitalopram as well as its inactive stereoisomer R-citalopram after, during, or throughout binding of purified recombinant $G\alpha_s$ with purified Tubulin resulted in a near universal abrogation of the His-$G\alpha_s$:Tubulin complex (Figure 20). This suggests that a relatively nonspecific physicochemical recognition of binding surface(s) on either $G\alpha_s$ and/or Tubulin exists. While these observations are in an isolated environment with purified proteins, it is possible that different classes of antidepressant affect $G\alpha_s$ in a similar fashion. Regardless, one or more of these proteins is key to establishing the utility of $G\alpha_s$ association with lipid rafts as a barometer of antidepressant efficacy and hallmark of antidepressant action, but also for exploitation in the development of adjunct therapies that reduce the time to onset of therapeutic action and/or more finely tune the pharmacology for the patient.

Figure 18. Representative Ion Fragmentation of Gα_s immunoprecipitated with NB35 from lipid rafts of C6 cells.
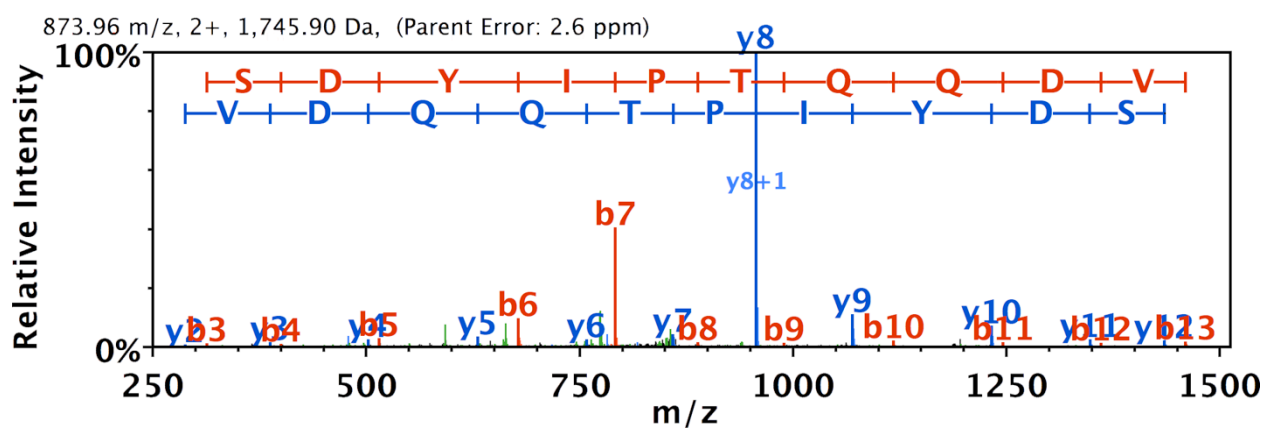
C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and Gα_s complexes were immunoprecipitated using NB35. Precipitations were digested with Trypsin and identifications made by LC-MS/MS analysis.

Figure 19. Chronic treatment of C6 cells with the antidepressants escitalopram and desipramine mediates the translocation of Gα$_s$ from lipid rafts via altering the protein anchors of Gα$_s$.
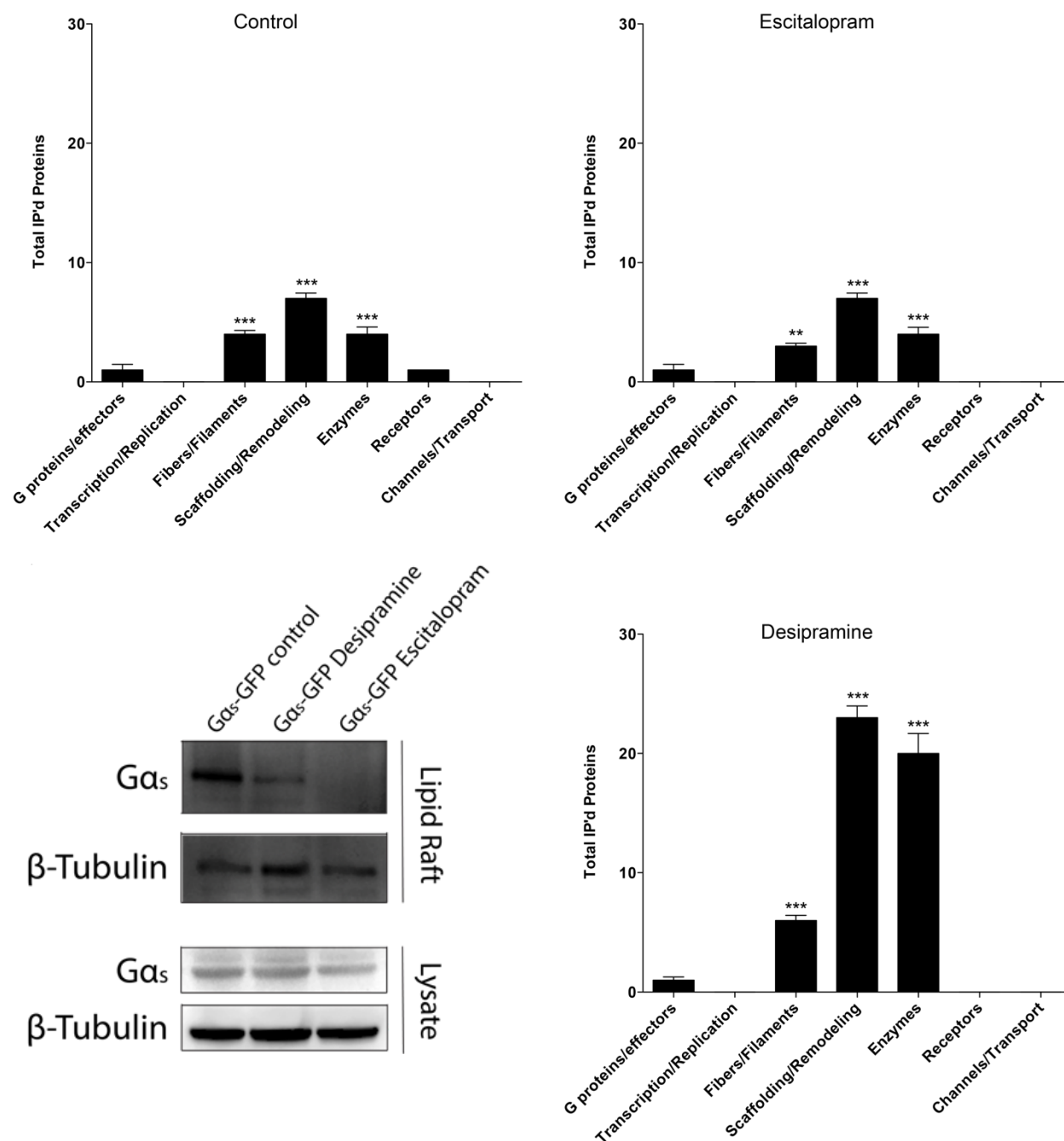
C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and Gα$_s$ complexes were immunoprecipitated using NB35. Protein identifications were made by LC-MS/MS analysis and grouped according to function. Under each condition, the predominant classes of proteins found in association with Gα$_s$ are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

Figure 20. Antidepressants and R-citalopram disrupt the interaction between recombinant Gα$_s$ and purified Total Tubulin from sheep brain.
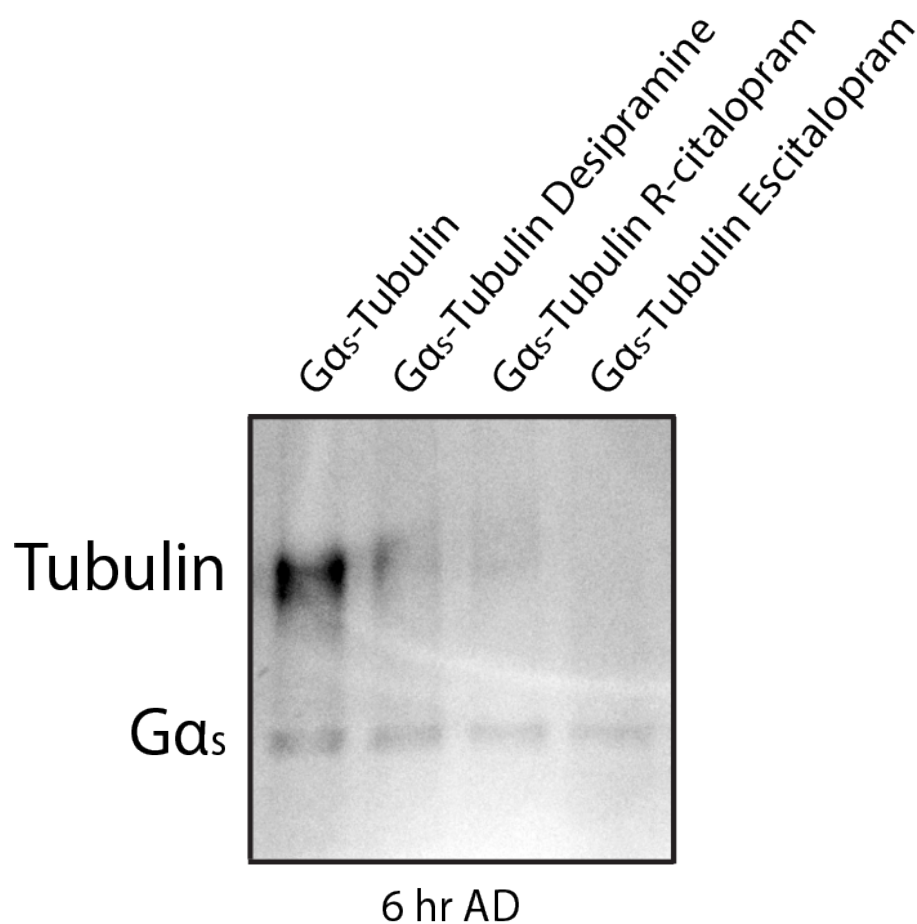
Pull down of Gα$_s$ and Tubulin suggests that antidepressants may interfere directly with the physical interactions in which Gα$_s$ participates. Recombinant Gα$_s$ was incubated with total Tubulin purified from sheep's brain for 6 hours and the interaction was disrupted with either the antidepressants desipramine or escitalopram or its inactive stereoisomer R-citalopram. Precipitation via the 6xHis tag on Gα$_s$ with Ni-NTA resin and silver staining of the gel resulted in a strong interaction between Gα$_s$ and Tubulin in the absence of any compound. Moreover, drug addition did not appreciably affect the precipitation of Gα$_s$.

TABLE IV. **Proteins in association with Gα$_s$ in Lipid rafts of C6 cells natively and following chronic antidepressant treatment.**

NO TREATMENT	*CHRONIC ESCITALOPRAM*	*CHRONIC DESIPRAMINE*
Receptors	**Receptors**	**Receptors**
GPCR 19		
Fibers/Filaments	**Fibers/Filaments**	**Fibers/Filaments**
		Tubulin alpha-1B
		Tubulin beta-2C
		Tubulin beta-5
Scaffolding/Remodeling	**Scaffolding/Remodeling**	**Scaffolding/Remodeling**
14-3-3 alpha	Junction Plakoglobin	14-3-3 delta
14-3-3 beta	Protocadherin-3	14-3-3 theta
		14-3-3 zeta
		Caprin-1
		Caveolin-1
		Caveolin-2
		Ceruloplasmin
		Chondroitin sulfate proteoglycan 4
		Complement component 1 Q
		Ezrin
		Flotillin-1
		Flotillin-2
		Neurabin-1
		Protocadherin-3
		Septin-10
		Septin-11
		Septin-7
		Septin-8
		Transmembrane protein 43
		Vinculin
Enzymes	**Enzymes**	**Enzymes**
	Disintegrin and metalloproteinase 7	Aconitate hydratase
		Aldehyde dehydrogenase
		Arginase-1
		Arginine N-methyltransferase 1
		Citrate synthase
		Creatine kinase B
		Cytochrome c oxidase
		Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase
		Disintegrin and metalloproteinase 7
		Fructose-bisphosphate aldolase C
		Guanylate Cyclase
		Liver carboxylesterase 1

NO TREATMENT	CHONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Enzymes	Enzymes	Enzymes
		Long-chain specific acyl-CoA dehydrogenase
		NADH dehydrogenase [ubiquinone]
		NADH-ubiquinone oxidoreductase
		Superoxide dismutase [Mn]
		Tyrosine-protein kinase JAK2

Table 4: Protein identifications made by LC-MS/MS on the associations of $G\alpha_s$ in lipid rafts of C6 cells, grouped according to function. C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and $G\alpha_s$ complexes were immunoprecipitated using NB35. Proteins know to associate with sepharose beads and those that were common across all treatments were subtracted to arrive at the protein profile changes upon differential drug treatment. Under each condition, the predominant classes of proteins found in association with $G\alpha_s$ are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

3.2.3 Conclusions

It is naïve to think that all of newly identified proteins are integral to the retention of $G\alpha_s$ in lipid rafts as well as in the development of depression and the antidepressant response. However, it is worth noting that the largest category in each case (no treatment control, chronic escitalopram, and chronic desipramine) is that of the cytoskeletal scaffolding/remodeling proteins. Furthermore, the relative absence of a receptor associated with $G\alpha_s$ in lipid rafts chronically treated with either escitalopram or desipramine suggests that the retention of $G\alpha_s$ and resultant phenomenon of chronic antidepressant mediated translocation from lipid rafts may be receptor independent (TABLE IV). Therefore, in agreement with my hypothesis and previous findings, remodeling of the cytoskeleton appears necessary for translocation of $G\alpha_s$ out of lipid rafts.

3.2.4 Implications

Cell fractionation and real time imaging of GFP tagged $G\alpha_s$ has revealed that chronic antidepressant treatment, but not acute, mediates its translocation into non-raft membrane domains where $G\alpha_s$ increasingly interacts with and activates AC (139, 152-154, 160, 161, 163, 168). As antidepressants have been observed to accumulate in lipid rafts over time and mediate translocation of a significant portion of $G\alpha_s$ from the lipid raft, a fraction remains, which is likely to have an entirely new panel of associated factors anchoring it to the membrane and trapped in lipid rafts. It is naïve to think that all newly identified proteins are integral to the retention of $G\alpha_s$ in lipid rafts as well as in the development of depression and the antidepressant response. Many of which are purely nugatory to this phenomenon, it is worth noting though that the largest percentage of proteins found in association with $G\alpha_s$, in each case (no treatment control, chronic escitalopram, and chronic desipramine) were cytoskeletal scaffolding (mediators of cytoskeletal attachment) and remodeling proteins (enzymes and factors recruiting cytoskeletal destabilizing agents).

Remodeling the cytoskeleton may be necessary for translocation of $G\alpha_s$ out of lipid rafts and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades (193). For example, changes in hippocampal neurofilament assembly have been observed in depressed animals (194) and the time required for such a rearrangement to occur would be in line with the hysteresis in therapeutic efficacy as well as the lack of response in ~70% of patients that take antidepressant drugs. Earlier studies from our lab found that antidepressant treatment diminishes association between $G\alpha_s$ and Tubulin (152, 153). Whether this

association is specific to GTP bound Tubulin is unclear, but GTP-bound $G\alpha_s$ appears to preferentially bind Tubulin (156, 191). Moreover, Tubulin may actually exchange GTP with $G\alpha_s$ (29, 199) and can inhibit the intrinsic GTPase activity of GTP bound $G\alpha_s$ (156, 191). Tubulin appears to be a potential molecular anchor holding $G\alpha_s$ in lipid rafts, but the question becomes what is happening to this interaction upon chronic antidepressant treatment?

At first glance, these findings appear to be incongruous, as membrane-associated Tubulin may activate $G\alpha_s$ and GTP-$G\alpha_s$ preferentially binds Tubulin, but antidepressants do induce dissolution of the $G\alpha_s$:Tubulin complex (192, 200). This actually makes sense when considering the ultimate molecular target of $G\alpha_s$, AC, which $G\alpha_s$ binds through its switch II segment (Sw II) in its Ras-like domain (201). This domain is in very close proximity to the bound GTP (202) and the nearby $\alpha3$-$\beta5$ loop involved in the direct association of $G\alpha_s$ with Tubulin (191). While these are not the exactly same regions of $G\alpha_s$, they are in close enough proximity to sterically hinder the simultaneous association of AC and Tubulin with $G\alpha_s$. Moreover, increased accumulation of cAMP is responsible for degrading the microtubule structure (203, 204), which appears to be holding $G\alpha_s$ in the lipid raft. Therefore, antidepressant induced translocation of $G\alpha_s$ from the lipid raft necessarily must proceed without shuttling on the membrane with bound Tubulin. Furthermore, the absence of a receptor associated with $G\alpha_s$ in lipid rafts chronically treated with either escitalopram or desipramine suggests that retention of $G\alpha_s$ is dependent upon other factors, thus leaving palmitoylation of $G\alpha_s$ as the only currently identified anchor to the plasma membrane.

AIM 2: Determine the effects of acylation state of Gα$_S$ on its subcellular localization and molecular associations that maintain lipid raft localization.

Aim 2A. Acylation state directs the subcellular localization of Gα$_s$ and molecular associations in which Gα$_s$ participates.

3.3.1 Background

G protein alpha subunits are acylated in the N-terminus with myristate and/or palmitate. Myristoylation by itself actually provides barely enough energy to attach a protein to the plasma membrane (137), but palmitoylation is more than capable even though it is reversible (104). Palmitoylation of Gα$_s$ is a post-translational modification that occurs under native wild type conditions and is important for membrane targeting and anchoring (3, 125). This is significant, as active Gα$_s$ dissociates from either its coupled GPCR and/or heterotrimeric Gβγ subunits, it should possess a mechanism for maintaining membrane localization; unless the signaling pathway necessitates intracellular translocation of Gα$_s$. Although, translocation of Gα$_s$ from the plasma membrane suggests activation-induced depalmitoylation of Gα$_s$ (88, 107, 128). It is worth noting that Gα$_s$ is the only Gα that internalizes when activated (129-131) and switches between raft and non-raft domains. Gα$_i$ is both myristoylated and palmitoylated in the N-terminus (205-207), which presumably impairs activation induced subcellular redistribution. Thus, it may be possible that one, or a companion, of the potential mechanism(s) of antidepressant-induced translocation of Gα$_s$ is via attenuating its N-terminal palmitoylation. Furthermore, generation of mutants of Gα$_s$ that mimic Gα$_i$ provide further insight into the importance of acylation in antidepressant mediated Gα$_s$ activation.

3.3.2 Results

We have prepared acylation mutants of $G\alpha_s$ as previously described (3), ensured GFP is monomeric (169), selected stable clones, and imaged by confocal microscopy to determine the subcellular localization of $G\alpha_s$. Mutation of $G\alpha_s$ at an N-terminal Glycine residue ($G\alpha_i$ like modification state), $G\alpha_s$ is both palmitoylated and myristoylated, whereas mutation of the N-terminal cysteine residue makes $G\alpha_s$ acylation deficient (Figure 21). Cell fractionation studies of stably transfected C6 cells with each acylation mutant suggests that the singly palmitoylated and palmitoylated/myristoylated $G\alpha_s$-GFP versions are raft associated, but only $G\alpha_s$-GFP is removed upon chronic escitalopram-mediated activation. Altered acylation does not affect total solubility even though the distribution between the fractions is distinct from the singly palmitoylated "normal" version. Myristoylated/palmitoylated $G\alpha_s$ does not translocate from lipid rafts, even after chronic escitalopram treatment and palmitoylation deficient $G\alpha_s$ does not even appreciably localize to lipid rafts as it is cytosolically distributed (Figure 22). Since N-terminally modified $G\alpha_s$–GFP retains a cytosolic and/or plasma membrane association, $G\alpha_s$ becomes effectively "antidepressant–insensitive." Therefore, the reversible nature of the palmitoylation event appears to enable $G\alpha_s$ to translocate from lipid rafts in response to chronic antidepressant treatment and suggests the importance of both acylation state, but also suggests the importance of its removal upon activation.

However, the issue of how a depalmitoylated internalized $G\alpha_s$ is able to associate with membrane imbedded AC remains? Our lab has hypothesized that Tubulin association is a molecular event that locks $G\alpha_s$ into lipid rafts and that chronic antidepressant treatment disrupts this association. However, we have also observed

that GTP bound $G\alpha_s$ appears to be the preferential state bound to Tubulin (156, 191), even though Tubulin is able to activate $G\alpha_s$ through GTP exchange (29). The resulting "GTP-cap" allows for increased microtubule dynamics with $G\alpha_s$ remaining in association (192). Different isoforms of Tubulin do in fact remain in association with $G\alpha_s$ following activation by chronic antidepressant treatment (TABLES IV-VII), which appears to correlate with the acylation state and subcellular localization of $G\alpha_s$ (Figures 21-23). Mutation of $G\alpha_s$ at its N-terminal Glycine residue ($G\alpha_i$ like modification state), $G\alpha_s$ is both palmitoylated and myristoylated and localizes to cytosolic structures, whereas mutation of the N-terminal cysteine residue makes $G\alpha_s$ acylation deficient and retain no defined localization pattern. However, dually acylated $G\alpha_s$ appears to be raft localized independent of Tubulin, which fits with the hypothesis that secondary myristoylation is sufficient to lock $G\alpha_s$ in the lipid raft (TABLE VII). Under chronic desipramine treatment, associations between $G\alpha_s$ and Tubulin isoforms return. Tubulin itself is palmitoylated (101-103, 106, 108, 109), which might enable $G\alpha_s$ to translocate to non-raft regions of the plasma membrane while depalmitoylated and capping the plus end of the microtubule. Regardless, the predominant functional class of proteins found in association with $G\alpha_s$ in lipid rafts is the scaffolding/remodeling protein class. As certain antidepressants gradually accumulate over time in lipid rafts, and depalmitoylation appears to be integral to fully mediating the antidepressant response through $G\alpha_s$, this suggests the coupling of each antidepressant to an as of yet unidentified protein target.

Figure 21. N-terminal amino acid sequences of G-proteins used for Gα$_s$-GFP acylation mutant generation.

$Gα_s$ | M-G**C**LG**N**-SKTE

$Gα_{i1}$ | M-**GC**TLS-AEDK

$Gα_{i2}$ | M-**GC**TVS-AEDK

$Gα_{i3}$ | M-**GC**TLS-AEDK

GXXXS/T is the N-myristoyltransferase motif

G-Myristoylation site

C-Palmitoylation site

Lipid Modification		Enzyme
Protein—N—C (O) ...chain	Myristoylation	N-myristoyl transferase
Protein—S—C (O) ...chain	Palmitoylation	Palmitoyl transferase

N-terminal amino acid sequences for G-proteins used to build Gα$_s$-GFP acylation mutants for analyzing the subcellular localization and molecular associations of Gα$_s$ relative to its acylated status. Gα$_s$ is palmitoylated and Gα$_i$ is both palmitoylated and myristoylated. Palmitoylation occurs on cys3, which is ΔC3S in the palmitoylation deficient mutant and the dual acylation mutant (Gα$_i$ like) is ΔN6S.

Figure 22. Acylation state of Gαs determines whether it lipid raft localizes and if Gαs translocates in response to chronic antidepressant treatment.
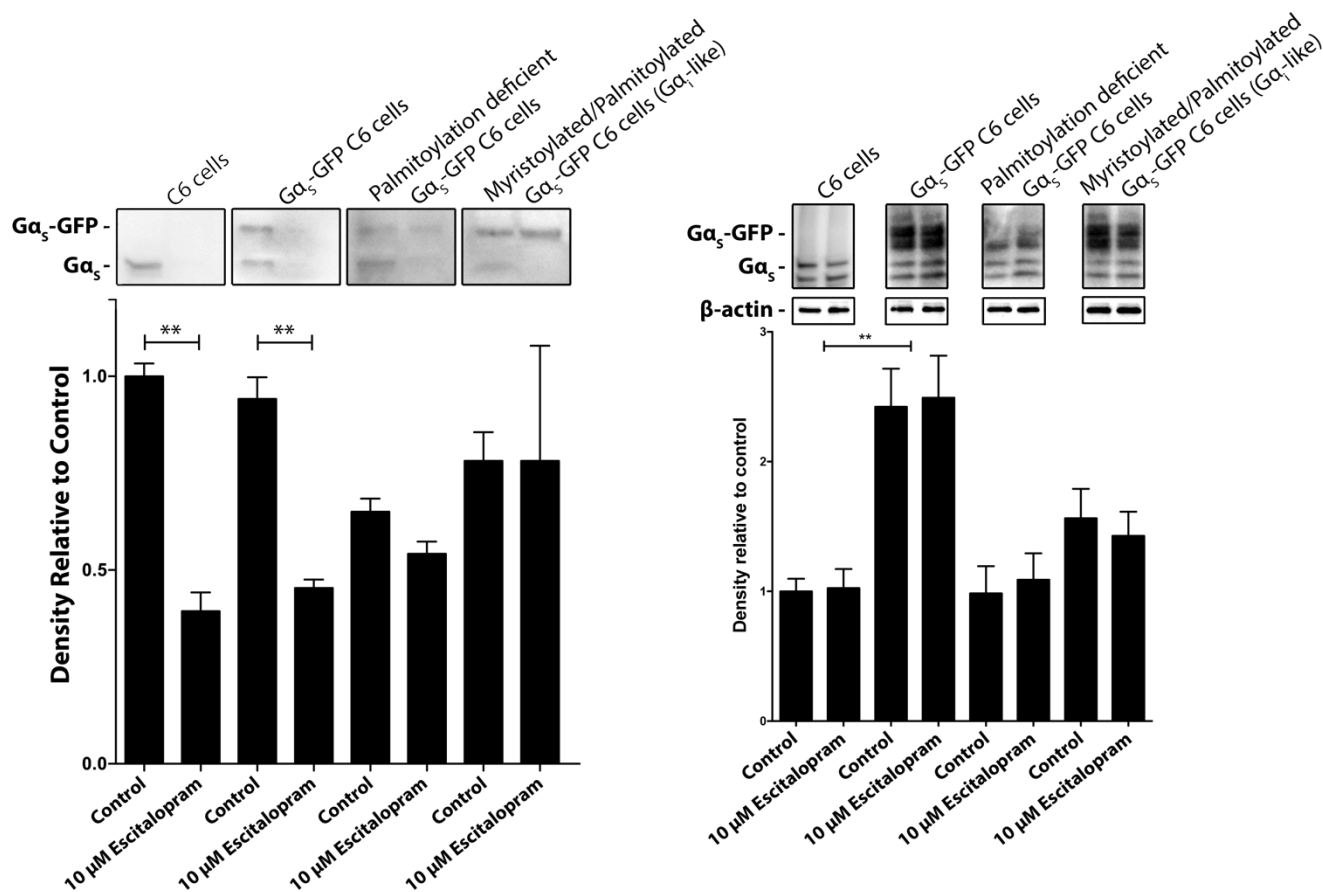
Gα$_s$ acylation mutants localize to lipid rafts to varying degrees. Normally modified Gα$_s$, as well as depalmitoylated Gα$_s$, responds to chronic antidepressant treatment (72 hrs) by moving out of lipid rafts, but when dually modified (Myr/Palm) Gα$_s$ does not respond to antidepressants as assessed by biochemical fractionation (*i.e.* sucrose gradient) **(Left panel).** Moreover, antidepressant treatment does not appreciably affect the expression of either endogenous Gα$_s$ or GFP-Gα$_s$ **(Right panel).**

Figure 23. Acylation dependent Protein Association Profiles for Gα$_s$-GFP immunoprecipitated with NB35 from C6 cells Lipid Rafts.
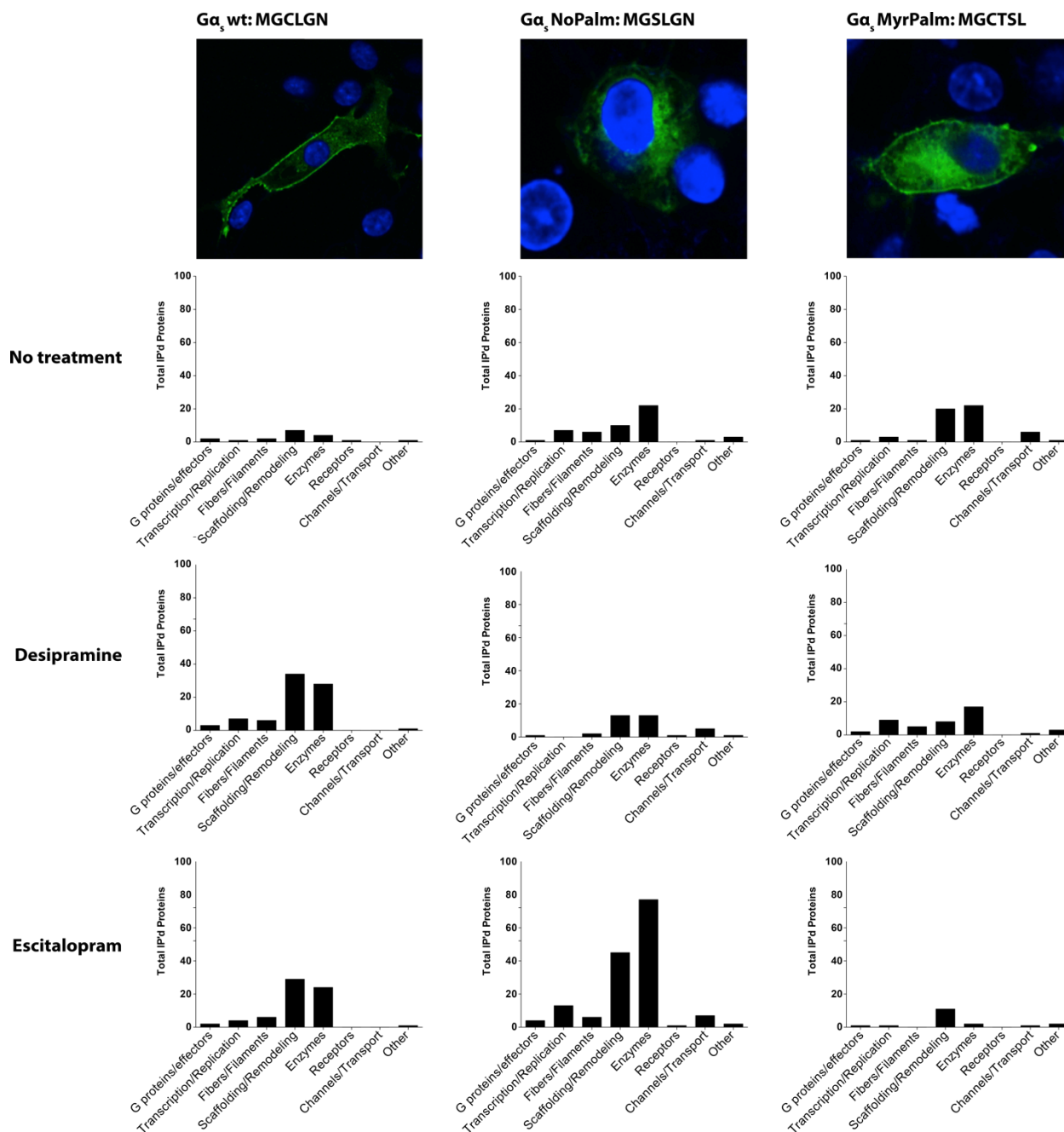
C6 cells were treated with either 10 μM escitalopram or 10 μM desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and Gα$_s$ complexes immunoprecipitated using NB35. Protein identifications were made by LC-MS/MS analysis and grouped according to function. Under each condition, the predominant classes of proteins found in association with Gα$_s$, regardless of acylation status, are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

TABLE V. Proteins in association with Gαs-GFP in Lipid rafts of C6 cells.

NO TREATMENT	CHRONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Receptors	**Receptors**	**Receptors**
GPCR 19		
Fibers/Filaments	**Fibers/Filaments**	**Fibers/Filaments**
	Tubulin alpha-1C chain	Tubulin alpha-1C chain
Scaffolding/Remodeling	**Scaffolding/Remodeling**	**Scaffolding/Remodeling**
	A-kinase anchor protein 12	14-3-3 protein zeta/delta
	Brain acid soluble protein 1	A-kinase anchor protein 12
	Ceruloplasmin	Brain acid soluble protein 1
	Endoplasmin	Ceruloplasmin
	Ezrin	Ezrin
	Gelsolin	Galectin-1
	Microtubule-associated protein 4	Gelsolin
	Moesin	Microtubule-associated protein 4
	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Moesin
	Non-muscle Caldesmon	Phosphatidylethanolamine-binding protein 1
	Protocadherin-3	Protocadherin-3
	Septin-11	Septin-10
	Septin-7	Septin-8
		Testis-specific gene 10 protein
		Vacuolar protein sorting-associated protein 33A
Enzymes	**Enzymes**	**Enzymes**
	2',3'-cyclic-nucleotide 3'-phosphodiesterase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
	Alpha-enolase	Alpha-enolase
	Beta-enolase	Beta-enolase
	Creatine kinase B-type	Creatine kinase B-type
	Disintegrin and metalloproteinase domain-containing protein 7	Disintegrin and metalloproteinase domain-containing protein 7
	Fructose-bisphosphate aldolase A	Fructose-bisphosphate aldolase A
	Fructose-bisphosphate aldolase C	Fructose-bisphosphate aldolase C
		L-lactate dehydrogenase A chain
		Malate dehydrogenase
		Protein kinase C theta type
		Superoxide dismutase [Cu-Zn]

Table 5: Protein identifications made by LC-MS/MS on the associations of Gαs in lipid rafts of C6 cells, grouped according to function. C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and Gαs complexes were immunoprecipitated using NB35. Proteins know to associate with sepharose beads and GFP as well as those that were common across all treatments were subtracted to arrive at the protein profile changes upon differential drug treatment. Under each condition, the predominant classes of proteins found in association with Gαs are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

TABLE VI. Proteins in association with acylation deficient Gα$_s$-GFP mutants in Lipid rafts of C6 cells.

NO TREATMENT	CHRONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Receptors	**Receptors**	**Receptors**
		GPCR 19
Fibers/Filaments	**Fibers/Filaments**	**Fibers/Filaments**
Tubulin alpha-1A	Tubulin alpha-1B	
Tubulin alpha-1C	Tubulin beta-5	
Tubulin beta-2B		
Scaffolding/Remodeling	**Scaffolding/Remodeling**	**Scaffolding/Remodeling**
14-3-3 protein theta	14-3-3 protein epsilon	Chondroitin sulfate proteoglycan 4
14-3-3 protein zeta/delta	Alpha-2-macroglobulin receptor-associated protein	Flotillin-1
	Alpha-soluble NSF attachment protein	
	Ameloblastin	Golgi apparatus protein 1
	Caveolin-1	Junction Plakoglobin
	Cell division control protein 42 homolog	Protocadherin-3
	Chondroitin sulfate proteoglycan 4	
	Coatomer subunit delta	
	Flotillin-1	
	Flotillin-2	
	Galectin-3	
	Golgi apparatus protein 1	
	Immediate early response 3-interacting protein 1	
	Integrin beta-1	
	Kinase D-interacting substrate of 220 kDa	
	Lactadherin	
	Leukocyte surface antigen CD47	
	Moesin	
	Protocadherin-3	
	Sideroflexin-1	
	Sorting and assembly machinery component 50 homolog	
	Syntaxin-6	
	Tetraspanin-12	
	Tetratricopeptide repeat protein 35	
	Vesicle-trafficking protein SEC22b	

NO TREATMENT	CHONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Enzymes	Enzymes	Enzymes
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase-like protein	3,2-trans-enoyl-CoA isomerase, mitochondrial	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
78 kDa glucose-regulated protein	Alpha-enolase	Disintegrin and metalloproteinase domain-containing protein 7
Alsin	Alpha-1,6-mannosyl-glycoprotein 6-beta-N-acetylglucosaminyltransferase	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
Arsenite methyltransferase	Aspartate aminotransferase	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
Cofilin-1	Beta-enolase	
Endothelin-converting enzyme-like 1	Cytochrome b5 type B	Glutamyl aminopeptidase
Phosphoglycerate kinase 1	Cytochrome c oxidase	NADH dehydrogenase [ubiquinone] flavoprotein 2
Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	Oxidation resistance protein 1
T-complex protein 1 subunit gamma	Disintegrin and metalloproteinase domain-containing protein 7	TANK-binding kinase 1-binding protein 1
Ubiquinone biosynthesis protein COQ4 homolog, mitochondrial	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase	
Ufm1-specific protease 2	Endoplasmic reticulum metallopeptidase 1	
	Erlin-2	
	Glutamate dehydrogenase 1, mitochondrial	
	Glutamyl aminopeptidase	
	Glycerol kinase	
	GPI transamidase component PIG-S	
	Hexokinase-1	
	Integrin-linked protein kinase	
	MOSC domain-containing protein 2, mitochondrial	
	NADH dehydrogenase [ubiquinone]	
	NADH-cytochrome b5 reductase 3	
	NADH-ubiquinone oxidoreductase	
	Oligosaccharyltransferase complex subunit OSTC	
	Ornithine aminotransferase	

NO TREATMENT	CHONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Enzymes	**Enzymes**	**Enzymes**
	Polypeptide N-acetylgalactosaminyltransferase 1	
	Signal peptidase complex catalytic subunit SEC11A	
	Succinate dehydrogenase [ubiquinone] flavoprotein	
	Trifunctional enzyme subunit alpha	
	UDP-glucuronosyltransferase 2B1	
Channels/Transport	**Channels/Transport**	**Channels/Transport**
	Golgin subfamily A member 7	
	Multidrug resistance protein 1	
	Multidrug resistance protein 2	

Table 6: Protein identifications made by LC-MS/MS on the associations of $G\alpha_s$ in lipid rafts of C6 cells, grouped according to function. C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and $G\alpha_s$ complexes were immunoprecipitated using NB35. Proteins know to associate with sepharose beads and GFP as well as those that were common across all treatments were subtracted to arrive at the protein profile changes upon differential drug treatment. Under each condition, the predominant classes of proteins found in association with $G\alpha_s$ are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

TABLE VII. Proteins in association with dually acylated Gα$_s$-GFP mutants in Lipid rafts of C6 cells.

NO TREATMENT	CHRONIC ESCITALOPRAM	CHRONIC DESIPRAMINE
Fibers/Filaments	**Fibers/Filaments**	**Fibers/Filaments**
		Tubulin alpha-1A
		Tubulin alpha-1C
		Tubulin beta-2C
Scaffolding/Remodeling	**Scaffolding/Remodeling**	**Scaffolding/Remodeling**
Chondroitin sulfate proteoglycan 4	Flotillin-2	14-3-3 protein theta
ER Lipid Raft Associated 2 (Erlin-2)	Islet cell auto antigen 1-like protein	Complement component 1 Q
Golgi apparatus protein 1		
Integrin beta-1		
Lactadherin		
Moesin		
Tetratricopeptide repeat protein 35		
UPF0510 protein INM02		
Enzymes	**Enzymes**	**Enzymes**
Aspartate aminotransferase	Disintegrin and metalloproteinase domain-containing protein 7	Arsenite methyltransferase
Cytochrome b5 type B		Disintegrin and metalloproteinase domain-containing protein 7
Cytochrome c oxidase subunit 4 isoform 1		Endoplasmin
Dihydrolipoyllysine-residue succinyltransferase		Endothelin-converting enzyme-like 1
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase		Mitogen-activated protein kinase 8
Glutamate dehydrogenase 1		Phosphoglycerate kinase 1
Glutamyl aminopeptidase		Ubiquinone biosynthesis protein COQ4 homolog
NADH dehydrogenase [ubiquinone] flavoprotein 2		
NADH-ubiquinone oxidoreductase		
Tyrosine-protein phosphatase		
Channels/Transport	**Channels/Transport**	**Channels/Transport**
Multidrug resistance protein 1		
Multidrug resistance protein 2		

Table 7: Protein identifications made by LC-MS/MS on the associations of Gα$_s$ in lipid rafts of C6 cells, grouped according to function. C6 cells were treated with either 10 µM escitalopram or desipramine for 72 hrs with daily media changes. Lipid rafts were isolated via sucrose density gradient and Gα$_s$ complexes were immunoprecipitated using NB35. Proteins know to associate with sepharose beads and GFP as well as those that were common across all treatments were subtracted to arrive at the protein profile changes upon differential drug treatment. Under each condition, the predominant classes of proteins found in association with Gα$_s$ are scaffolding or those proteins that might alter the cytoskeleton or cytoskeletal associated proteins.

3.3.3 Conclusions

As mentioned in the previous section, it cannot be said that all newly identified proteins are integral to the retention of $G\alpha_s$ in lipid rafts as well as in the development of depression and the antidepressant response. Moreover, the panel of proteins found in association with $G\alpha_s$ for the generated mutants is likely relevant for native $G\alpha_s$ upon differential acylation status, with the exception of the dually acylated mutant, this is a negative control for antidepressant effects. Thus, there exists a panel of important lipid raft anchors for $G\alpha_s$ that are dependent upon the acylation of $G\alpha_s$. Significantly, there does not appear to be a large contribution to retaining $G\alpha_s$ in the lipid raft when it is secondarily myristoylated. This is significant, as $G\alpha_i$ does not respond in a similar fashion to antidepressant treatments. Furthermore, when considering the wild type $G\alpha_s$ and upon removal of commonly associated cytoskeletal factors between treatments, it appears as though palmitoylation is sufficient for lipid raft localization and that upon chronic treatment cytoskeletal factors maintain a fraction in the raft (TABLE V). This suggests that chronic treatment with antidepressants mediates depalmitoylation of $G\alpha_s$.

3.3.4 Implications

$G\alpha_s$ is a membrane-associated protein that tends to exist in cholesterol rich lipid raft micro-domains (151). Lipid rafts contain many of the anchoring cytoskeletal-associated membrane structures and facilitate molecular association(s) of a vast array of different membrane-embedded and associated proteins to initiate intracellular signaling. While lipid rafts can facilitate this clustering of signaling molecules, the rigid structure afforded by increased cholesterol content appears to have a globally dampening effect on $G\alpha_s$ signaling by inhibiting association(s) between raft and non-raft based molecules (132, 150). Dampened signaling, through $G\alpha_s$ and/or $G\alpha_s$ coupled receptors, is consistent with the observed increase in $G\alpha_s$ association with rafts as well as damped cAMP signaling seen in MDD (151). Accordingly, $G\alpha_s$ content within lipid rafts is diminished after chronic treatment with fluoxetine, desipramine, and escitalopram (152, 153) and lipid raft disruption through cholesterol depletion or cytoskeletal disruption displaces many raft proteins, but activation or antidepressant treatment displaces only $G\alpha_s$ (139, 153).

Displacement of $G\alpha_s$ from lipid rafts could mean intracellular translocation. However, increased physical coupling between $G\alpha_s$ and AC after chronic, but not acute antidepressant treatment (157) suggests $G\alpha_s$ remains membrane associated. Moreover, the overall amount of $G\alpha_s$ is unchanged in response to chronic antidepressant treatment and neither the intrinsic GTP binding nor intrinsic AC activity was altered (154). All of which suggest a significant role for $G\alpha_s$ in depression and in mediating the physiological effects of antidepressants.

That being said, translocation of $G\alpha_s$ from the plasma membrane correlates with activation-induced depalmitoylation (88, 107, 128), which is exclusive to $G\alpha_s$ (129-131), whereas $G\alpha_i$ is both myristoylated and palmitoylated in the N-terminus (205-207). The secondary myristoylation presumably impairs activation induced subcellular redistribution even though myristoylation by itself actually provides barely enough energy to attach a protein to the plasma membrane (137). This is significant, as active $G\alpha_s$ dissociates from its coupled GPCR and $G\beta\gamma$ subunits, but $G\alpha_s$ needs to possess a mechanism for maintaining membrane localization. Mutation of the N-terminus of $G\alpha_s$ affects acylation state and prevents translocation from lipid rafts following chronic escitalopram treatment. Both myristoylated/palmitoylated and palmitoylation deficient $G\alpha_s$ do not appreciably localize to lipid rafts, effectively making thusly modified $G\alpha_s$ "antidepressant–insensitive." Therefore, the reversible nature of palmitoylation of $G\alpha_s$ appears to be integral to the antidepressant response.

Aim 2B. Chronic treatment with some, but not all antidepressants mediates depalmitoylation of Gα$_s$, which affects lipid raft anchoring of Gα$_s$.

3.4.1 Background

A single, reversible palmitoylation catalyzed by a DHHC motif containing protein (DHHC3/7) (124) anchors Gα$_s$ to the plasma membrane and may control its anchoring with lipid rafts (3, 104, 125), in part because it regulates the association between Gα and Gβγ, the latter associating with the membrane via prenylation.   However, the precise molecular association(s) anchoring Gα$_s$ to lipid rafts is not entirely clear, even considering the determination of proteins found in association with Gα$_s$ in the lipid rafts of C6 cells expressing each acylation mutant. Acylation of G protein alpha subunits appears important for membrane anchoring (3, 124, 125), in part because it regulates the association between Gα and Gβγ, the latter associating to the membrane via prenylation. Moreover, Gα$_s$ is the only Gα subunit that exhibits activation-induced translocation coupled with depalmitoylation (88, 107, 128-131).

Gα$_s$ is the most difficult Gα to extract from the membrane with detergent (139), likely owing to the fact that palmitate, in contrast with myristate (137), is more than capable of mediating strong association with the plasma membrane even though it is reversible (104).   However, secondary myristoylation impairs activation induced subcellular redistribution (Figures 22 and 23). Taken together, it may be possible that one, or a companion, of the potential mechanism(s) of antidepressant-induced translocation of Gα$_s$ is via attenuating its N-terminal palmitoylation. Although antidepressants mediate translocation of Gα$_s$ from lipid rafts, it is unknown if they activate Gα$_s$. Antidepressants do however induce dissolution of the Gα$_s$:Tubulin

complex (200), which may mean that Gα$_s$ is activated because Tubulin transfers its GTP to Gα$_s$ (199).

3.4.2 Results

Gα$_s$ is the most difficult Gα to extract from the membrane with detergent (139), likely owing to the fact that palmitate (104), in contrast with myristate (137), is capable of mediating strong association with the plasma membrane. A single, reversible palmitoylation catalyzed by a DHHC motif containing protein (DHHC3/7) (124) anchors Gα$_s$ to the plasma membrane. Although the precise molecular association(s) anchoring Gα$_s$ to lipid rafts is not entirely clear, palmitoylation controls its anchoring with lipid rafts (3, 104, 124, 125) and regulates the association between Gα and Gβγ, the latter associating to the membrane via prenylation. Moreover, Gα$_s$ is the only Gα subunit that exhibits activation-induced depalmitoylation (88, 107, 128, 130, 131). However, secondary myristoylation impairs antidepressant mediate subcellular redistribution (Figures 22 and 23). Taken together, it may be possible that one, or a companion, of the potential mechanism(s) of antidepressant-induced translocation of Gα$_s$ is via attenuating its N-terminal palmitoylation.

Although antidepressants mediate the translocation of Gα$_s$ from lipid rafts, it is unknown if they its depalmitoylation. We tested the effects of chronic treatment (72 hrs) with 10 µM phenelzine, desipramine, fluoxetine, escitalopram, R-citalopram, and olanzapine on Gα$_s$ palmitoylation using C6 glioma cells. Lipid rafts were extracted from purified membranes via sucrose density gradient and Gα$_s$ immunoprecipitated. We determined the palmitoylation status of Gα$_s$ in response to each pharmacological

treatment using an established protocol with some minor, but important modifications. Immunoprecipitations of Gα$_s$ were concentrated and free sulfhydryls blocked with N-ethylmaleimide (NEM). Irreversible covalent coupling of free cysteine sulfhydryls with NEM allows for differentiation between antidepressant-mediated versus alternatively induced depalmitoylation of Gα$_s$. This is an important designation because subjecting Gα$_s$ to enzymatic digestion and subsequently LC-MS may in fact induce depalmitoylation, generating a false positive. To further control for false positive detection, we cleaved sites of S-palmitoylation with hydroxylamine as well as retained a sample omitting this step. The resulting protein profiles in each fraction contains the nascent cysteine residues covalently modified with NEM (antidepressant mediated depalmitoylation), whereas the cysteine residues modified by S-acylation (i.e. Cys3 in the N-terminus of Gα$_s$) are free following reduction with hydroxylamine (antidepressant did not mediate depalmitoylation). Thus, when analyzing the response to chronic antidepressant via LC-MS, the resulting peptides from Tryptic digestion will produce ions corresponding to NEM-Gα$_s$ if the drug mediates de-palmitoylation of Gα$_s$, or SH-Gα$_s$ /palmitoyl-Gα$_s$ if the drug does not mediate de-palmitoylation of Gα$_s$.

Palmitoylation of wild type Gα$_s$ occurs on the third cysteine residue, and digestion with Trypsin results in a peptide sequence: MGCLGNSK or the longer MGCLGNSKTEDQR if the first cleavage is missed. Although it is not necessary, during LC/MS analysis other modification(s) may occur. Methionine may be singly or doubly oxygenated, asparagine and glutamine may be deamidated, and if the particular drug mediates a depalmitoylation of Gα$_s$, there will be a conjugated NEM on cysteine. When combining these modification possibilities, with the possibility that cysteine can be

conjugated to NEM, palmitate, or depalmitoylated, as well as be doubly, triply, or quadrupally charged creates several possible mass to charge (m/z) peak profiles. However, an overwhelming number of peptides carry a charge state of two or three (208). Stated another way, the maximum number of charges a peptide carries correlates well with the number of amino acid residues present able to accept a proton at low pH (K, R, H, and the N-terminus). Therefore, we identified only the two or three charge states for the putative $G\alpha_s$ peptides.

The treatment naive control sample produced doubly charged, deamidated, oxidized, and palmitoylated $G\alpha_s$ peptides without hydroxylamine (847.3 m/z, MGC*LGNSKTEDQR) and triply charged, doubly oxidized, deamidated, and depalmitoylated when treated with hydroxylamine (486.2 m/z, MGC*LGNSKTEDQR) (Figure 24). As expected, $G\alpha_s$ retains its acylation status in the absence of antidepressant treatment. By contrast, chronic treatment with phenelzine resulted in a doubly charged, doubly deamidated, NEM conjugated peptide (783.62 m/z, MGC*LGNSKTEDQR) (Figure 25), desipramine a triply charged, doubly deamidated, doubly oxidized, NEM conjugated peptide (533.2 m/z, MGC*LGNSKTEDQR) (Figure 26), fluoxetine a doubly charged, deamidated, oxidized, NEM conjugated peptide (791.8 m/z, MGC*LGNSKTEDQR) (Figure 27), and escitalopram a doubly charged, doubly deamidated, doubly oxidized, NEM conjugated peptide (799.32 m/z, MGC*LGNSKTEDQR) (Figure 28). Whether the peptide is secondarily modified is physiologically not relevant, but the fact that NEM conjugated peptides were produced from samples in each of these cases clearly indicates that these antidepressants mediated the depalmitoylation of $G\alpha_s$. Chronic treatment with either R-citalopram or the

antipsychotic olanzapine do not produce NEM conjugated peptides, but rather retain palmitoylated peptides in the absence of hydroxylamine treatment. Chronic treatment with R-citalopram resulted in a doubly charged, deamidated, oxidized, palmitoylated peptide (847.3 m/z, MGC*LGNSKTEDQR) (Figure 29) and olanzapine a triply charged, deamidated, doubly oxidized, palmitoylated peptide (570.64 m/z, MGC*LGNSKTEDQR) (Figure 30). All peak identifications were within ±10 ppm of expected.

In order to obtain relative rates of depalmitoylation, the palmitoylated peaks must also be identified under each antidepressant treatment. NEM conjugated peaks represent the depalmitoylated versions for chronically treated samples and hydroxylamine treatment produces the depalmitoylated peak in the treatment naïve, R-citalopram, and olanzapine samples. Again, the depalmitoylated peak produced in the control sample was triply charged, doubly oxidized, deamidated, and depalmitoylated when treated with hydroxylamine (486.2 m/z, MGC*LGNSKTEDQR), whereas chronic treatment with R-citalopram resulted in a doubly charged peptide (719.82 m/z, MGC*LGNSKTEDQR) and olanzapine a doubly charged and oxidized peptide (727.81 m/z, MGC*LGNSKTEDQR). A palmitoylated peak for chronic treatment with phenelzine resulted in a quadrupally charged deamidated peptide (420.15 m/z, MGC*LGNSKTEDQR), desipramine a doubly charged peptide (838.82 m/z, MGC*LGNSKTEDQR), fluoxetine a quadrupally charged, deamidated, doubly oxidized peptide (428.15 m/z, MGC*LGNSKTEDQR), and escitalopram a quadrupally charged, deamidated, doubly oxidized peptide (428.15 m/z, MGC*LGNSKTEDQR). The relative depalmitoylation of $G\alpha_s$ mediated by chronic treatment with each antidepressant is produced by normalizing the depalmitoylated peptide peak intensity (NEM for

antidepressants and hydroxylamine for other conditions) to both the palmitoylated peak intensity as well as the protein content before digestion.

Peptides conjugated to NEM were identified in only antidepressant treated samples. It is important to note that calculated depalmitoylation values are more qualitative than quantitative as there is not an internal standard to compare peaks to in a this quasi label free system. Regardless, chronic treatment with phenelzine, desipramine, fluoxetine, or escitalopram mediates the depalmitoylation of $G\alpha_s$ (Figure 31). Therefore, drugs that mediate the translocation of $G\alpha_s$ from lipid rafts (161), appear to do so through accumulating in lipid rafts and mediating the depalmitoylation of $G\alpha_s$. By contrast, drugs that do not mediate redistribution of $G\alpha_s$ (R-citalopram and olanzapine) do not accumulate in lipid and do not mediate the depalmitoylation of $G\alpha_s$. Antidepressant-mediated depalmitoylation of $G\alpha_s$ appears to be an important mechanism of action that may explain on a biochemical level the hysteresis of their action.

However, to fully determine whether antidepressant mediated depalmitoylation of $G\alpha_s$ is integral to antidepressant function, pharmacological inhibition of depalmitoylating enzymes must also be combined with chronic antidepressant treatments. For example, APT1 depalmitoylates $G\alpha_s$ (140), for which the inhibitor Palmostatin B (209) could be used to confirm the importance of depalmitoylation of $G\alpha_s$ in the chronic antidepressant response. Due to the effects on other palmitoylated proteins, Palmostatin B would likely have little therapeutic application, but a short-lived acute activator of APT1, or direct inhibitor of $G\alpha_s$ palmitoylation could be therapeutically useful in combination with chronic antidepressant treatment.

Figure 24. Gα_s is natively palmitoylated and is not depalmitoylated via preparation or the mass spectrometry instrumentation.
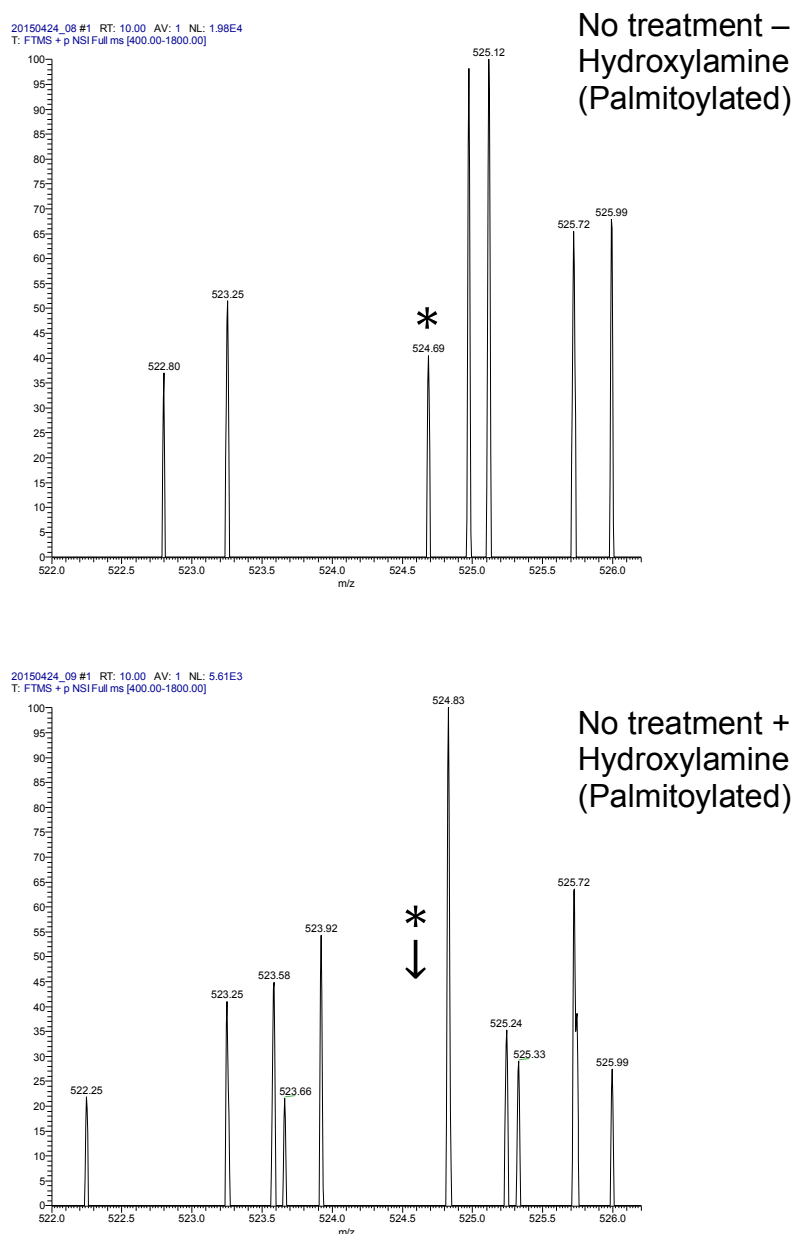
Gα_s was immunoprecipitated with an anti Gα_s mAb from plasma membranes of treatment naïve C6 cells. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated MGCLGSNKTEDQR peptide without hydroxylamine (524.69 m/z) and depalmitoylated peptide with hydroxylamine treatment (422.88 m/z) confirmed the subsequent depalmitoylation results are drug mediated and not through instrumental analysis.

Figure 25. Chronic treatment with phenelzine mediates depalmitoylation of Gα$_s$.
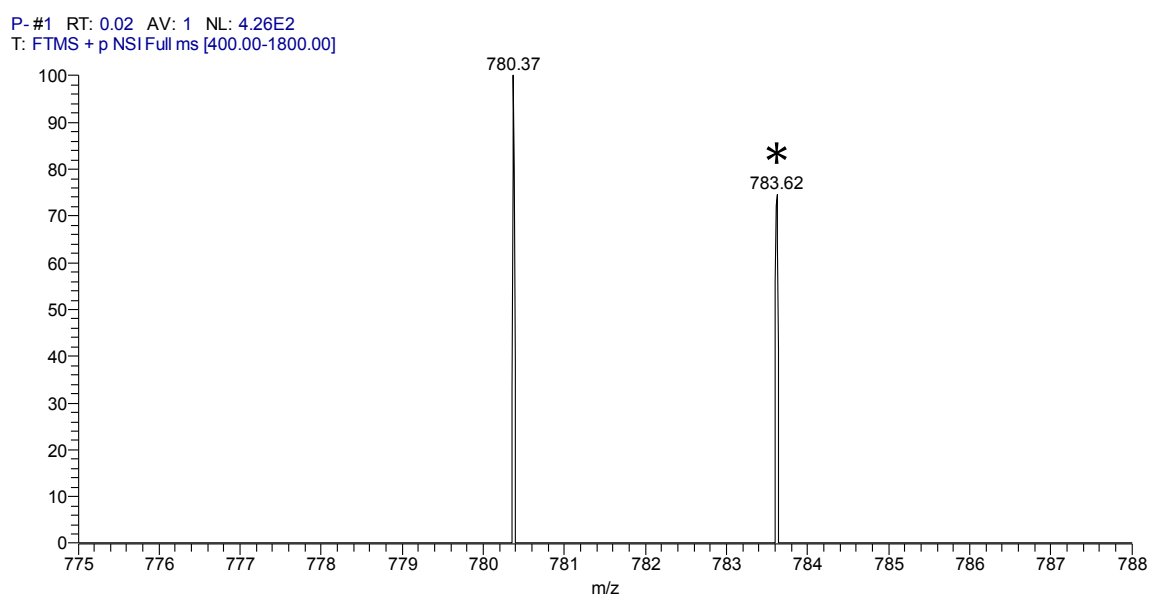
P- #1 RT: 0.02   AV: 1   NL: 4.26E2
T: FTMS + p NSI Full ms [400.00-1800.00]

Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with phenelzine. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated MGC*(NEM)LGNSKTEDQR peptide (783.32 m/z) confirmed that phenelzine mediates depalmitoylation of Gα$_s$.

Figure 26. Chronic treatment with desipramine mediates depalmitoylation of Gα$_s$.
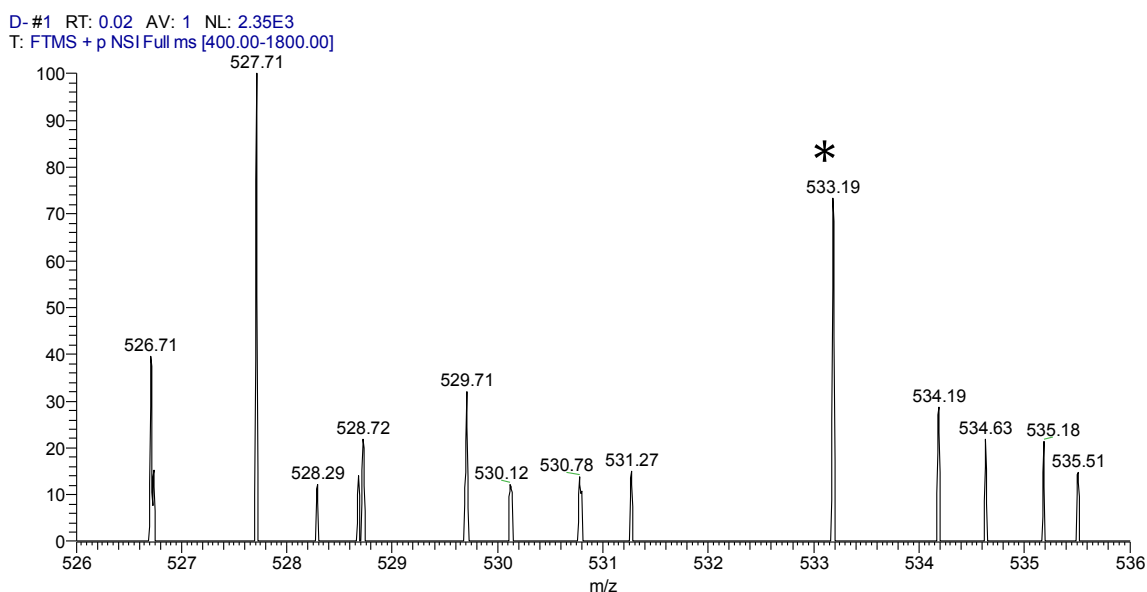
Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with desipramine. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated M*GC*(NEM)LGN*SKTEDQ*R peptide (533.2 m/z) confirmed that desipramine mediates depalmitoylation of Gα$_s$.

Figure 27. Chronic treatment with fluoxetine mediates depalmitoylation of Gα$_s$.
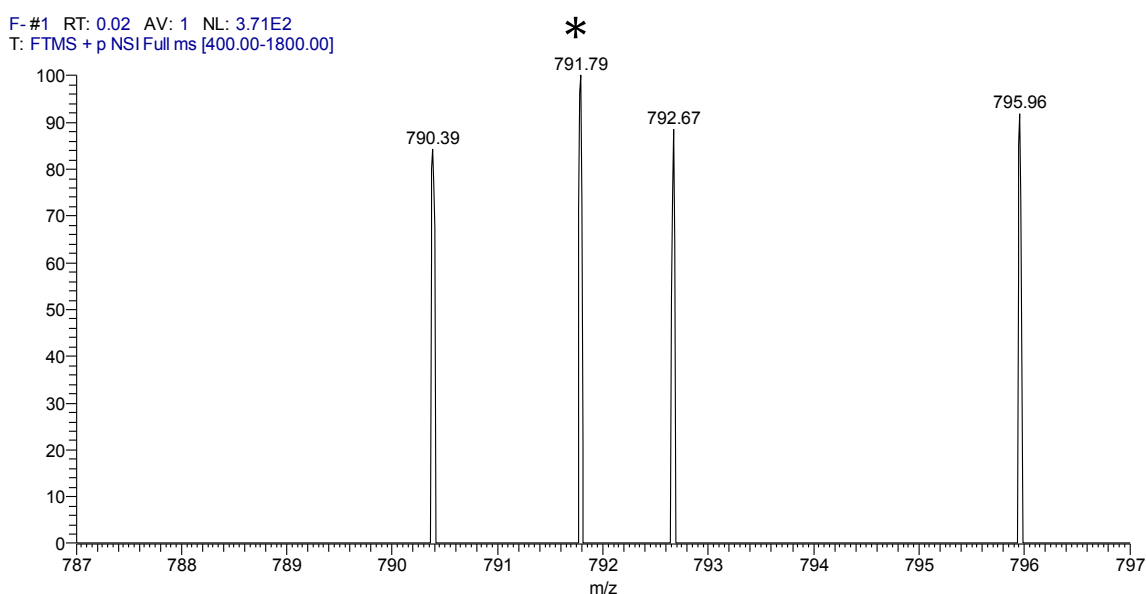
Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with Fluoxetine. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated M*GC*(NEM)LGNSKTEDQR peptide (791.8 m/z) confirmed that Fluoxetine mediates depalmitoylation of Gα$_s$.

Figure 28. Chronic treatment with escitalopram mediates depalmitoylation of Gα_s.
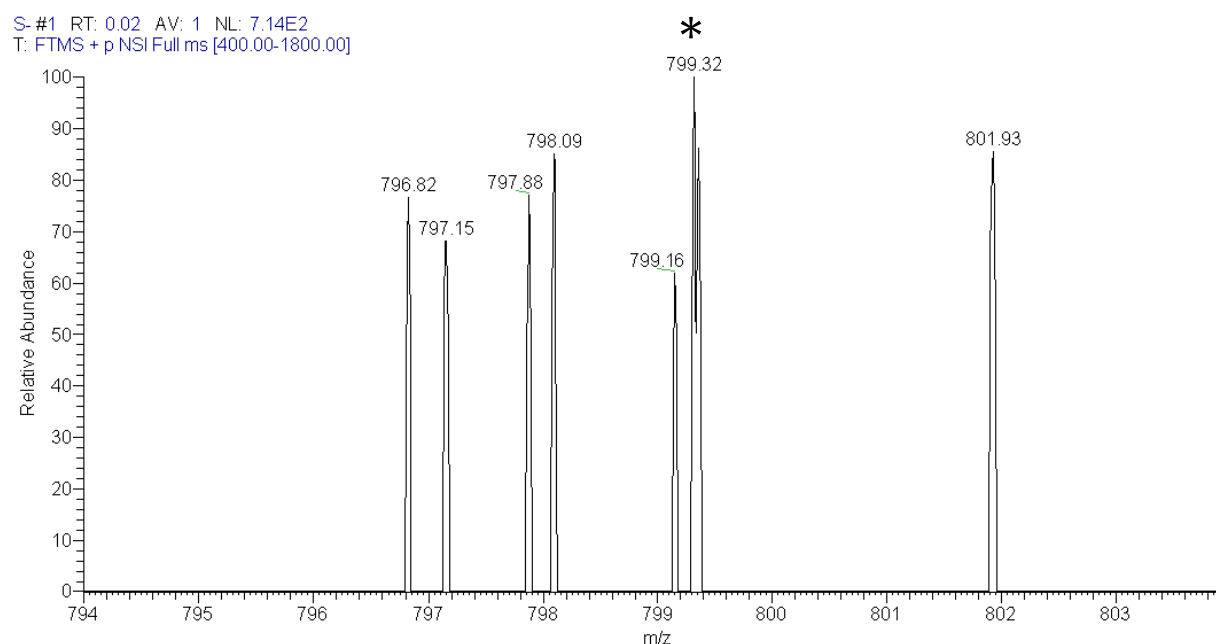
Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with escitalopram. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated M*GC*(NEM)LGN*SKTEDQR peptide (799.32 m/z) confirmed that escitalopram mediates depalmitoylation of Gα$_s$.

**Figure 29. Chronic treatment with R-citalopram does not mediate depalmitoylation of Gα$_s$.**
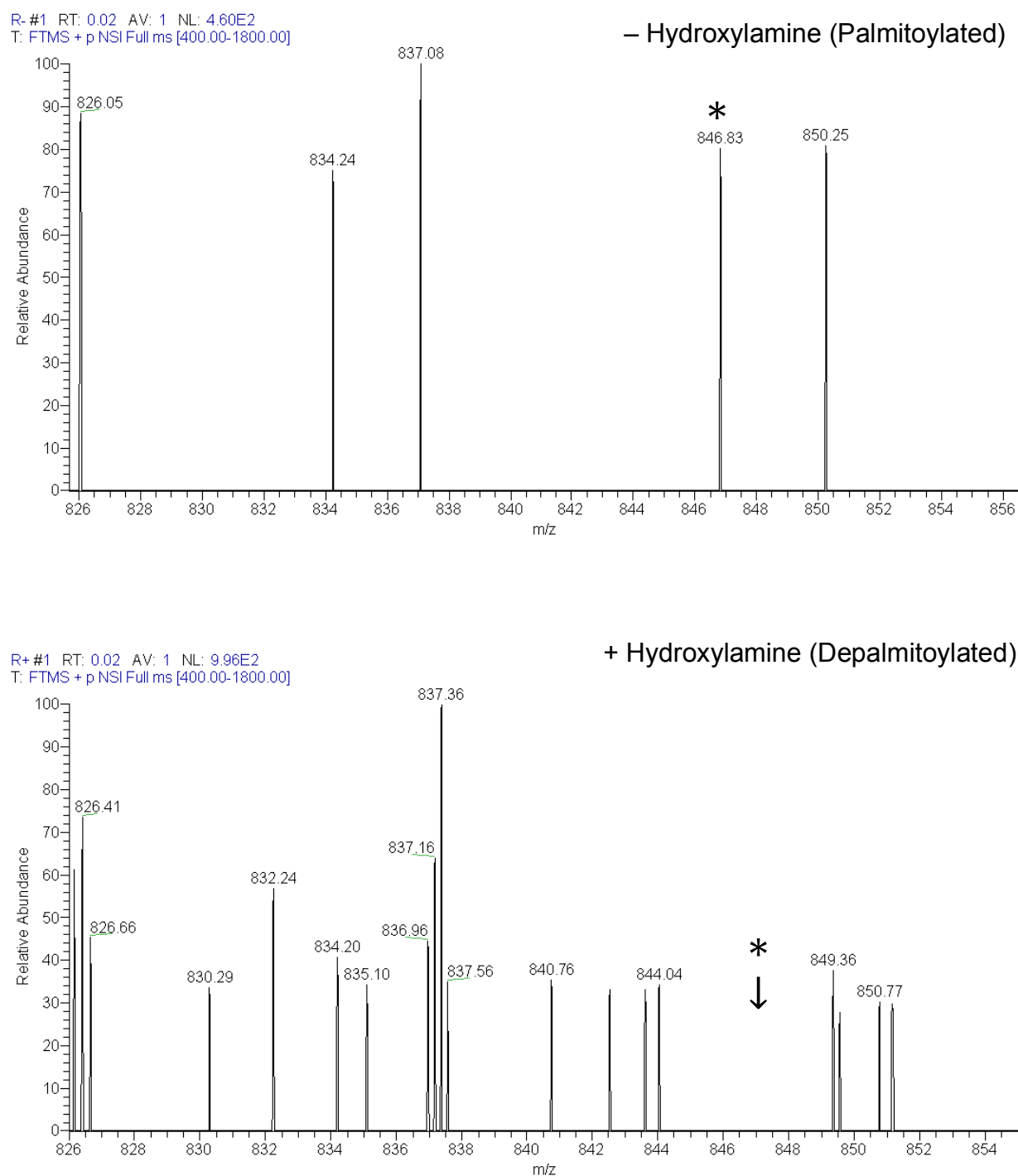
Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with R-citalopram. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated M*GC*LGN*SKTEDQR peptide (847.3 m/z) confirmed that R-citalopram does not mediate depalmitoylation of Gα$_s$.

Figure 30. Chronic treatment with Olanzapine does not mediate depalmitoylation of Gα$_s$.
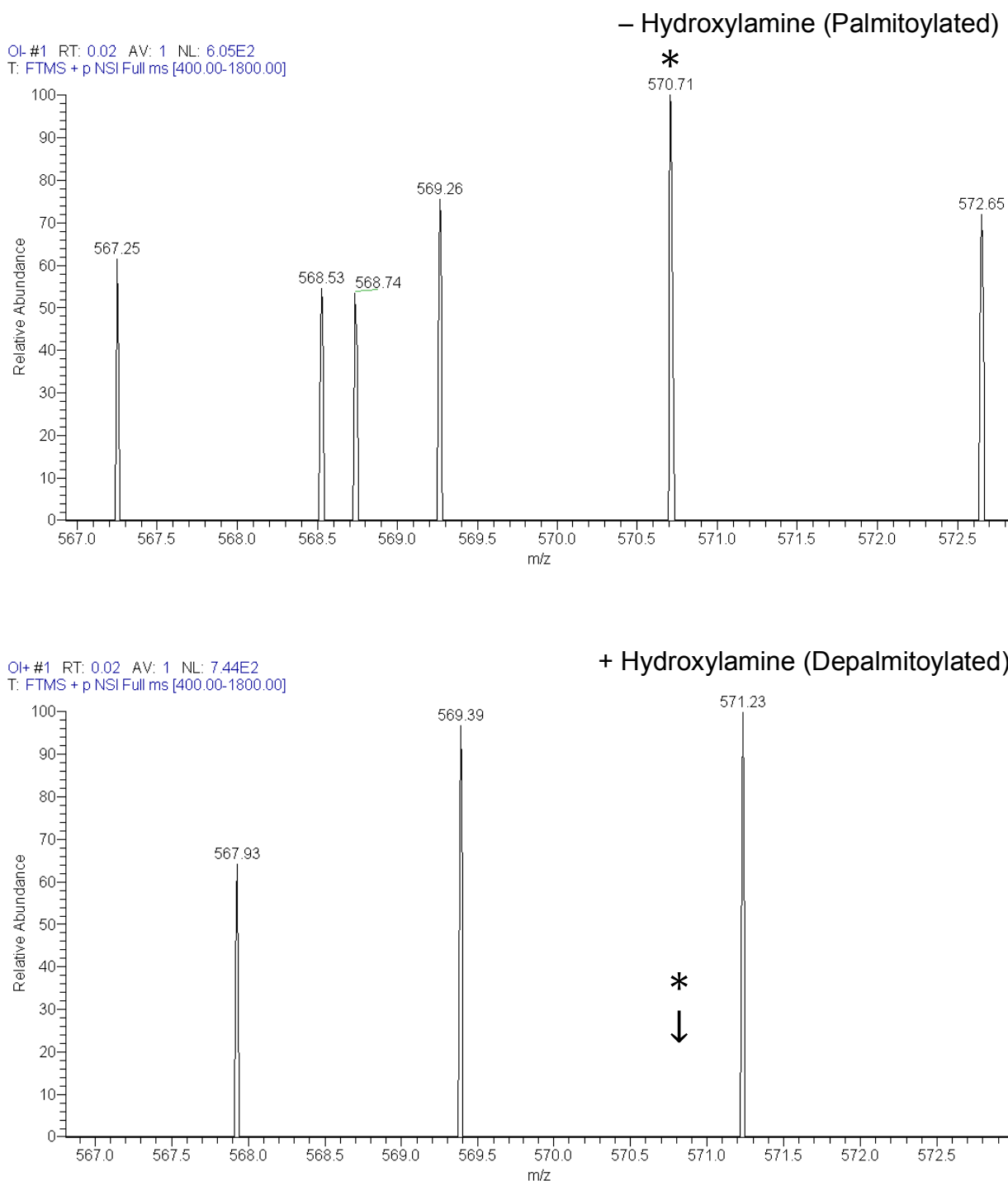
Gα$_s$ was immunoprecipitated with an anti Gα$_s$ mAb from plasma membranes of C6 cells chronically treated with Olanzapine. Precipitates were treated with NEM, divided in half and half was treated with hydroxylamine, and the other retained as control. Samples were digested with Trypsin, run through reverse phase and size exclusion columns, and subjected to ESI-MS/MS analysis. Detection of peaks corresponding to a palmitoylated M*GC*LGN*SKTEDQR peptide (570.64 m/z) confirmed that Olanzapine does not mediate depalmitoylation of Gα$_s$.

Figure 31. Chronic treatment of C6 glioma cells with antidepressant drugs affects the palmitoylation status of Gα_s.
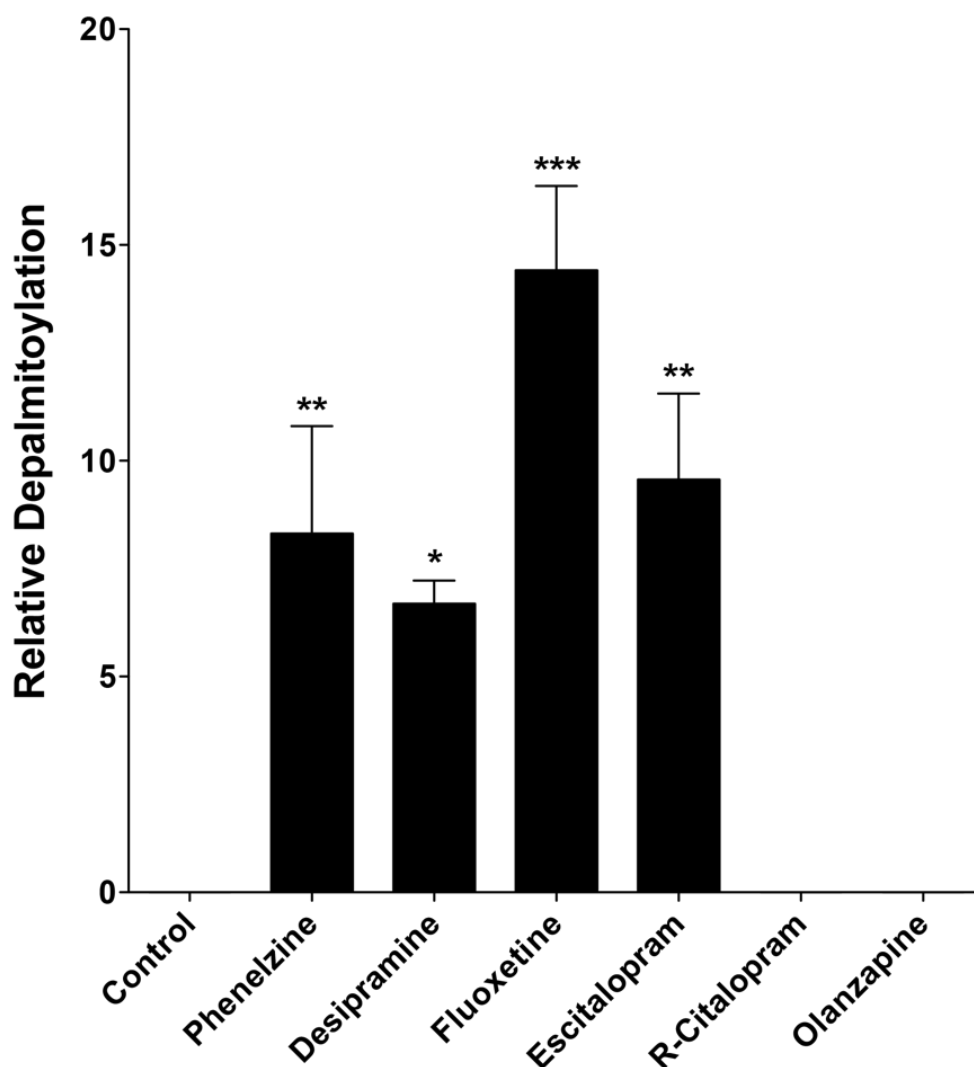
LC-MS detection of N-ethylmaleimide (NEM) conjugated peptides MGCLGNSK$^{\downarrow}$TEDQR$^{\downarrow}$ revealed phenelzine (MAOI), desipramine (TCA), fluoxetine (SSRI), and escitalopram (SSRI), but not its stereoisomer R-citalopram or the antipsychotic olanzapine mediate the depalmitoylation of Gα$_s$. The ability to depalmitoylate appears to correlate with accumulation capacity of each drug in the lipid raft and suggests that antidepressant-mediated depalmitoylation of Gα$_s$ is a potential biochemical mechanism explaining antidepressant hysteresis. (n=3; *, $p<0.05$; **, $p<0.01$; ***, $p<0.01$).

3.4.3 Conclusions

The implication of these experiments is that acylation state of Gα$_s$ is a significant

indicator of subcellular localization, may direct the molecular associations in which Gα$_s$

participates, and is a significant molecular event that plays a role in the delayed

therapeutic response to antidepressants. As expected, antidepressants enrich Gα$_s$ in

the non-raft fraction and this Gα$_s$ fraction is largely depalmitoylated. One possible

difficulty with these experiments is that not all of the Gα$_s$ is immunoprecipitated. To

address this possibility, I employed conformation specific nanobodies directed at

different regions of Gα$_s$ (NB35 and NB37) (164). The reasoning behind such an

approach lies in the fact that nanobodies are derived from only the antigen recognition

region of camelid antibodies, minimizing nonspecific associations, are very small at 10-

15-kDa, reducing steric restrictions during precipitation, and are available as cDNA

encoding a 6His tag for expression in and purification from *E.coli*. Precipitation with

NB35 restricts the detected associations to those found only with activated Gα$_s$. This

distinction is important for identifying those proteins that maintain Gα$_s$ in lipid rafts, but

restricting the population of Gα$_s$ immunoprecipitated to only the active state is

counterproductive to the intended purpose of identifying antidepressant induced

depalmitoylation of Gα$_s$. This is because activated Gα$_s$ is depalmitoylated (105), but

largely cytosolic (159). For this principal reason, I precipitated Gα$_s$ using a monoclonal

antibody for Gα$_s$.

Our lab has previously shown that antidepressants mediate their effects through

modulating the subcellular localization of Gα$_s$ (132, 139, 151-153, 157, 159-163).

However, precise transmission of these effects from outside of the cell to Gα$_s$ on the

inside of the cell remains relatively obscure. Lipid rafts tightly coordinate cholesterol,

saturated membrane lipids, and acylated proteins, which have the effect of trapping

inactive $G\alpha_s$ until it GTP bound (active) and depalmitoylated. The observations of this

dissertation demonstrating that antidepressants accumulate in lipid rafts and

depalmitoylate $G\alpha_s$ in cells that do not express the proteins (reuptake transporters) for

which the later generation antidepressants are designed to bind, suggests that

alternative targets exist that are likely more important for their therapeutic action than

are the reuptake transporter proteins. Moreover, the drugs that accumulate in lipid rafts

are those that depalmitoylate $G\alpha_s$ (Aims 1 and 2 herein) and increase its FRAP recovery

half time (161), whereas as those that do not accumulate, do not depalmitoylate and do

not increase the FRAP recovery half time of $G\alpha_s$ (161). These results would seem to

indicate that instead of binding the targets they were designed for, antidepressants are

perhaps binding and activating a depalmitoylating enzyme(s) that has yet to be

identified or inhibiting the palmitoylating enzyme of $G\alpha_s$, DHHC3/7 (124). Alternatively,

antidepressants could be activating a $G\alpha_s$ coupled receptor or even directly activating

$G\alpha_s$, both of which would induce its activation dependent depalmitoylation.

3.4.4 Implications

It is possible that one, or more, of the potential mechanism(s) of antidepressant-induced translocation of $G\alpha_s$ is via attenuating its N-terminal palmitoylation. However, the issue of how a depalmitoylated internalized $G\alpha_s$ is able to associate with membrane imbedded AC remains? We have hypothesized that Tubulin association is a molecular event that locks $G\alpha_s$ into lipid rafts and have shown that chronic antidepressant treatment disrupts this association (192, 200). However, we have also observed that GTP bound $G\alpha_s$ appears to be the preferential state bound to Tubulin (156, 191), even though Tubulin is able to activate $G\alpha_s$ through GTP exchange (29, 199). Different isoforms of Tubulin do in fact remain in association with $G\alpha_s$ following activation by chronic antidepressant treatment, which appears to correlate with the acylation state and subcellular localization of $G\alpha_s$. Tubulin itself is palmitoylated (101-103, 106, 108, 109), which might enable $G\alpha_s$ to translocate to non-raft regions of the plasma membrane while depalmitoylated, but this complex would need to dissociate upon proximal localization with AC as the binding sites of $G\alpha_s$ are in too close of proximity. The preceding presumes that antidepressants themselves activate $G\alpha_s$ as opposed to another protein that mediates translocation from lipid rafts. In light of the unlikely event of forming a ternary complex between AC, $G\alpha_s$, and Tubulin in response to chronic antidepressant treatment, it is probable that the palmitoylation remains long enough for $G\alpha_s$ to associate with AC before activation (i.e. depalmitoylated).

However, presented herein I have demonstrated that antidepressants accumulate in lipid rafts and depalmitoylate $G\alpha_s$ in cells that do not express the proteins (reuptake transporters) for which the later generation antidepressants are designed to

bind. This suggests that alternative targets exist that are likely more important for their therapeutic action than are the reuptake transporter proteins. Moreover, the drugs that accumulate in lipid rafts are those that depalmitoylate $G\alpha_s$ (Aims 1 and 2 herein) and increase its fluorescence recovery after photobleaching (FRAP) recovery half time (161), whereas as those that do not accumulate, do not depalmitoylate and do not increase the FRAP recovery half time of $G\alpha_s$ (161).

These results would seem to indicate that instead of binding the targets they were designed for, antidepressants are perhaps binding and activating a depalmitoylating enzyme(s) that has yet to be identified or inhibiting the palmitoylating enzyme of $G\alpha_s$, DHHC3/7 (124). Alternatively, antidepressants could be activating a $G\alpha_s$ coupled receptor or even directly activating $G\alpha_s$, both of which would induce its activation dependent depalmitoylation. Regardless, one or more of the proteins in association with $G\alpha_s$ is likely key to establishing the utility of $G\alpha_s$ association with lipid rafts as a barometer of depression and its translocation a metric for antidepressant efficacy.

Our lab has previously shown that antidepressants mediate their effects through modulating the subcellular localization of $G\alpha_s$ (132, 139, 151-153, 157, 159-163). However, the precise transmission of these effects from outside of the cell to $G\alpha_s$ on the inside of the cell remains relatively obscure. Lipid raft microdomains tightly coordinate cholesterol, saturated membrane lipids, and acylated proteins, which has the effect of trapping inactive and palmitoylated $G\alpha_s$. The predominant functional class of proteins found in association with $G\alpha_s$ in lipid rafts is the scaffolding/remodeling protein class. As certain antidepressants gradually accumulate over time in lipid rafts, and

depalmitoylation appears integral to fully mediating the antidepressant response through Gα$_s$, the coupling of an unidentified protein(s) is of paramount significance to fully understanding depression, antidepressant pharmacology, and to develop more targeted therapies. Once identified, this target-drug interaction is available for exploitation in the development of adjunct therapies that reduce the hysteresis of therapeutic action and/or more finely tune the pharmacology of antidepressant drugs.

Chapter 4 – General Discussion, Significance, and Future Directions

4.1. Antidepressant modes of action and novel receptor identification

Depression is the leading cause of long term disability in the industrialized world (6) and it is estimated that ~15% of the population is affected at some time in their life (7). Although depression is a significant health problem in the United States and antidepressants are the most widely prescribed class of drugs, the precise mechanisms by which each function may vary from the transporters normally described as their targets. Moreover, the STAR*D trial taught us that nearly 70% of sufferers of MDD fail to respond to an initial trial of citalopram and 40% of those relapse within a year (45, 48, 49). Previous studies from our laboratory have shown that chronic antidepressant treatment mediates movement of $G\alpha_s$ out from under the inhibitory effects of lipid rafts. However, the precise biochemical mechanisms leading to the manifestation of symptoms as well as the molecular mechanisms that account for this phenomenon are not well defined and present a significant knowledge gap in our understanding of the complex pharmacology antidepressants display.

Older theories regard depression as an imbalance (i.e. deficiency) in monoamine neurotransmitters in the brain, which is termed the monoamine hypothesis of depression. However, the monoamine hypothesis fails to address the fact that antidepressants exhibit delayed therapeutic action (hysteresis), of at least a week and often longer, whereas the increase in monoamine density occurs relatively soon after treatment begins (40-43). Antidepressant hysteresis suggests that mechanism(s) apart from inhibiting the serotonin, dopamine, and/or adrenergic receptors exist (40-43), which is an indictment of the prevailing dogma that antidepressants work via a

presynaptic mechanism. Currently available antidepressants enhance the density of serotonin, norepinephrine, or a combination of the two in the synaptic cleft; targeting either the reuptake transporters or monoamine oxidase (MAO). As the low hanging fruit has essentially all been picked, it is not surprising that treatment efficacies are not improving, as each new drug is essentially a derivative of the one that preceded it.

It is necessary to understand the pharmacological mechanism(s) by which current antidepressants act, so that we can design novel adjunct therapies for the treatment of MDD.   Evidence from our laboratory and others, including the data contained within this dissertation, suggest that long-term (chronic) antidepressant treatment also engages signaling pathways apart from increasing monoamine density in the synaptic cleft. Moreover, chronic antidepressant treatment results in an increased accumulation of cellular cAMP (34) and recent PET evidence has shown that cAMP is globally diminished in depressed patients, but rebounds in response to antidepressants (39), which suggests involvement of the cAMP generating system: $G\alpha_s$–AC–cAMP in mediating the chronic antidepressant response. The increase in monoamines as a result of antidepressant therapy cannot necessarily be discounted, but the fact that such a substantial hysteresis exists in their therapeutic action suggests other pharmacological actions are being conducted simultaneously or perhaps even apart from the increased monoamine densities.

The next logical step is to identify the receptor(s) for which antidepressants are mediating their effects in cells that lack a monoamine transport system. There are several ways to identify a target receptor of a drug, but the highest utility option for a system in which the receptor might also be internalizing, suggested by the accumulation

of escitalopram at 120 hrs treatment, is to fluorescently tag the drug. Much of the work

in this dissertation focuses on the apparent differences between escitalopram and its

inactive isomer, R-citalopram, binding in lipid rafts. Fluorescently tagged drugs could

then be followed microscopically and localization correlated with $G\alpha_s$-GFP. The

presence of the comparatively large fluorophore however could sterically restrict this

particular application. For example, screening of insect (Sf9) cells stably expressing the

human β_2-adrenoceptor with bordifluoropyrromethene (BODIPY), fluorescein, and

related derivatives of the beta-adrenergic ligand CGP 12177 showed that only BODIPY-

CGP gave a signal sufficient for measuring equilibrium rate constants by photon

counting or spectrofluorometry. Moreover, the cell-bound fluorescence was restricted to

the cell surface at both 4 and 30 ^{o}C (210) and the fluorophore could be active at

alternative sites that complicate the pharmacology (211).   Perhaps, quantum dots are a

more viable option for observing trafficking of antidepressants and for the identification

of the as of yet unidentified binder of Citalopram. This is because the surface chemistry

of quantum dot nanocrystals allows them to be functionalized with targeting ligands,

antidepressants in this case, and their optical properties make them suitable for both *in

vitro* as well as *in vivo* tracking (212). Quantum dots have been developed for

antidepressant drug development via a SERT competition assay (213), but may also be

used for identifying the unidentified target in monoamine transport system deficient C6

cells (214).

Citalopram contains a nitrile group that when reduced would be a perfect site for

the addition of an N-hydroxyl succinimide (NHS) fluorophore. While seemingly a simple

straightforward process, reduction of nitriles with lithium aluminum hydrate ($LiAlH_4$) to a

primary amine is not particularly easy for several reasons. The first of which is the reactivity of $LiAlH_4$ with water, necessitating reaction under inert gas, and the second being that dimerization of Grignard reagents is an issue (215); both are managed through careful preparation of all reactants. Alternatively, the nitrile can be biologically converted to a primary amine with nitrile hydratase (216). Regardless, rhodamine labeled citalopram exists for cellular imaging (217), but since the nitrile is gone due to the labeling with rhodamine, the fluorine on the opposite end of citalopram could be used for photo affinity labeling of the target protein(s). Photo affinity labeling is necessary to make sure the drug and target do not separate during the reducing conditions of electrophoretic transfer and the gel purification of the red bands prior to mass spectrometric analysis of the identified target (218). Therefore, obtaining fluorescent-citalopram, or as described previously, developing quantum dot labeled antidepressant, binding the unidentified target(s), and cutting gel bands corresponding to the quantum dot's particular fluorescence, would be extremely useful for identifying the target(s) of escitalopram in lipid rafts.

4.2. Antidepressant-mediated activation of Gα_s.

Although, a simplistic explanation for the observed effects of antidepressants on Gα_s localization and depalmitoylation is to speculate that the antidepressants directly bind and activate Gα_s. In order to do so, the drug must cross the plasma membrane of a target cell before coming into contact with Gα_s. Based solely upon the partitioning coefficients for the drugs that I used in this dissertation, with the exception of olanzapine, this could be possible as they are more hydrophilic at physiologic pH (Figure 17); olanzapine does not have the observed effects through accumulation, depalmitoylation, nor the ability to mediate the translocation of Gα_s from lipid rafts. At face value, the direct association with and activation of Gα_s by antidepressants logically makes sense, but this raises the issue of a transport protein(s).

As presented in this dissertation, antidepressants gradually accumulate in the plasma membrane of C6 cells and mediate the depalmitoylation of membrane associated Gα_s. The depalmitoylation of Gα_s also occurs upon activation (88, 107, 128). Gα_s has several sites for which antidepressants might bind. There are two distinct Mg^{2+} binding sites (219) as well as the GTP binding GTPase domain in Gα_s that could potentially be sites of interaction. Presumably, interaction with one or more of these sites would induce a conformational change in Gα_s. Specifically, conformational rearrangement associated with activation of Gα_s results in a rotation of Tryptophan residues in its Switch domains outward, which allows for a fluorescent emission at 340 nm (182, 183). Addition of sodium fluoride and aluminum tetrachloride to purified Gα_s, activates it, results in such a rearrangement, and serves as a positive control.

The results contained herein suggest that antidepressants mediate the depalmitoylation of $G\alpha_s$, which is a portion of the biochemical aspects of activation. However, we do not know if antidepressants also mediate the activation of $G\alpha_s$. To test this, I used purified recombinant $G\alpha_s$ expressed in *E. coli* to determine if antidepressants directly activate $G\alpha_s$. I used $G\alpha_s$ alone and $G\alpha_s$ combined with 10 mM NaF and 30 μM $AlCl_3$ as negative and positive controls respectively for assessing the capacity of each drug to directly activate $G\alpha_s$; all experiments contained 200 nM $G\alpha_s$. As expected, there was no fluorescence detected for $G\alpha_s$ alone and an increase when adding AlF_4^-. However, every drug, including those that do not mediate the translocation of $G\alpha_s$ from lipid rafts (220), that do not accumulate in lipid rafts (Aim 1), and that do not mediate the depalmitoylation of $G\alpha_s$ (Aim 2), appear to directly activate $G\alpha_s$ (Figure 32). However, this observation is only significant with fluoride addition and for olanzapine, which is significant for several reasons. 1) Olanzapine is very hydrophobic (Figure 17), which suggests that it will imbed in and not cross the plasma membrane. 2) Olanzapine does not mediate translocation of $G\alpha_s$ from the lipid raft (161). 3) Olanzapine does not accumulate in lipid rafts (Figures 4, 5, and 10). 4) Olanzapine does not mediate depalmitoylation of $G\alpha_s$ (Figures 30 and 31). The latter three characteristics are because the biochemical effects on $G\alpha_s$ are an antidepressant-mediated process and olanzapine is not an antidepressant.

I subtracted the fluorescence for each drug at 340 nm, the emission wavelength of the tryptophan residues of $G\alpha_s$ (182, 183) (nearly zero), from the $G\alpha_s$ plus drug samples to determine the change in emitted fluorescence; the percent error is between 37 (+ Fluoride) and 50% for all drugs. Regardless, diffusion of drugs across the plasma

membrane of target cells may account for the time required for therapeutic efficacy, but the fact that each drug is apparently able to activate $G\alpha_s$ suggests that each mediates the depalmitoylation of $G\alpha_s$ as well. The results presented in this dissertation are in opposition to the idea that antidepressants bind and activate $G\alpha_s$ directly. Since $G\alpha_s$ translocates in response to chronic antidepressant treatment, via an accumulation of antidepressants (not R-citalopram or olanzapine) and antidepressants mediate depalmitoylation of $G\alpha_s$, it is rather unlikely that direct binding and activation is a way by which antidepressants exert their effects through $G\alpha_s$. Thus, finding the target(s) of antidepressant drugs that is/are in addition to the reuptake transport proteins is paramount to elucidating their full mechanism(s) of action and for developing better treatment options for those suffering with depression.

The results presented within this dissertation regarding the accumulation of antidepressants over time in the membranes of C6 cells, which lack the monoamine transport system, and the resulting depalmitoylation of $G\alpha_s$ as well as the antidepressant mediated effects on the subcellular localization of $G\alpha_s$ that our lab has published $G\alpha_s$ (132, 139, 151-153, 157, 159-163), suggest another target exists. If $G\alpha_s$ itself is not the direct target of antidepressants, the next logical consideration is the class of GPCRs that couple to $G\alpha_s$. More specifically, the receptors of the monoamines serotonin and norepinephrine that couple to $G\alpha_s$ need investigation.

Norepinephrine binds the adrenergic receptors, which includes both α and β receptors. There are two subtypes of α receptors, α1 and α2, which are $G\alpha_q$ (221) and $G\alpha_i$ (222) coupled respectively. While the α receptor might be involved, since it does not couple to $G\alpha_s$ and $G\alpha_q$ and $G\alpha_i$ do not respond to antidepressants, its not a likely

place to start looking for targets of antidepressants. The β receptor by contrast has three subtypes, β1, β2, and β3, which are all coupled to Gα$_s$ (50, 53-55, 159, 164, 222, 223). On the surface, it seems that the β receptor is involved in the chronic antidepressant response, since it couples to Gα$_s$ and antidepressants function to increase monoamine density, including norepinephrine. However, chronic treatment of rats with reboxetine and imipramine, but not citalopram, produced a down-regulation of β receptor density and no significant modulation of CREB or BDNF (224). Moreover, others have reported that antidepressants do not depend on functionally responsive β-2

Figure 32. Antidepressant dependent conformational change of Gα$_s$.
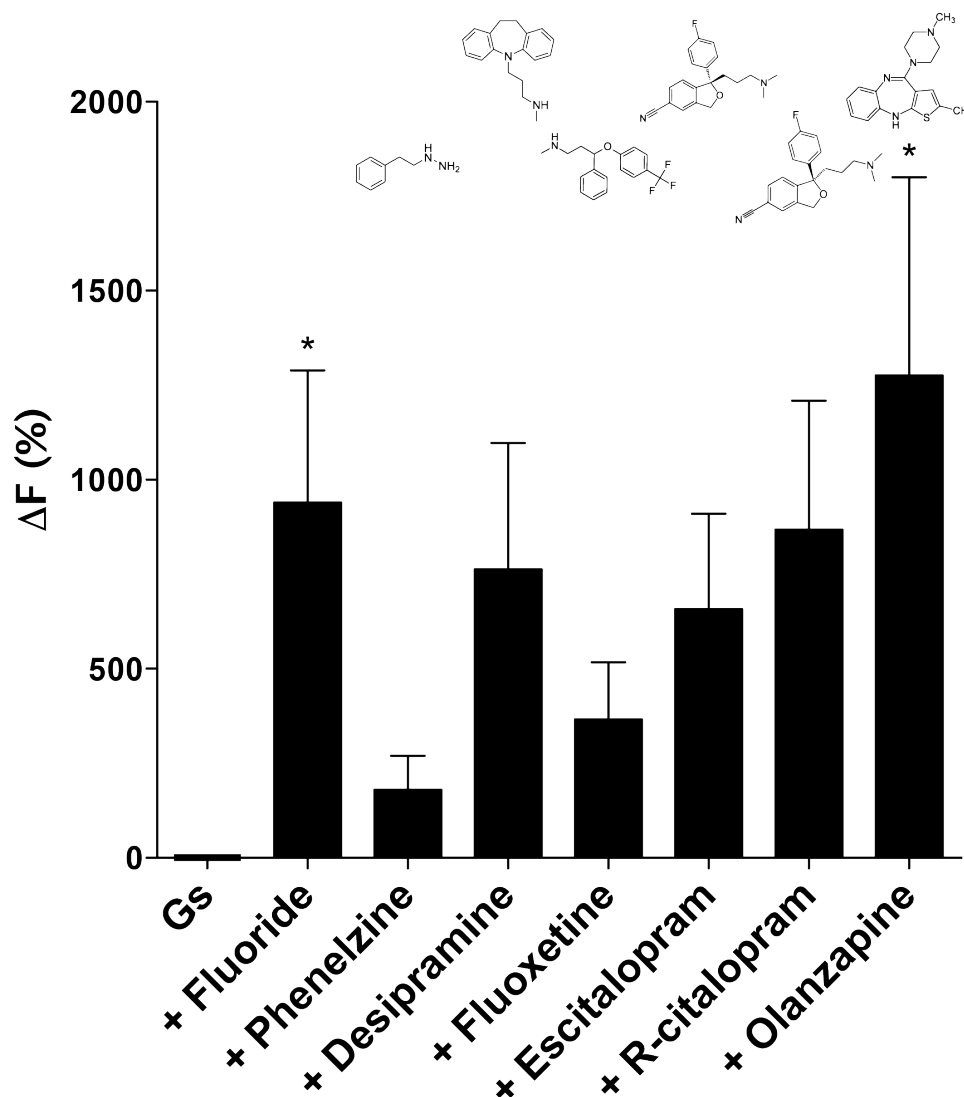
Recombinant GST-Gα$_s$ was purified from E. coli and the tag cleaved with Thrombin. Two hundred nM of purified Gα$_s$ was incubated with AlF4- at room temperature for 5 minutes before excitation at 280 nm and reading emission at 340 nm. Similarly, AlF4- was replaced with each drug and the change in fluorescence recorded.   ΔF (%) = (F−F$_o$)/F$_o$ × 100.

adrenergic receptors (225). Still others have shown that certain antidepressant drugs potentiate isoproterenol stimulated accumulation of cAMP in human leukocytes, suggesting that antidepressants potentiate the effects of neurotransmitters through β adrenergic receptors (226). The lack of consensus does not discount the involvement of adrenergic receptor signaling the antidepressant response, but also does not support its involvement either.

Serotonin, or 5-hydroxytryptamine (5-HT), signaling is similar to norepinephrine in that there are multiple isoforms of the serotonin receptor that couple to a variety of Gα subunits. There are 7 5-HT receptor isoforms and 14 total subtypes, but only 5-HT_4, 5-HT_6, and 5-HT_7 couple to $G\alpha_s$ (227, 228). Thus, these three 5-HT receptor isoforms are a good place to start. Indeed, 5-HT_4 receptor agonists are reported to be putative rapid acting antidepressants (229). However, stimulation of 5-HT_{1A}, which is $G\alpha_i$ coupled, and blockade of the $G\alpha_s$ coupled 5-HT_6 and 5-HT_7 receptors augments the antidepressant effects of SERT inhibition (230). Again, the lack of consensus as well as the contradictory evidence in the literature with regard to $G\alpha_s$ coupled 5-HT signaling pathways and antidepressant actions suggests that the 5-HT receptor might not be a potential binding target of antidepressants either. However, the delay between treatment and therapeutic action could be due to an auto inhibition of Raphe neurons, which suggests that auto receptor blockade may decrease the hysteresis in antidepressant action (231). Raphe neurons are composed of the *nucleus raphe obscurus, nucleus raphe magnus, nucleus raphe pontis, nucleus raphe pallidus, median raphe nucleus, dorsal raphe nucleus nuclei linearis intermedius,* and *linearis rostralis* and considered the main site of serotonin release in the brain as well as a principle site

of antidepressant action (232). Serotonin treatment appears to reverse depression related reductions in neurogenesis (14, 233, 234). However, rodent studies suggest that this decrease in neurogenesis is actually an inescapable shock that does not result in an overall decrease in hippocampal volume (235). Regardless, similar to observations with adrenergic signaling, the accompanying activation of 5-HT receptors with the inhibition of the reuptake of 5-HT during the chronic action of antidepressants remains inconclusive.

4.3. Inflammation, Depression, and Antidepressants.

While the precise biochemical causes of MDD remain unknown, genetics, trauma, and stress are potential mediators (236, 237). This may be a significant factor in the manifestation of MDD symptoms as exposure to stressful stimuli does increase hippocampal glutamatergic neurotransmission and trigger excitotoxic changes that influence some aspects of cognitive processing (238). Moreover, the antidepressants escitalopram, mirtazapine, tianeptine, and venlafaxine reduce cellular stress (237), which may suggest that they have anti-inflammatory properties. However, others suggest that antidepressants exhibit both pro- and anti-inflammatory effects (239).

Chronic antidepressant treatment mediates the translocation and depalmitoylation of $G\alpha_s$. $G\alpha_s$ activates AC, which catalyzes the conversion of ATP to cAMP and results in increased PKA mediated signal transduction. PKA activation is indicative of inflammatory signaling activation via mediating the release of potent transmitters of inflammatory stimuli, substance P (SP) and calcitonin gene-related peptide (CGRP) (240-242). SP binds the neurokinin 1 receptor (NK1), which is $G\alpha_q$ coupled, for which antagonism through chemical blockade or NK1 receptor genetic deletion produces an antidepressant-like effect (243-245). By contrast, CGRP may innately have antidepressant like properties (246). Given the results presented in this dissertation and previous reports from our laboratory regarding the involvement of $G\alpha_s$ in the chronic antidepressant response, this is not surprising since the CGRP receptor is $G\alpha_s$ coupled (247). Perhaps this is why some report that antidepressants are anti-inflammatory and others that antidepressants are inflammatory.

Activation of PKA is suggested to be a readout for the induction of inflammatory pathways and chronic antidepressant treatment results in an activation of PKA. This suggests that the anti-inflammatory effects of antidepressants are actually acutely mediated via the inhibitory effects of lipid rafts on $G\alpha_s$ signaling, but that chronic antidepressant treatments result in inflammatory signaling through PKA when chronically administered. Although the precise link(s) between inflammation and depression is unknown, serum levels of inflammatory cytokines, for example, tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and IL-1 beta (IL-1β), are elevated in subjects with major depressive disorder (MDD) (248-250) and antidepressant therapies reduce the concentration of inflammatory markers (251). Ultimately though, inflammatory stimuli result in the rearrangement of the cytoskeleton as elevated cAMP is responsible for degrading the microtubule structure (203, 204).

Based upon the aforementioned observations and the fact that $G\alpha_s$ predominantly associates with cytoskeletal remodeling proteins, suggests that antidepressants might exert anti-inflammatory properties through modulating inflammatory effects upon the cytoskeleton. Although, the fact that this is more pronounced upon chronic treatment with antidepressant, could also suggest that antidepressants and inflammatory cytokines have similar effects upon cytoskeletal dynamics. Regardless, it seems prudent to investigate the mechanisms by which antidepressants modulate inflammatory signaling and vice versa. Peripheral exposure of animals to lipopolysaccharide (LPS) induces the expression of IL-1β, IL-6, and TNFα in the brain (252-255), promoting inflammatory signaling, and consequently rendering tubulin incapable of microtubule formation (256). Moreover, the microtubule-associated

protein and membrane anchor for tubulin, 2',3'-Cyclic nucleotide 3'-phosphodiesterase (257), may play a role in this localization through degradation of cAMP responsible for degrading the microtubule structure (203, 204); intact microtubules may be responsible for holding $G\alpha_s$ in the lipid raft.

Chronic exposure to antidepressants induces the dissolution of the $G\alpha_s$:Tubulin complex (200), from which Tubulin transfers its GTP to $G\alpha_s$ (199), resulting in activation of $G\alpha_s$. Active $G\alpha_s$ necessarily results in the accumulation of more cAMP that further leads to the dissolution of the microtubule structure and further translocation of $G\alpha_s$ from the lipid raft; thus accounting for the hysteresis of antidepressant action. The effects on Tubulin resulting from exposure to the inflammatory agents LPS and Trypsin, as well as the ability of antidepressants to inhibit these effects, was assessed in C6 cells (Figure 33). Trypsin mediates inflammatory signaling via selective targeting of protease activated receptor 2 (PAR_2) (258-263). The effects of each agent upon Tubulin expression is presented for total and β1-Tubulin, but warrants exploration of additional isoforms of beta as well as alpha Tubulin. Desipramine and escitalopram do not impair the LPS nor Trypsin mediated reduction in Tubulin as I expected they would, but rather appear to augment the inflammatory mediated modulation of the Tubulin cytoskeleton.

Figure 33. Antidepressants modulate expression of Tubulin isoforms and are not Anti-inflammatory with respect to LPS and Trypsin.
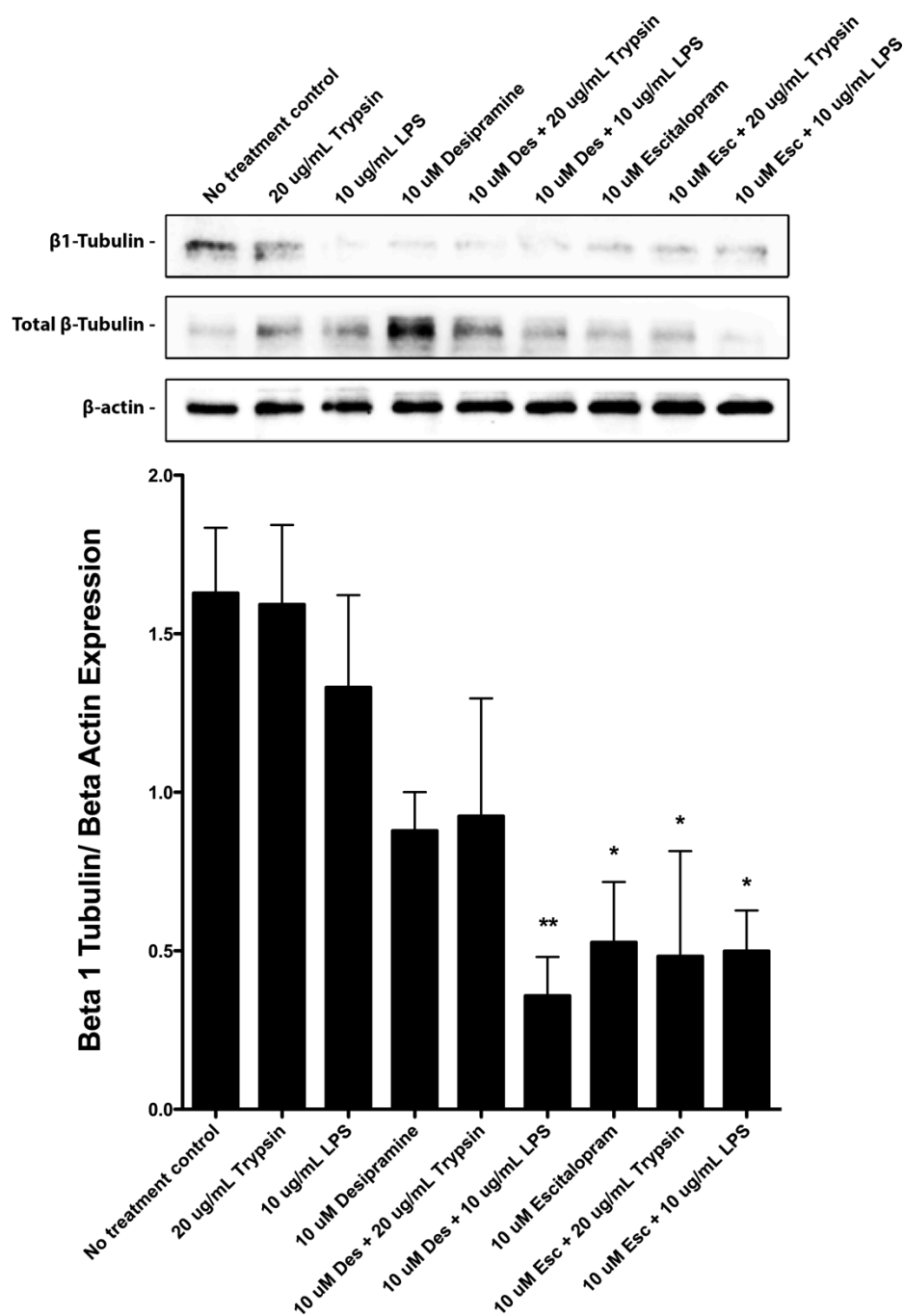
Antidepressants are not anti-inflammatory with regard to Lipopolysaccharide (LPS) or Trypsin mediated signaling. C6 cells were chronically treated (72 hrs) with 10 µM of either desipramine or escitalopram, the inflammatory agents 10 µg/mL LPS or 20 µg/mL Trypsin for 15 min., or with antidepressant and subsequently the inflammatory agent.

4.4. Antidepressants and disruption of the Gα$_s$:Tubulin complex in Lipid Rafts.

We have hypothesized that Tubulin association is a molecular event that locks Gα$_s$ into lipid rafts and have shown that chronic antidepressant treatment disrupts this association (192, 200). However, we have also observed that GTP bound Gα$_s$ is the preferential state bound to Tubulin (156, 191) and that Tubulin can activate Gα$_s$ through GTP exchange (29, 199). Results presented within this dissertation demonstrate that different isoforms of Tubulin remain in association with Gα$_s$ following chronic antidepressant treatment. Further study is required to confirm that Tubulin is a major mediator of retention of Gα$_s$ in the lipid raft, and that chronic antidepressant treatment disruption of this or other interactions is a mechanistic action, but the fact that Tubulin is able to transfer its GTP to Gα$_s$ and activate it suggests that antidepressants might bind directly to Tubulin. There is evidence to suggest that microtubule disruption is involved in the chronic antidepressant response as studies with colchicine or vinblastine, both inhibitors of microtubule polymerization, resulted in increased interaction of Gα$_s$ and AC in the soluble fraction (264). However, colchicine and vinblastine do not cross the blood brain barrier (265, 266), but this creates another biochemical conundrum: How is Gα$_s$ localization maintained at the plasma membrane?

AC is a membrane imbedded protein and chronic antidepressant treatment leads to increased coupling between it and Gα$_s$. The results contained herein suggest that depalmitoylation of Gα$_s$, which necessarily impairs membrane targeting and association of Gα$_s$, is a hallmark of antidepressant action. So, if Gα$_s$ is depalmitoylated, removing its mechanism of membrane anchoring (3, 88, 104, 107, 128-132, 137-139), Tubulin transfers its GTP to Gα$_s$ (29), and Gα$_s$ remains membrane localized in order to interact

with and activate AC (139, 152-154, 160, 161, 163, 168), what is preventing $G\alpha_s$ from internalizing? Tubulin itself is palmitoylated (101-103, 106, 108, 109), which might enable $G\alpha_s$ to translocate to non-raft regions of the plasma membrane while depalmitoylated, but this complex would need to dissociate upon proximal localization with AC as Tubulin's binding site of $G\alpha_s$ is in too close of proximity with AC's. The preceding presumes that antidepressants themselves activate $G\alpha_s$ as opposed to another protein that mediates translocation from lipid rafts. This possibility is previously discussed and not likely to occur based upon the evidence at hand. Moreover, in light of the unlikely event of forming a ternary complex between AC, $G\alpha_s$, and Tubulin in response to chronic antidepressant treatment, it is probable that the palmitoylation remains long enough for $G\alpha_s$ to associate with AC before activation (i.e. depalmitoylated), unless another molecular "shuttle" for $G\alpha_s$ is identified.

If the molecular target of antidepressants is not a GPCR, is not $G\alpha_s$ itself, is not a Tubulin isoform, nor a cytoskeletal remodeling protein, the latter three are likely restricted due to partitioning, perhaps the chronic antidepressant response is as simple as activating the depalmitoylating enzyme of $G\alpha_s$. APT1 depalmitoylates $G\alpha_s$ (140), for which inhibitors exist (209). An inhibitor of APT1, such as Palmostatin B, could be used to study the involvement of APT1 in the chronic antidepressant response through $G\alpha_s$. However, due to the effects that Palmostatin B would have on other palmitoylated proteins, it likely would have little application as an actual companion therapy in depression. Although, a short-lived acute activator of APT1, or direct inhibitor of $G\alpha_s$ palmitoylation could be useful in combination with chronic antidepressant treatment in order to induce and maintain the depalmitoylation of $G\alpha_s$ and alleviate the

antidepressant hysteresis. Acutely, ethanol abrogates the palmitoylation of $G\alpha_s$ (267), which seems to fit with the notion of, "drinking your sorrows away." Moreover, a clinical trial did not show any significant counter indication between alcohol and escitalopram (268). However, mixing alcohol with an antidepressant is probably not the best idea. Alternatively, ketamine is a short acting antidepressant (269), but the fact that ketamine prevents the reuptake of dopamine (270) makes it not an attractive choice either as addiction becomes an issue. In and interesting aside, ketamine has been reported to aid in the treatment of alcoholism (271). Regardless, determination of the target(s) of antidepressants, apart from the monoamine transport proteins (SSRIs/SNRIs/TCAs), will shed light on the biochemical mechanism(s) that mediate the depalmitoylation and subcellular translocation of $G\alpha_s$ in response to chronic antidepressant treatment.

In conclusion, one or more of the proteins in association with $G\alpha_s$ that are identified in the preceding chapters is key to developing a more complete understanding of how antidepressants behave. Over time, antidepressants accumulate in lipid raft microdomains of target cells, **binding an unknown target**, mediate the remodeling of the cellular architecture maintaining $G\alpha_s$ localization, and mediate the depalmitoylation of $G\alpha_s$ (Figure 34). The latter event allows $G\alpha_s$ to translocate into the non-raft membrane, interact with AC, and result in the accumulation of cAMP that is characteristic of antidepressant therapy.

Figure 34. Model of Antidepressant Action on Gα$_s$ and its Molecular Associations.
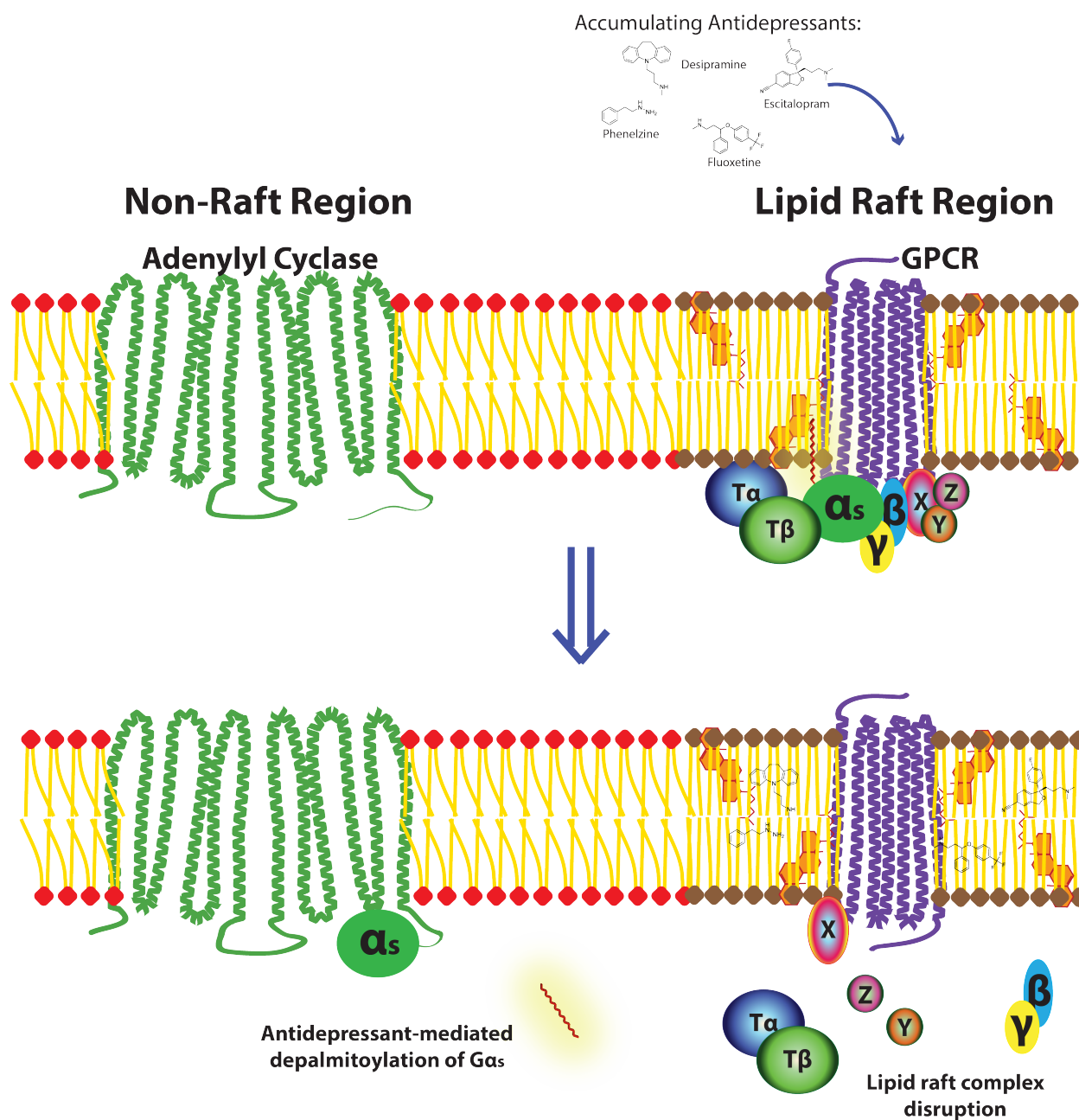
N-terminal palmitoylation directs Gα$_s$ to the plasma membrane, preferentially to lipid raft regions. Over time, antidepressants accumulate in lipid rafts, mediate remodeling of the molecular architecture around Gα$_s$, leading to its dissociation from the unidentified molecular anchors X, Y, and Z, further resulting in the depalmitoylation of Gα$_s$. This allows greater interaction with/activation of AC.

4.5. Conclusions.

Many factors are likely at play in causing someone to suffer from the symptoms of depression. Herein, I have presented a potentially significant biochemical event to explain both the hysteresis of effect as well as a possible reason accounting for the fact that some sufferers of depression do not respond to antidepressant therapies. The accumulation of antidepressants, but not other psychoactive drugs, in the lipid rafts of cells that do not express the receptors for which they were designed to bind (SERT) itself suggests one or more of the proteins identified in this dissertation may be an unknown target. Moreover, the observation that these drugs mediate the depalmitoylation and translocation of $G\alpha_s$ in these cells suggests one or more of these proteins in association with $G\alpha_s$ are integral to the biochemical etiology of depression and antidepressant therapy. Further elucidation of the proteins integral to the translocation and depalmitoylation of $G\alpha_s$ has the potential to allow for novel and/or adjunct therapy development.

Chapter 5 – Literature Cited

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Curriculum Vitae

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PROFESSIONAL SUMMARY:

Protein biochemist with extensive experience designing, developing, and executing methods for the purification and biochemical characterization of drugs and proteins. An extensive research career that includes experience using *in vitro* and *in vivo* proteins immuno and affinity purified followed by C18 reversed-phase, anion/cation-exchange, or size exclusion chromatography. Extensive experience in mass spectroscopy based methods for the characterization of protein-protein and drug-protein interactions, protein identification and confirmation of modifications, as well as the identification of impurities and quantification of products. I also have significant knowledge and hands-on experience in the physicochemical characterization of proteins and peptides with calorimetry and circular dichroism.

CORE SKILLS AND TECHNICAL EXPERTISE:

- Recombinant Protein Expression and Purification
- Protein Biochemistry/Proteomics
- Assay Design and Development
- Genetic Manipulation/Silencing
- Cloning/Gene Editing
- Mass Spectrometry (GC/MS, LC/MS, MALDI-TOF)
- Column chromatography (affinity tagged, reversed-phase, anion and cation-exchange, and size exclusion)
- High Performance Liquid Chromatography (HPLC)
- Mammalian Cell/Tissue Culture
- Calorimetry (ITC/DSC)
- Public Speaking

EDUCATION:

Doctor of Philosophy in Biopharmaceutical Sciences   2015
  University of Illinois at Chicago, Chicago IL
  Dissertation: Antidepressants Accumulate in Lipid Rafts and Modify the Acylation State of G alpha S

Master of Science in Biochemistry   2010
  University of Nebraska Medical Center, Omaha NE
  Thesis: Modulation of p53 Oligomerization through Differential Phosphorylation of the MUC1CT

Bachelor of Science in Biological Sciences 2007
 University of Nebraska-Lincoln, Lincoln NE
 Project: Similarities between PGK1 and PPR1 mRNAs for Nonsense Mediated mRNA Decay

PROFESSIONAL EXPERIENCE:

Graduate Research Assistant/Research Associate 08/10-present
 University of Illinois at Chicago Dept. of Biopharmaceutical Sciences
- Designed, developed, optimized, and troubleshot methods to study post-synaptic signaling mechanisms of antidepressants to understand their hysteresis of action at a molecular level.
- Evaluated the effects of membrane microdomains on the accumulation of psychoactive drugs via GC/MS and their associated effects upon the acylation status and molecular interactions of G proteins using LC/MS.
- Developed and optimized methods for assessing antidepressant effects upon G protein intracellular trafficking by stably expressing engineered GFP tagged acylation mutant variants in different cell lines.

Graduate Research Assistant 08/07-07/10
 University of Nebraska Medical Center Dept. of Biochemistry and Molecular Biology
- Evaluated the effects of transcriptional modulation of p53 to better understand tumorigenesis using a combination of vector design for recombinant protein production or for stable cell line overexpression.
- Designed and developed bacterial expression vectors to express and purify recombinant fragments of p53 for biophysical association analyses with engineered phosphomimetic proteins.
- Performed the biophysical characterization of differential phosphorylation of the MUC1 cytoplasmic tail upon physical interaction with specific domains of p53 using Mass spectrometry, ITC, CD, and SPR.

Undergraduate Research Assistant 01/05-05/07
 University of Nebraska-Lincoln Dept. of Biological Sciences
- Designed and developed strategies to clone upstream elements of the PGK1 and PPR1 genes and fuse these with GFP to study mRNA decay pathways in *S. cerevisiae*.

LEADERSHIP AND ADMINISTRATION:

Chair, Pharmaceutics Graduate Student Research Meeting (*PGSRM*) Planning Committee
 2013
Elected Rep., Graduate Employees Organization Bargaining/Steering Committee
 2012 - 2013
Treasurer, American Association of Pharmaceutical Sciences Student Chapter
 2011 - 2014
Manager/Coordinator, Biopharmaceutical Sciences Journal Club
 2011 - 2015

TECHNICAL EXPERTISE:

Molecular Biology: Cloning/gene-editing: Primer design, Restriction digest, plasmid prep, gel extraction, ligation, transformation, PCR, Point mutation PCR, cDNA synthesis, and qRTPCR; Mammalian cell and tissue culture: transient and stable transfection/adenoviral infection; Bacterial culture; Cell migration and invasion assays; Animal Models of Pain; EMSA; Genomic DNA, RNA, and Protein isolation; Western, Southern, and Northern Blotting; Fluorescent activated cell sorting (FACS).

Biochemistry: Mass Spectrometry (Agilent GC-MS, Thermo LC/MS/MS, and MALDI-TOF); Xcalibur and Chemstation software; Recombinant protein expression and purification; Column chromatography (Affinity, C18 Reversed Phase, Anion and Cation Exchange, and Size exclusion); Immunohistochemistry; In situ hybridization; *in vitro* Binding assays: co-IP, Surface Plasmon Resonance, Isothermal Titration Calorimetry, and Circular Dichroism; Subcellular fractionation; glucose density gradient fractionation; Protein Chemical labeling.

Neuro-chemistry: Radiolabeling; cAMP assays; primary neuron and glial cell culture; Human and Animal Tissue Dissection; enzyme linked immunosorbent assay (ELISA). Identification and characterization of neurotransmitters, neuromodulators, receptors, and second messengers. Immunocytochemistry and fluorescent imaging of cultured cell lines using fluorescent and confocal microscopy (Immunofluorescence staining).

MANUSCRIPTS:

Manuscripts in Preparation:

Samuel J. Erb, Andrew Czysz, and Mark M. Rasenick. Antidepressants accumulate in lipid rafts and modify the acylation state of G alpha S ($G\alpha_s$), promoting translocation of $G\alpha_s$ from cholesterol-rich plasma membrane microdomains. (*In preparation*)

Manuscripts in Revision:

Erb, S. J., Lee, H. T., Band, V., Marky, L. A., and Hollingsworth, M. A. Specific Phosphorylation of the MUC1 Cytoplasmic Tail Modulates association with and oligomerization of p53.

Acknowledgements:

Jahan, I., Fujimoto, J., Alam, S. M., Sato, E., and Tamaya, T. (2008) Role of protease activated receptor-2 in lymph node metastasis of uterine cervical cancers, BMC Cancer 8, 301.

ABSTRACTS AND PRESENTATIONS:

Presented research findings at the following conferences on the following topics:

"Chronic antidepressants accumulate in lipid rafts and modify the acylation state of G alpha S ($G\alpha_s$), promoting translocation of $G\alpha_s$ from cholesterol-rich plasma membrane microdomains" at Society for Neuroscience Annual Meeting 2015, Pharmaceutics Graduate Student Research Meeting 2015, and Chicago Symposium on Cell Signaling Symposium 2015; "Protease activated receptor 2 (PAR_2)-mediated activation of protein kinase A" at Chicago Symposium on Cell Signaling 2012, Society for Neuroscience - Chicago Chapter - Annual Meeting 2012; "Serum Proteins Mediate Cell Attachment within Poly(ethylene glycol) diacrylate (PEGDA) Superporous Hydrogels" at Pharmaceutics Graduate Student Research Meeting 2011 and 2012; and "Two Potential Similarities between PGK1 and PPR1 mRNAs for Nonsense Mediated mRNA Decay" at the Undergraduate Creative Activities and Research Experiences (UCARE) symposium 2007.