Dynamic interactions between G proteins and plasma membrane

nanodomains: effects of antidepressants and PUFAs

BY

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THESIS
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AA	Arachidonic acid
AC	Adenylyl cyclase
ATP	adenosine 5'-triphosphate
AlF_4^-	Aluminum tetrafluoride
βAR	β-adrenergic receptor
BDNF	Brain-derived neurotrophic factor
cAMP	adenosine 3',5'-cyclic monophosphate
CAV	caveolin
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CTXB	cholera toxin B subunit
dDPPC	Deutrated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DHA	Docosahexaenoic acid
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DTT	dithiothreitol
EDTA	ethylene diamine tetracetic acid
EPA	Eicosapentaenoic acid
FP	Fluorescent protein
FRAP	Fluorescent Recovery After Photobleaching
Gα	G-protein alpha subunit
$Gα_s$	Alpha subunit of the stimulatory G-proteinf or adenylyl cyclase
Gβ	G-protein β subunit

Gγ   G-protein γ subunit

GDP   Guanosine 5'-diphosphate

GFP   Green fluorescent protein

GPCR   G-protein coupled receptor

GTP   Guanosine 5'-triphosphate

HEPES   4-(2-hydroxyethyl)-1-piperazineethanesuflonic acid

ISO   Isoproternol

kDa   kiloDalton

LA   Linoleic acid

M   Molar

μg   Microgram

μL   Microliter

μM   Micromolar

PBS   Phosphate buffered saline

PKA   Protein kinase A

PUFA   Polyunsaturated fatty acids

RGS   Regulators of G-protein signaling

RPM   Rotations per minute

SEM   Standard error of the mean

SDS   Sodium dodecyl sulfate

TrkB   Tyrosine receptor kinase type B

TX-100   Triton X-100 detergent

TX-114   Triton X-114 detergent

wt   Wild type

SUMMARY

Cellular neurotransmission is a complex array of processes vital for transforming chemical signals into behavior. Aside from ion channels, no other class of receptors is more prominent to this process than G-protein coupled receptors. These receptors transmit signals in cell throughout the body, constitute the largest family of genes and are a target for a plurality of drugs. Most GPCRs, however, would be at least partially neutralized without their coupled G-proteins for which their name derives. This dissertation focuses on the complex regulation of the G alpha stimulatory subunit.

In particular, lipids are vital mediators of $G\alpha_s$ signal transduction. In a simple sense, lipids are necessary for $G\alpha_s$ function since GPCRs and adenylyl cyclase are both transmembrane proteins. Delving deeper however, membranes are not simple environments – hundreds of lipid species exist in complex patterns that cell biologists are only beginning to understand. Among these exists the concept of lipid rafts, signaling centers organized by hydrophobic saturated phosopholipids, cholesterol and cytoskeleton corrals that concentrate or segregate signaling pathways. In the case of $G\alpha_s$, lipid rafts are thought to dampen coupling to adenylyl cyclase.

The nature of $G\alpha_s$ raft association is still poorly understood. Part of this stems from the controversial existence of lipid rafts themselves. Most studies of protein regulation by rafts, including those of $G\alpha_s$, rely of lipid raft extractions. This thesis

SUMMARY (continued)

complements this single approach with GFP-Gα_s diffusion studies to better

understand the relationship between Gα_s and lipid raft association. First, a

monomeric variant of the previously characterized eGFP-Gα_s was developed. Unlike

eGFP-Gα_s, monomeric GFP-Gα_s properly localizes to lipid rafts similar to

endogenous Gα_s. Next this tool was used in a fluorescent recovery after

photobleaching (FRAP) assay under conditions that altered Gα_s raft localization. In

particular, antidepressant treatment decreases Gα_s localization to rafts. FRAP

reveals after chronic, but not acute antidepressant treatment, GFP-Gα_s diffuses

significantly slower. Results also suggest this decrease in diffusion could be

attributed to increased scaffolding to slow diffusing transmembrane proteins.

Chapter 1: Introduction to G-protein Signaling in Depression

1.1. Depression

1.1.1 Introduction to depression and societal impact

Depression is an often severely debilitating psychiatric syndrome that, despite decades of research, remains a major public health concern and whose origins, symptom profiles, and complete physiological effects remain unknown to neuroscientists. The underlying pathology is unclear, although altered regulation of monoamine neurotransmission was a significant target of research and therapeutic intervention(Schildkraut 1965). An improved understanding of depression is necessary, as an estimated 1 out of 6 people will experience clinical depression in their lifetime(Kessler et al. 2005). In addition, depression correlates with a poorer prognosis in other comorbidities including other psychiatric disorders, cardiovascular disease, and diabetes (Musselman, Evans, and Nemeroff 1998; Ciechanowski, Katon, and Russo 2000). Continued research into the causes and treatment is therefore critical, and should extend beyond the traditional focus of monoamine dysregulation.

1.1.2 Therapeutic interventions for depression

The era of modern pharmacologic therapy for depression began with the serendipitous observation that iproniazid, a then novel anti-tuberculosis compound, acted as a "psychic energizer (LOOMER, SAUNDERS, and KLINE 1957)." Shortly thereafter a derivative of the antipsychotic chlorpromazine was also demonstrated to have anti-depressive properties(KLERMAN and Cole 1965). Further study revealed that both compounds regulated monoamine neurotransmission through different mechanisms. Iproniazid was classified as a MAOI – monoamine oxidase inhibitor. The chlorpromazine relative, imipramine, became known as a tricyclic antidepressant because it of its chemical structure. It was shown to inhibit monoamine reuptake at the synaptic cleft. Together these drugs, and their derivatives, closed the door on antiquated therapies like opiates and harsh anticholinergics(Ban 2001).

The burgeoning field quickly became dominated by monoamine reuptake inhibitors (MAUIs) and biochemists focused on creating drugs selective to particular monoamine transporters. This included other TCAs that were more selective for norepinephrine relative to serotonin and later, drugs that lacked tricyclic structure, but still inhibited various monoamine transporters. In particular selective-serotonin reuptake inhibitors (SSRIs) became a huge commercial success, largely because their side-effect profile was more attractive than TCAs. Interestingly however, meta-analysis comparing SSRIs versus TCAs reveals similar efficacy for treating depression (MacGillivray et al. 2003). In addition, selective noradrenergic and selective dopaminergic reuptake inhibitors have also been developed.

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Despite dozens of drugs to choose from, many clinically depressed patients either fail to respond, or relapse after treatment. Electroconvulsive therapy is efficacious in some of these cases, and more recently, highly controlled, low dosed ketamine has shown some promise. Nonetheless, these interventions are only practical as a last resort. Drug resistance suggests that depression is not a homogenous disease, and that multiple etiologies are likely to play a role.

1.1.3 Anatomical, animal and cellular models of depression

Mental illness research is unique relative to other pathologies. In contrast to illnesses like heart disease, cancer and infections, there is a lack of empirical lab tests to assess symptom severity and track progress. This complicates research because it compromises the validity of non-human models. Presently, there is no universally accepted anatomical, animal or cellular model for depression. Existing models rely heavily on either correlational data or treatment of non-depressed subjects with antidepressants.

1.1.4 Defining depression

The management of depression is hindered because of a lack of objective assessments of disease severity. With only subjective measurements - mostly patient-reported affect - a comparison between patients or even within the same patient at different time points becomes less valuable. As a result, the collection of symptoms that attract the label "major depressive disorder" may in fact be different

pathologies among which cannot accurately discriminate(Nestler et al. 2002). This may account for why a single cause for the etiology for depression has not been identified and why patients exhibit a variety of responses to treatment.

The current criteria for major depression make diagnosis imprecise. Most of the symptoms are challenging to quantify, such as low self-esteem or feelings of hopelessness. Even to the extent that questionnaires may be useful, since screening is not universally routine, and we rarely have an established baseline from before the patient became depressed for comparison. Other criteria for depression, such as weight or sleep changes, are much more objective but are non-specific to depression.

Further complicating quantification efforts, depression can be a comorbid with a host of diseases (Kessler et al. 1997). In many cases, depression is believed to be a secondary illness, since people often resolve their depression with treatment of their other symptoms. Unfortunately the most common comorbidities include other psychiatric disorders, which are also a challenge to define. The most common of these are anxiety and pain. In addition, depression can be a risk factor for developing future disease, most significantly impacting the cardiovascular system(Roose, Glassman, and Dalack 1989; Rugulies 2002).

These reasons have motivated scientists to search for new ways to define depression. Refining diagnostic criteria for major depression between the DSMIV

and V was minor compared to where researchers and clinicians would like to head(Donati and Rasenick 2008). For both prevention and treatment, an objective test for mood disorders would be invaluable. Although it is estimated that 20% of the population is suffering from some form of depression, screening is not routine because it requires a highly skilled professional(Nestler et al. 2002). Even if depression is suspected, the treatment plan can become convoluted if the patient does not respond to first-line drugs or the illness is mistaken for another mood disturbance such as bipolar disorder(Kaufman and Charney 2000).

In an effort to investigate new criteria, researchers are employing a variety of anatomical, animal, and cellular models for depression. As with other pathologies of the brain such as Parkinson's or Alzheimer's, scientists believe there are hallmark anatomic and tissue changes of the brain linked to depression. Unfortunately these models are limited because tissue biopsy is not practical in humans. Therefore animal models are a popular choice for researchers. Using animals additionally allows for a more rigorous application of the scientific method, as it is considered more ethical to induce stressful behavioral conditions and test uncharacterized drugs on animals. Finally, researchers study cellular models for depression despite the significant difference in physiology and behavior of cells compared to intact animals.

1.1.5 Anatomical findings in depression

Research has not identified a single region of the brain that is primarily responsible for the symptoms of depression. Nonetheless, many regions have received special

focus in research because their functions are related to some of the symptoms in depression. For example, the amygdala is believed to be significant because of its key role in emotional behavior. The striatum may also be critical in cases of depressed subjects because reward circuitry seems to be deficient (namely a lack of motivation to engage in formerly pleasurable activity). The hypothalamus might also be involved since patients commonly exhibit weight and sleep changes. In fact, depression research has focused on almost every major nucleus of the brain(Nestler et al. 2002).

Initial work to isolate a region of the brain in search of cause for depression was motivated by observation that inhibitors of serotonin or norepinephrine degradation can treat a depressed phenotype. Therefore, regions rich in production or receptors of these neurotransmitters were investigated. The subgenual cingulate, for example, is very rich in serotonin receptors and is thought to act as a gateway to many parts of the brain. This target is attractive since depression seems to affect many different aspects of brain function. Unfortunately, it is unlikely that these monoamines account for all of the pathology in depression. Most striking is the fact that while reuptake inhibitors should act instantaneously, relief from depression takes weeks to months. Therefore, simply studying serotonergic or adrenergic circuits in the brain is probably insufficient to create a complete model. While reuptake inhibitors relieve depression in many cases, there is still a strong demand for new classes of antidepressants.

An alternative to the traditional monoamine-based approach is to look at other

regions of the brain implicated in the symptoms of depression. Much of this research has targeted the hippocampus and hypothalamic-pituitary-adrenal (HPA) axis. As part of the limbic system, the hippocampus plays a role in emotion, but it is best known for its impact on memory formation(Nestler et al. 2002). Nonetheless, because of the abundance of glucocortocoid receptors in this region, researchers speculate that it might be especially sensitive to stress. Stress and anxiety are two common features of depression, but alone do not define it. Unlike other symptoms, however, researchers are able to quantify it. The most important glucocortocoid, cortisol, is well known for having a diverse array of effects including cardiovascular, immunological, and metabolic modulation. This gives researchers a variety of options to monitor stress by examining at any of these systems or simply blood cortisol levels.

Cortisol production is controlled at least partially by the brain. While the aderenal cortex produces cortisol, the anterior pituitary stimulates the adrenal cortex via a release of adrenocorticotropin (ACTH). Upstream of this, corticotrophin-releasing factor (CRF) is produced from the paraventricular nucleus of the hypothalamus. In the brain, the operation is never as simple as one or two connections; in this case, the hypothalamus receives many other connections influencing CRF release, including an inhibitory one from the hippocampus.

Some researchers believe pathologic stress may be caused by decreased inhibition of the hypothalamus due to deficiency in the hippocampus. *In vivo* and *in vitro* studies suggest that sustained elevated levels of glucocorticoids can have a lethal

effect on hippocampal neurons(Sapolsky 2000). In addition, postmortem analysis of

hippocampal tissue shows a decreased volume in depressed subjects (Sheline et al.

1999; Bremner et al. 2000). This would create a positive feedback loop, since loss of

inhibition of the hypothalamus would result in increased cortisol, and thus further

hippocampal damage. This may also explain why prolonged exposure to stressful

situations can cause a depressed phenotype to persist even after the stressful

stimuli are removed. Along these lines, some data support the idea that

antidepressants reverse this process by either offering protection to the remaining

hippocampal neurons or by stimulating neurogenesis. Unfortunately, since the field

of adult neurogenesis is still relatively new, the role of hippocampal neurogenesis in

depression remains contested(Malberg et al. 2000; Santarelli et al. 2003; Bessa et al.

2008; Vollmayr, Mahlstedt, and Henn 2007). In fact, some of the behavioral effects of

antidepressants may be linked to neurogenesis whereas others are not(David et al.

2009).

In addition to research in the hippocampus and HPA axis, study is also being

directed toward the brain's reward pathways. One of the most common symptoms

of depression is a lack of motivation or an inability to derive pleasure from

previously pleasurable activities. Through study of the mechanisms of addiction, the

mesolimbic dopamine pathway has been identified as a major player in mood

regulation(Nestler, Carlezon, others 2006). Currently, much of the evidence of a

mesolimic role in depression relies on clinical data indicating that several

antidepressants, such as bupropion, also inhibit reuptake of dopamine in addition to

either serotonin or norepinephrine. Curiously, very selective dopamine reuptake

inhibitors like cocaine are not widely reported to have antidepressant effects. Of course, the acute effect of cocaine may confound this observation. Nonetheless, CREB (cAMP response element binding protein), a downstream effector of certain serotonin, norepinephrine and dopamine receptors, has been implicated in depression with diminished expression in numerous brain regions. In the case of the nucleus accumbens, overexpression seems to be linked to a depressed phenotype(Newton et al. 2002).

This role of CREB in depression highlights the importance of studying depression at the anatomical level. In the nucleus accumbens, increases in CREB are demonstrated to increase depressive symptoms, whereas the opposite is shown in the hippocampus (as discussed below). Unlike many other systems, brain regions receive important connections from its neighbors that cannot be replicated in cell culture. Even though experimentation at this level may be more expensive and challenging to control for variables, it offers the most physiologic relevance.

1.1.6 Overview of animal models

The value of animal models is unique in depression compared to other diseases. Since depression is largely characterized as a set of subjectively communicated symptoms, the relevance of non-verbal animals is particularly questionable. Furthermore, certain aspects of depression in humans may not be homologous to other animals, especially if the frontal cortex is involved. Nonetheless, like all pathology, they afford a more ethical outlet for experimental manipulation. This is especially crucial in depression because our current understanding is deficient in

reproducible, objective findings.

Since we do not communicate verbally with experimental animals, researchers rely on non-verbal behavioral paradigms(Leonard 1999). Most of these use some type of manipulation followed by a quantifiable behavioral reaction to a situation. Alternatively, researchers may invoke the manipulation and then sacrifice the animal to look at protein levels, gene transcription or anatomical changes. This is especially valuable because brain biopsy of living human subjects is not practical. Finally, some researchers also examine live animals using less-invasive imaging to better understand the value of similar tests in humans.

The manipulations employed by researchers in animal studies are diverse. The most common is pharmacologic intervention, where the animals receive any number of drugs, including widely accepted antidepressants or placebos to serve as controls. In a similar manner, intervention can be anatomical, through surgical modifications of relevant brain regions. Aside from surgery, anatomical intervention is also achieved through electroconclusive or transcranial magnetic stimulation.

Since the effects of antidepressants in non-depressed specimens may not be physiologically relevant, researchers may also attempt to invoke a depressed phenotype. The most common method is through behavioral intervention, almost always involving some degree of chronic stress. Typically this involves unpredictable, unpleasant stimuli such as odor, temperature change, or electrical shock(Deussing 2007). Alternatively, social stress can be invoked by removing young pups from their mother or by exposing the animal to dominant males. Many

of these result in learned helplessness or fear conditioning(Vollmayr, Mahlstedt, and Henn 2007). Perhaps less significant to humans, researchers have shown that olfactory bulbectomies in mice may also be a means of inducing a depressed phenotype(Lumia et al. 1992).

Post-manipulation a variety of behavioral assays may be employed. While most are very different from how depression is diagnosed in humans, they are still designed to measure symptoms like anxiety, lack of motivation, decreased concentration, and hopelessness. A common test involves a rodent struggling either in a water bath or during tail suspension. Researchers measure the length of the time before the mouse gives up as a metric for despair. Anxiety is routinely measured by a rodent's general movement, but especially in and outside of dark or light areas. Motivation is assessed by the reproducibility an animal's behavior in response to a stimuli linked to a powerful pharmacologic reinforcer. Together, these tests reliably produce behavioral effects to both antidepressants and induced depressed phenotypes(Nestler et al. 2002; Berton and Nestler 2006).

A criticism of non-human animal models is that they are neither totally physiologically relevant to humans nor as reproducible as cell models. Indeed, humans and animals are diagnosed as depressed by very different criteria. While behavioral tests are more standardized than a patient interview, this is meaningless if they are not measuring comparable data. Furthermore, if depression is primarily a disease of abnormal chemical signaling, it might be a better use of resources to reserve animal work for experiments that have been extensively verified in more

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basic systems.

1.1.7 Cellular and biochemical models

As with most biological research, cellular models of depression are much less physiologically relevant than animal models. Researchers are able to examine significantly more specimen at lower cost and greater speed, as well as begin new projects with fewer administrative requirements. The biggest disadvantage is the inability to perform behavioral manipulation with cells. Therefore, research is mostly focused on cells that are believed to be a non-depressed phenotype(Berton and Nestler 2006). Moreover, as with all cultured cell lines, they are either from oncogenic origin or virally transformed.

Despite these issues, cellular models are excellent for isolating details of important signaling pathways. Particular cell lines express only proteins relevant to their function, allowing one to investigate that pathway without the background of other cellular processes. In addition, if a desired gene is not expressed in a cell line, it is relatively simple to transfect it in. While this is possible in animal models, it often relies on viral transfection, which is much less consistent. Together, cells are simpler systems and therefore more easily controlled.

Research on cellular models has focused on two related pathways: monoamines and BDNF/CREB(Altar 1999). The monoamine hypothesis of depression is based on the observation that drugs, which raise the levels of monoamine in the synapse, can alleviate symptoms of depression. Brain derived neurotrophic factor, BDNF, is also

observed to be decreased in depressed patients. Some groups hypothesize that BDNF promotes dendritic arborization and that this might reverse some of the pathology of depression. Antidepressants have been shown to increase cAMP signaling in cell models, which in turn activates CREB, a transcription factor that can lead to increased BDNF production(Conti et al. 2002; Chen and Rasenick 1995a).

While much of the work of these pathways can also be examined on the animal level, the cellular level is ideal for drug discovery. The high-throughput nature of cellular research is likely to drive the search for new targets of antidepressants. For example, some groups are studying downstream effectors of BDNF such as TrkB, the Ras-Raf-ERK and PI3K-Akt pathways to act as targets for novel antidepressants(Nibuya, Morinobu, and Duman 1995; Shirayama et al. 2002). This includes phosphodiesterase inhibitors, especially the PDE4 inhibitor rolipram(Houslay 2001). In cell culture rolipram increases cAMP and induces BDNF in a similar fashion to antidepressants(Takahashi et al. 1999). This initial finding then lead to more extensive animal and eventually clinical trials demonstrating that cell culture can indeed be an initial stepping stone for depression research even though cultured cells are so far removed from their complete host.

1.1.8 Future models

Anatomic, animal and cellular models show a promising future. Less expensive and improved resolution of clinical imaging technologies will accelerate the data collected in anatomic models. Especially exciting research techniques include two-photon imaging of live animals and CLARITY to visualize neuronal networks in fixed

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tissue(Chung et al. 2013). Hopefully as this allows the collection of more data on

human subjects, it will help validate and correct animal models(Airan et al. 2007;

Drevets 2001; Liotti and Mayberg 2001). Meanwhile, advances in genetics may lead

to animal models with particular genotypes(Sanders, Detera-Wadleigh, and Gershon

1999; Overstreet et al. 2005). While there has not been a single gene identified as

predominant player in depression, having access to animals with depressed

phenotypes that are not behaviorally derived will add diversity to the available

models(Nestler et al. 2002). Additionally, cellular models are becoming more

attractive with advances in -omics: genomics, proteomics and even lipidomics are

likely to provide insight onto even more signaling pathways related to depression.

1.2 G-protein signaling

1.2.1 Cellular neurotransmission

Cell signaling is classically communicated through ligand-receptor interactions or

changes in voltage across membranes. Neurons and glia are no different, but there

are particular ligands, receptors and ion channels enriched in these cells. While not

exclusive to the brain, monoamines and their receptors are especially important,

including serotonin, dopamine and norepinephrine.

Monoamines can act on both ion channels and G-protein coupled receptors, but

there is a greater diversity of GPCRs relative to ion channels. Serotonin, for example,

acts on at least 13 different GPCRs, whereas the 5-HT$_3$ receptor is the only ion channel gated by serotonin(Barnes and Sharp 1999). It should be noted that receptor nomenclature after a ligand is also somewhat arbitrary, as receptors are not exclusive to a given chemical ligand. Instead, receptors are sensitive to ligands at different concentrations, and often have more than one binding site for ligand interaction. Transient receptor oligiomerization can further complicate receptor signaling, as can downstream regulation of GPCR's canonical effector, the heterotrimeric G-protein complex.

1.2.2 Heterotrimeric G-proteins

By the traditional definition, G-proteins are essential for GPCRs to signal. G-proteins are a diverse group of proteins that are named for their ability to hydrolyze GTP to GDP. These proteins are often grouped as either heterotrimeric G-proteins or small G-proteins, but other proteins such as tubulin are technically G-proteins, even though this role is frequently not emphasized. Among the four families (Ras,Rab, Rho and ARF), small G-proteins regulate a variety of cellular processes including growth/differentiation, actin polymerization, and vesicle trafficking(Takai, Sasaki, and Matozaki 2001). Small G-proteins and tubulin are indirect targets of some GPCRs, but only heterotrimeric G-proteins act directly with almost every GPCR. This family is named as such because, when bound to the GPCR, it is in complex of three different subunits: α, β, and γ. After agonist binding, GPCRs undergo a structural change that facilitates exchange of GDP for GTP on the alpha subunit. The structural

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changes due to the GTP binding alter G-protein-receptor interactions (typically

disassociation) as well as alter α, β, and γ association. These changes permit all of

these proteins to associate with new signaling partners.

The Gα subunit is especially interesting because it possesses the capacity to

dramatically amplify a signal. A single activated Gα binds to a single effector

protein at a time, but this complex can catalyze the production of thousands of

second messenger molecules, leading to quick and powerful changes in signal

transduction.

The Gα family is typically divided into four groups based on sequence homology and

effector specificity: s, i, q and 12/13. $G\alpha_s$ is noted "s" for its ability to stimulate the

protein adenylyl cyclase, while $G\alpha_i$ is noted "i" for its ability to inhibit it. Activation

of adenylyl cyclase leads to the conversion of ATP to cAMP, a second messenger

with a diverse range of cellular functions. $G\alpha_{olf}$ is closely related to $G\alpha_s$ but is mostly

expressed in the olfactory organ and frontal cortex. The $G\alpha_i$ family contains many

sub-members, $G\alpha_{i1\text{-}3}$, as well as some others noted by different letters but still

closely related: $G\alpha_o$, $G\alpha_t$, $G\alpha_{gust}$ and $G\alpha_z$.

Nomenclature becomes more confusing when addressing the remaining Gα families.

The $G\alpha_q$ subfamily includes $G\alpha_q$ and $G\alpha_{11,14\text{-}16}$. Alongside $G\alpha_{11,14\text{-}16}$, $G\alpha_q$ activates

Phospholipase C leading to the production of the second messenger PIP_2. $G\alpha_{12/13}$ are

the least-characterized family, but are implicated in actin cytoskeleton remodeling.

Gα proteins have many additional targets, which may include several that are still unknown. $G\alpha_s$ and $G\alpha_i$ interact with tubulin to modulate its own GTPase activity and alter microtubule dynamics(Roychowdhury and Rasenick 2008). However, the closely-related $G\alpha_t$ does not have this capacity, and $G\alpha_t$-$G\alpha_s$ chimeras have been generated to identify the specificity of this activity(Layden et al. 2008). It should also be noted that tubulin is capable of "transactivating" $G\alpha_s$ by donating its GTP.

1.2.3 Regulation of G-proteins

The ultimate regulator of the heterotrimeric G-protein complex is the GPCR, but many other proteins also influence signaling. Aside from GPCRs, regulation by other proteins is largely mediated by the aptly named family of proteins, Regulators of G-protein Signaling (RGS). Members of this family act through an RGS domain, which accelerates the hydrolysis of GTP to GDP in the G-protein, terminating its activity. In addition to the RGS domain, RGS proteins may also contain others with additional functions, such as scaffolding domains.

Other proteins may regulate G-proteins in the opposite sense – facilitating the transition from GDP to GTP. Abbreviated as GEFs, guanine nucleotide exchange factors are more frequently associated with small G-proteins, but do exist for Gα as well. This is not surprising since GPCRs are technically GEFs for Gα.

1.2.4 Gα_s signaling

Among the various Gα subunits, the stimulatory Gα is perhaps the most researched and consequently best understood. Its canonical downstream product, cAMP, was initially described as a heat stable compound from liver homogenates(Berthet, Sutherland, and Rall 1957; Rall and Sutherland 1958). This finding helped develop the concept of hormones and corresponding receptors leading to 1971 Nobel Prize to Sutherland(Robison, Butcher, and Sutherland 1971).

Later work by Martin Rodbell and Alfred Gilman identified the necessity of GTP for receptor-mediated cAMP production, suggesting the need of an unknown stimulating factor, described as N$_s$. The generic N (for nucleotide) was later replaced with a G for GTP. Gilman noted that mutant S49 lymphoma cell lines had both β-adrenergic receptors and adenylyl cyclase, but were unable to produce cAMP. Ultimately their work identified Gα_s as a protein, and their work was recognized with the 1994 Nobel Prize.

Rodbell and Gilman's successors have continued to characterize the regulation of Gα_s. In addition to both β-adrenergic receptor, numerous additional receptors have been identified that couple and activate Gα_s. Of special importance to physiology are the D1 dopamine, 5-HT4 serotonin, TSH thyroid stimulating hormone, LH leutinizing hormone and PGE1 prostaglandin hormone receptors.

Researchers also better understand activation of $G\alpha_s$ by the receptor. A crystal structure of the $G\alpha_s$-β-adrenergic receptor reveals exactly how the two proteins interact and what key amino acids negotiate the exchange of GDP for GTP in $G\alpha_s$. Artificial activation of $G\alpha_s$ and other Gα has also been demonstrated using aluminum terafluoride, which binds to GDP to mimic the structure of a third phosphate. This has been a valuable tool for probing receptor-agnostic effects of $G\alpha_s$ signaling. Mutagenesis of in the Gln227 position can also lock $G\alpha_s$ into a constitutively active confirmation.

Aberrant constitutive activation of $G\alpha_s$ is occasionally seen in nature too. The bacteria *vibrio cholera* produces a cholera toxin, which causes ADP-ribosylation of $G\alpha_s$. Similar to aluminum tetrafluoride, this modification alters the conformation of $G\alpha_s$ to an active state. This activation is likely advantageous to the bacteria, especially in the gut where cAMP signaling leads to diarrhea and therefore increased bacterial transmission. Over-activation of $G\alpha_s$ is also seen in McCune-Albright syndrome, leading to precocious puberty, hormonal dysregulation and bone dysplasia. Conversely Albright hereditary oseteodystrophy is caused by an activating mutation in $G\alpha_s$. Both hereditary disorders are seen in heterozygous carriers of the mutation, since a normal functioning copy of $G\alpha_s$ is probably essential for human life. Interestingly, severity varies among patients due to imprinting of the $G\alpha_s$ gene, GNAS.

In addition to GTP/GDP binding, $G\alpha_s$ is also regulated by trafficking between various cellular compartments. Unlike GPCRs and AC, $G\alpha_s$ is a peripheral membrane protein and can dissociate from the membrane into the cytosol. Its cytosolic role is relatively unclear, but regulation of microtubule dynamics is one of its roles(Yu et al. 2009). Membrane scaffolding is largely attributed to protein binding: GPCRs, adenylyl cyclase, $G\beta\gamma$, as well as direct membrane associations.

Direct membrane association of $G\alpha_s$ is mediated by two factors, both present in the $G\alpha_s$ N-terminus. First is a stretch of polybasic amino acids that facilitate association through electrostatic interactions with negatively charged phospholipid head groups. Second is a reversible covalent palmitoyl group added to the second amino acid, cysteine. This contrasts to other $G\alpha_i$ proteins, which also have a non-reversible myristol group. The non-reversible nature of myristoylation is often cited as the reason why $G\alpha_i$ is much less cytosolic relative to $G\alpha_s$.

The significance of $G\alpha_s$ palmitoylation and the role of palmitoylation in biology in general remains unclear. More so than the most well-studied covalent protein modification – phosphorylation - palmitoylation is challenging to study. Palmitoylation is less antigenic than phosphorylation and antibodies selective to palmitoylated proteins are very rare. Furthermore, phosphorylation is easily probed using radioactive phosphorus-32, but palmitic acid only contains oxygen, carbon and hydrogen. Carbon-14 and hydrogen-3 have been used to assay palmitoylation, but signal from these isotypes is significantly less potent than ^{32}P.

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Furthermore, this requires cells to artificially uptake the radioactive palmitic acid, which limits its utility.

While still lagging behind the numerous tools for assaying phosphorylation, additional palmitoylation assays are slowly closing this gap. A biotin-switch assay is also a qualitative analysis for the presence of S-acylation, but it is not specific to palmitic acid relative to other fatty acids. This is likely significant, as mass spectroscopy suggests other fats compete with palmitic acid for incorporation into $G\alpha_s$. Another mass spectroscopy experiment has also demonstrated N-acylation is possible in $G\alpha_s$ at the adjacent glycine, suggesting there is more than one site of $G\alpha_s$ lipid modification.

Much of what one can infer about $G\alpha_s$ palmitoylation is derived by mutagenesis experiments and speculation based on other proteins. Mutating the palmitoylated cysteine into an alanine causes a significant shift of $G\alpha_s$ from the membrane to the cytosol. Palmitoylation is not necessary for membrane association, as overexpression of some $G\beta\gamma$ isoforms can restore membrane association in palmitoylation-deficient $G\alpha_s$ (Evanko et al. 2001). Mutated $G\alpha_s$ constructs unable to bind to $G\beta\gamma$ fail to localize to the membrane, and are not palmitoylated (Evanko 2000).

1.2.5 G-protein regulation through lipid rafts

In addition to proteins, the influence of lipid signaling is an increasingly recognized modality of cellular signal transduction. In particular is the regulation of signaling by highly ordered cholesterol and cytoskeleton-rich membrane nanodomains called lipid rafts. Conceptually, these pockets of membrane heterogeneity are speculated to either promote signaling by scaffolding signaling partners in accessible proximity, or dampen signaling by segregating them(Allen, Halverson-Tamboli, and Rasenick 2006). Their nano-scale size and dynamic nature have made them challenging to study and their exact role in physiologic systems remains unclear.

The concept of lipid rafts began with the observation that particular lipids such as sphingolipids and cholesterol self-associate. Physicists noted the formation of liquid-ordered and liquid-disordered phases in model membrane systems and biologists began to speculate whether this separation could explain the membrane polarity of particular cell types. In the early 1990's, the concept of lipid rafts evolved to include detergent-resistant membranes – cholesterol and cytoskeleton-rich biologic membranes that are not extracted with mild detergents such as Triton-X100. Since then, the existence of lipid rafts *in vivo* has remained controversial, since model membranes are highly non-physiologic and detergent extraction may create artifacts. More recently however, super high-resolution microscopy has begun to clarify their nature in live cells(Lingwood and Simons 2010).

Regardless of whether the concept of lipid rafts is precise in the canonical sense, detergent-resistant membranes have demonstrated considerable insight into G-protein signaling. Most notably, G-protein or GPCR association with lipid rafts is

often a proxy for the protein's activity.   For example, $G\alpha_s$ signaling is dampened when concentrated in lipid rafts, but cholesterol chelation, which destroys the structure of rafts, greatly enhances $G\alpha_s$-AC coupling(Rybin et al. 2000; Allen et al. 2005). $G\alpha_q$-PLC coupling, on the other hand is dampened, with lipid raft disruption (Pešanová et al. 1999; Bhatnagar et al. 2004; P. Oh and Schnitzer 2001).

Rafts also play an interesting role in G-protein recycling. Unlike most GPCRs who internalize through clathrin-coated pits in an arrestin-dependent manner, $G\alpha_s$ internalization co-localizes with cholera-toxin labeled vesicles, a well-established marker of lipid rafts(Allen et al. 2005). Although it is speculated that $G\alpha_s$ may signal through GPCRs in cytosolic membranes(Irannejad et al. 2013), independent internalization suggests this type of signaling is differentially regulated from which occurs on the plasma membrane.
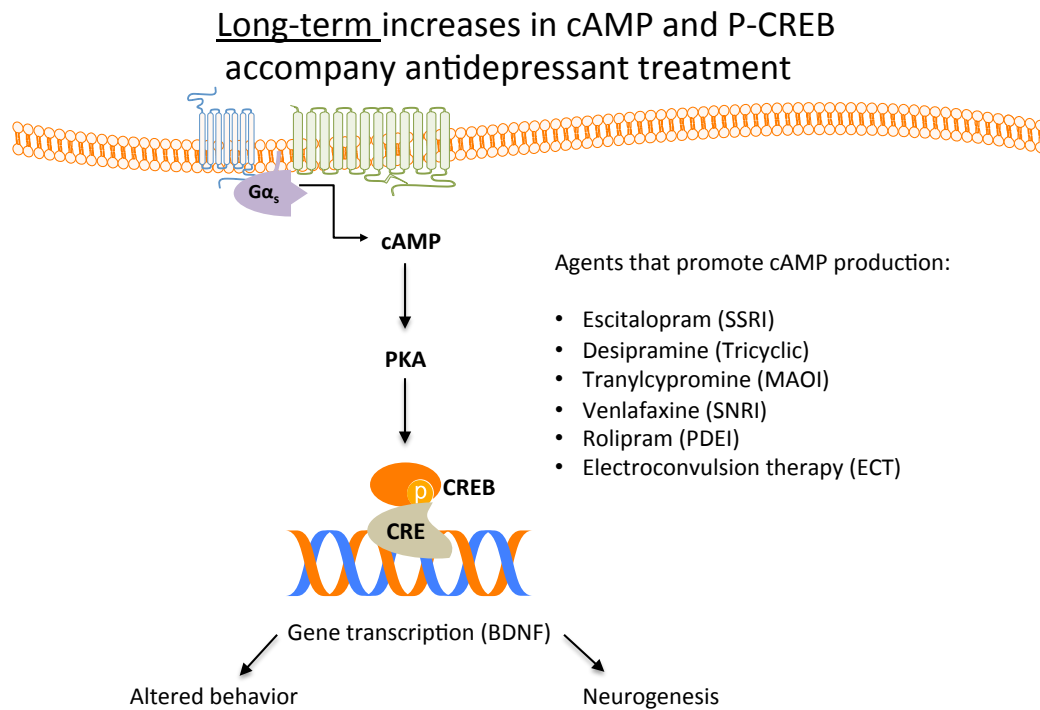
Figure 1. Antidepressants augment cAMP signaling.
All antidepressants tested, as well as ECT augment cAMP signaling. Downstream targets of cAMP, such as CREB, interact with BDNF to alter behavior and influence synaptic modeling.

1.2.6 G-protein signaling in depression

Monoamine neurotransmission occurs primarily on GPCRs and their coupled G-proteins. It follows that G-proteins play a significant role in decreased monoamine signaling in depression and increased monoamine signaling with antidepressant treatment. While monoamines activate GPCRs coupled to most $G\alpha$ subtypes, $G\alpha_s$ is of particular interest because cAMP is responsible for a host of downstream neurotropic signaling including BDNF. This downstream signaling is speculated to be responsible for at least some of the symptom relief offered by antidepressants.

Antidepressants require days to weeks for relief of depression, while increased monoamine signaling is relatively rapid. Therefore, it is suspected that additional effects of antidepressants are relevant. Among these is a monoamine-independent enhancement of cAMP signaling only seen after chronic antidepressant treatment (Menkes et al. 1983). This was first demonstrated in rat brains treated chronically or acutely with antidepressants. Washed membranes isolated from these animals, free from endogenous neurotransmitters, were then stimulated with either a fixed concentration of ligand (isoproterenol) or aluminum tetrafluoride. In both scenarios, chronically antidepressant treated brain homogenates produced significantly higher cAMP. Later work confirmed this effect for a variety of classes of antidepressants (TCAs, SSRIs and MAOIs) as well as electric-convulsive therapy(Ozawa and Rasenick 1991).

Extending these findings, it was demonstrated that increased $G\alpha_s$-AC coupling was mediated through a redistribution of $G\alpha_s$ out of lipid rafts(Chen and Rasenick 1995b;

Toki, Donati, and Rasenick 1999). Furthermore, post-mortem analysis of depressed patients revealed an increased distribution of $G\alpha_s$ in detergent insoluble domains(Donati et al. 2008) and decreased cAMP production from leukocytes of depressed patients(Pappalarado, Alpert, and Schildkraut 2013).

To date, however, a direct mechanism by which antidepressants alter $G\alpha_s$ raft association is unknown. The classic targets of antidepressants are neither necessary nor sufficient. C6 glioma demonstrate this effect after escitalopram, sertraline and fluoxetine treatment, despite lacking the serotonin transporter(L. Zhang and Rasenick 2010). Furthermore, transfection of non-responsive cell types with this transporter, such as HEK293, does not confer a responsive phenotype. The target however is highly selective, as *R*-citalopram, the structural isomer of escitalopram, does not affect $G\alpha_s$ signaling.

These observations make $G\alpha_s$ signaling a puzzling, but exciting area of antidepressant research. Since the timing of antidepressant augmented $G\alpha_s$ – adenylyl cylcase coupling much better matches the symptomatic relief of antidepressants relative to increased monoamine transmission, regulation of $G\alpha_s$ is an attractive therapeutic target for novel fast-acting antidepressants. First however, increased mechanistic understanding of $G\alpha_s$ regulation by lipid rafts is necessary.

1.3 Specific Aims

This thesis explores the dynamic interaction of G-proteins and membrane nanodomains. Specifically, the nature of $G\alpha_s$ regulation by antidepressants and

polyunsaturated fatty acids is explored. Through four aims, the relationship

between $G\alpha_s$ signaling and lipid rafts is elucidated through two novel approaches:

fluorescence recovery after photobleaching and small-angle neutron scattering.

1.3.1 To develop a live-cell imaging assay to evaluate $G\alpha_s$ membrane scaffolding

Controversy over the existence of lipid rafts stems largely from the lack of imaging

of lipid nanodomains in live cells. Lipid rafts are estimated to exist at sizes smaller

than the limit of fluorescent light refraction, and therefore cannot be visualized

directly by traditional light microscopy. Nonetheless, diffusion may act a proxy for

lipid raft association since rafts are thought to organize protein scaffolding and thus

movement. This aim seeks to develop a highly reproducible diffusion assay for GFP-

$G\alpha_s$.

1.3.2 To determine the effects of antidepressant treatment and lipid raft disruption on GFP- $G\alpha_s$ dynamics using Fluorescence Recovery After Photobleaching

After successful characterization of the FRAP diffusion assay, $G\alpha_s$ diffusion is studied

under a variety of conditions. This includes antidepressant treatment and lipid raft

disruption – two very different methods that both show similar augmentation of $G\alpha_s$

-adenylyl cyclase coupling and decreased $G\alpha_s$ raft association.

1.3.3 To evaluate the changes in $G\alpha_s$ signaling by polyunsaturated fatty acids

Clinical studies suggest polyunsaturated fatty acids, specifically omega-3 PUFAs,

demonstrate antidepressant properties, but mechanism is poorly understood. Given

the role of lipids in regulating $G\alpha_s$ signaling by traditional antidepressants, this

pathway is studied after PUFA treatment to evaluate similar effects.

1.3.4 To analyze the structural effects of antidepressants in model membranes.

Antidepressant accumulation in cell membranes suggests the possibility that

changes in membrane structure may play a role in their effects on cell signaling.

Chapter 2: Materials and Methods

Parts of this chapter were based on methods previously published:
Zhang, L., and M.M. Rasenick. 2010. "Chronic Treatment with Escitalopram but Not
R-Citalopram Translocates G Alpha S From Lipid Raft Domains and Potentiates
Adenylyl Cyclase: a 5-Hydroxytryptamine Transporter-Independent Action of This
Antidepressant Compound." *Journal of Pharmacology and Experimental Therapeutics*
332 (3). ASPET: 977.

2.1 Cell Culture and Drug Treatment

C6 cells were cultured in Dulbecco's modified Eagle's medium, 4.5 g of glucose/L,

10% newborn calf serum (Hyclone Laboratories, Logan, UT), 100 mg/ml penicillin

and streptomycin at 37°C in humidified 10% CO_2 atmosphere. The cells were

treated with 10μM antidepressants, or 50μM fatty acids for three days or as

otherwise specified. The culture media and drug were changed daily. There was no

change in morphology of cells during the period of exposure to antidepressants or

fatty acids.

Escitalopram and *R*-citalopram were gifts from Lundbeck, Copenhagen, Denmark.

Velafaxine and sertraline were gifts from Pfizer. Desipramine hydrochloride,

resperine, tianeptine sodium salt, and bupropion hydrochloride were purchased

from Tocris Bioscience, Ellisville, MO. Lithium chloride, chlorpromazine

hydrochloride, phenelzine sulfate, imipramine hydrochloride, colchicine, MβCD, 2-

bromopalmitate, stearic acid, palmitic acid and sodium valproic acid were

purchased from Sigma-Aldrich, St. Louis, MO. DHA, EPA, linoleic acid and

arachidonic acid were purchased from Nu-Chek Prep Inc, Elysian, MN.

2.2 Expression Plasmids

GFP-Gα_s A206K was constructed with Stratagene QuikChange mutagenesis using previously described GFP-Gα_s as a template(Yu and Rasenick 2002) and primers described elsewhere(Zacharias 2002). Palmitoylation deficient GFP-Gα_s was also constructed using Stratagene QuikChange mutagenesis as described before with HA-Gα_s(Thiyagarajan et al. 2002). The resulting constructs were verified by DNA sequencing to contain no mutations other than those desired.

2.3 Transfection and generation of stable cell lines

C6 glioma were cultured until 80% confluency and then trypsinized into suspension for electroporation with the Neon Transfection System. Approximately 15µg of DNA was used per one million cells. After transfection, cells were plated in an appropriate dish for 24 hours before further lysis, imaging or clonal selection. To isolate a stable expressing cell line, cells were treated with 1mg/mL G418 for at least three passages (approximately one week each) and individual clones were selected using Fluorescence-activated cell sorting (FACS). After sorting, G418 was not needed to maintain stable expression of transfected DNA.

2.4 Cell Membrane, Lipid Raft, and Detergent Extract Preparation

Cells were washed and harvested in ice-cold Phosphate-Buffered Saline (Mediatech Inc.). TX-100 insoluble membrane fractions were prepared as described by Li et al., with slight modification(Li et al. 1995; Donati and Rasenick 2005). In brief, C6 cells or tissue were scraped into 0.75 ml of HEPES buffer (10 mM HEPES, pH 7.5, 150 mM

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NaCl, 1 mM DTT, and protease inhibitors) containing 1% TX-100. Samples were homogenized with 10 strokes of a Potter-Elvehjem homogenizer. The homogenate was then mixed with an equal volume of 80% sucrose prepared in HEPES buffer to form 40% sucrose and loaded at the bottom of an ultracentrifuge tube. A step gradient was generated by sequentially layering 30, 15, and 5% sucrose over the homogenate. Gradients were centrifuged at 200,000g for 20 h in an SW55 rotor (Beckman, Palo Alto, CA). Two or three opaque bands were confined between the 15 and 30% sucrose layers. These bands were removed from the tube, diluted 3-fold with HEPES buffer, and pelleted in a microcentrifuge at 16,000 g. The pellet was resuspended in HEPES buffer and subsequently analyzed by immunoblotting.

In order to prepare TTX-100/114 detergent extracts, sample pellets were resuspended in TME lysis buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.5, protease inhibitors) and homogenized 10 strokes in a motor controlled Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). All procedures are carried out under ice-cold conditions. Total cell homogenates were centrifuged at 100,000 × g for 30 min. at 4°C to pellet the membranes. The cytosolic supernatant was aspirated and saved. The remaining crude membrane pellet was resuspended with a 10:1 volume per pellet size ratio of TME containing 1% TX-100 and 150mM NaCl, using the same homogenization method and rotated in centrifuge tubes for an additional 30 min. at 4°C. This sample was centrifuged as above and both the supernatant (TX-100 extract) and pellet were saved. This pellet was resuspended with a 10:1 volume per pellet size ratio of TME containing 1% TX-114 and 150mM NaCl, for 30 min. at 4°C

and homogenized as above. The sample was centrifuged as above and both the supernatant (TX-114 extract) and pellet (detergent-insoluble pellet) were saved.

2.5 SDS-PAGE and Western Blotting

Samples were assayed for protein via a bicinchoninic acid assay (Pierce Research, Rockford, IL) and equal quantities were loaded onto an acrylamide gel for SDS-PAGE. Gels were transferred to Immobilon-P PVDF membranes (EMD Millipore, Billerica, MA) for western blotting. The membranes were blocked with 5% nonfat dry milk diluted in TBS-T (10 mM Tris-HCl, 159 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h. Following the blocking step, membranes were washed with Tris-buffered saline/Tween 20 and then incubated with an anti-Gα_s monoclonal antibody (NeuroMab clone N192/12, Davis, CA) or anti-Gα_s polyclonal antibody (EMD Millipore, Billerica, MA) overnight at 4°C. Membranes were washed with TBS-T and incubated with a secondary antibody [HRP-linked anti-mouse antibody IgG F(ab′)2 or HRP-linked anti-rabbit antibody IgG F(ab′)2] (Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature, washed, and developed using ECL Luminata Forte chemiluminescent reagent (Millipore, Billerica, MA). Blots were imaged using Chemidoc computerized densitometer (Bio-Rad, Hercules, CA) and quantified by ImageLab 3.0 software (Bio-Rad, Hercules, CA). In all experiments, the original gels are visualized using BioRad's Stainfree technology to normalize protein loading.

2.6 Fluorescence recovery after photobleaching (FRAP)

A clonal stable C6 glioma cell line expressing GFP-Gα_s was selected using a combination of G418 resistance followed by clonal fluorescent cell sorting. The established line was then plated onto glass dishes for live cell imaging 4 days before an experiment. Cells were then treated as specified. Drug was washed out 1 hour before microscopy for chronic treatments. The media was also changed to 2.5% newborn calf serum in phenol-red free DMEM to decrease fluorescent background. For imaging, cells were kept at 37°C using a heated stage plate. All images were taken using a Zeiss LSM 710 at 512 x 512 resolution using an open pinhole to maximize signal but minimizing photobleaching. For each cell, 150 data points, including 10 pre-bleach values, were measured, approximately 300ms apart. In addition, background and total photobleaching were subtracted for each data point. Half-time to recovery and immobile fraction were calculated by a one-phase association curve fit.

2.7 Adenylyl cyclase assays

Adenylyl cyclase enzyme activity assays in cell membranes were also performed according to the method of Salomon with slight modifications(Salomon, Londos, and Rodbell 1974). Initially, membranes from cells or tissue were obtained by centrifugation as follows. Harvested samples were resuspended in 1mL cold HEPES/sucrose buffer (10mM HEPES, 1mM DTT, 0.25M sucrose, 1x protease inhibitor, pH 7.5) and homogenized with 10 strokes of a Potter-Elvehjam homogenizer. The homogenate was centrifuged at 600 x g for 10 min. The

supernatant was then ultracentrifuged at 100,000 x g for 30 min and membrane

pellets obtained and resupensed in 700uL HEPES buffer (15mM HEPES buffer, 1x

protease inhibitor cocktail, pH 7.5). Samples were stored under liquid nitrogen until

use.

Then, 25 μg of membranes was added into a reaction mixture with 10 μM GTPYS, 10

mM NaF (+20 μM $AlCl_3$), or 10 μM isoproterenol for 20 min at 30°C in 100 μl of

medium containing 15 mM HEPES, pH 7.5, 0.05 mM ATP, 2.5 μCi/ml [^{32}P]ATP, 5 mM

$MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.05 mM cAMP, 0.01 mM GTP, 0.25 mg/ml bovine

serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 mg/ml creatine

phosphate, and 0.14 mg/ml creatine phosphokinase. The reaction was terminated

by adding 0.1 ml of a solution containing 2% SDS, 1.4 mM cAMP, and 40 mM ATP.

[^{32}P]cAMP was isolated by the method of Salomon(Salomon, Londos, and Rodbell

1974) using [^{3}H]cAMP to monitor recovery. All assays were performed in triplicate.

2.8 Small Angle Neutron Scattering

SANS experiments described in this work were carried out at the ILL in Grenoble

France using the D22 instrument and at National Institute of Standards and

Technology (NIST) Center for Neutron Research (NCNR) on NG7-SANS. Small

unilamellar vesicles (SUV) were prepared using deuterated DPPC, hydrogenated

DOPC plus hydrogenated cholesterol and/or drugs. First, dry components were

mixed by dissolving in chloroform and then evaporated for at least four hours in

vacuum at 50°C. Samples were then resuspended in the approximate contrast

matched water (50/50 D_2O/H_2O) at a concentration of 25mg/mL. The suspension

was then sequentially extruded 42 passages through a 100nm and then 30nm filter

to result with stable, uniform vesicles. Aliquots of this were then diluted to 5mg/mL

in various ratios of D_2O/H_2O to perform a contrast-matching series at 37°C. The

resulting intensities generated from a brief SANS experiment were then plotted

against the corresponding D_2O/H_2O ratios and an ideal contrast match point was

estimated from the linear fit. The remaining sample was then diluted to 20mg/mL in

the corresponding D_2O/H_2O ratio and analyzed by SANS at indicated temperatures.

2.8 Statistical Analysis

All of the experiments were performed at least three times. Data were analyzed for

statistical significance using a one-way ANOVA followed by Tukey test for post hoc

multiple comparisons of means. Values of $p < 0.05$ were taken to indicate

significance.

Chapter 3: To develop a highly reproducible FRAP assay to probe Gα$_s$ raft association and signaling.

3.1 Introduction to fluorescent proteins and their uses

3.1.1 Discovery and initial characterization of GFP

The development of fluorescent fusion proteins in the late 20th century revolutionized molecular biology and Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien were awarded the 2008 Nobel Prize in chemistry for this contribution. The most famous fluorescent protein, GFP, was identified in *Aequorea victoria* in the 1960s, but not cloned into other species until 1994(Chalfie et al. 1994). Since then, countless GFP fusion proteins have been derived to probe a variety of biologic processes.

3.1.2 Evolution of FPs

The first variations of GFP focused on increasing its photostability, intensity and emission/excitation profile. Enhanced GFP or eGFP became so widely used that it is often simply abbreviated as GFP. This work was greatly accelerated by solving GFP's crystal structure and thus offering insight into the chromatophore (Ormö et al. 1996). An understanding of the chromatophore has allowed numerous spectral variants of GFP including the common cyan and yellow fluorescent proteins. Furthermore, the discovery of other naturally occurring fluorescent proteins in marine life has lead to a very large palette of FPs.

Choices now include the full visible light spectrum: violet, blue, cyan, green, yellow, orange, red and far red(Chudakov et al. 2010). Theses variants are classified by their peak emission profile, but in practice, emission can bleed into other colors. Therefore if using multiple FPs in one cell, it is critical to select colors that do not share overlapping emission profiles. Nonetheless, up to six simultaneous FPs have been measured at once(Kogure et al. 2006). Unfortunately altering the chromatophore structure to shift emission/excitations of profiles often changes more than the color of the protein. In particular, photostability, folding and intensity often suffer. Thus, fining tuning of each spectral variant is a complicated and on-going field of research(Goedhart et al. 2012).

Many initial problems of FPs have turned into interesting tools for researchers. For example, native GFP actually has two excitation peaks, which are usually undesirable for imaging. Researchers removed one of these peaks when designing the more commonly used eGFP, but the double peak phenomenon was later retooled to design a photoactivatable FP. This clever FP switches peak excitation after exposure to a particular wavelength of light, allowing researchers to track a particular population of activated FP as it traffics throughout a cell(Patterson and Lippincott-Schwartz 2002). Similarly, pH sensitivity was a problem of some FPs. Later this was exploited by generating FPs who emission will vanish or appear when the protein is moved into different pH environments(Miesenböck, De Angelis, and Rothman 1998).

Indeed, there exists an ever-growing library of applications for FPs beyond simply visualizing a protein's location. Förster resonance energy transfer (FRET) is particularly useful for creating a plethora of biosensors. Here, proximity of two fluorophores is detected because one FP's emission matches the excitation of another. Therefore the ratio of the two FP's emission profiles can be correlated to the interactions of two FPs. While useful for studying protein-protein interactions in fusion proteins, FRET is also used to detect the dynamics of small molecules like cAMP. In this sensor, CFP and YFP are fused to the cAMP-binding domain of the protein EPAC. This domain dramatically alters conformation when bound to cAMP, separating the FRET pair and thus changing the recorded emission profile(DiPilato, Cheng, and Zhang 2004). Additional biosensors and applications of FPs are extensively reviewed elsewhere(Chudakov et al. 2010).

3.1.3 FPs to study lipid rafts

Fluorescence microscopy is an attractive complementary approach for studying lipid raft domains because other approaches, such as cell fractionation, may produce non-physiologic artifacts. Unfortunately, the utility of FPs in studying lipid rafts remains challenging because of limitations in resolution of light microscopy. Lipid rafts are thought to be approximately 10-100nm in size, but light microscopy is limited by the refractive index of light to approximately 500nm.

As an alternative to directly visualizing rafts, the role of rafts in protein trafficking can be assessed indirectly. FRET for example has been used to demonstrate FP

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density when targeted to rafts(Zacharias 2002). Fluorescence recovery after photobleaching (FRAP) is another technique, but its value remains unclear(Kenworthy et al. 2004; Engel et al. 2010). Simple colocalization with raft markers like the GM1-labeling cholera toxin B is also used even though they cannot resolve individual rafts(Pinaud et al. 2009; Allen et al. 2005). New advances in super resolution microscopy such as Stochastic optical reconstruction microscopy (STORM) and fluorescence photoactivation localization microscopy (fPALM) are beginning to change this, but are still in their infancy(Sengupta et al. 2011; Betzig et al. 2006; Rust, Bates, and Zhuang 2006; Gunewardene et al. 2011).

3.1.4 GFP-Gα$_s$ as a tool to study Gα$_s$ association with lipid rafts

Gα$_s$ regulation by lipid rafts has been exclusively demonstrated by cell fractionation and cholera toxin B colocalization(Allen et al. 2005; Moffett, Brown, and Linder 2000). The latter was studied using an eGFP-Gα$_s$ fusion protein. Traditional FP fusions occur at the amino or carboxyl terminus in most constructs because these locations are least likely to affect the tagged protein's structure. Unfortunately Gα has important interactions in both regions and resulting fusions result in non-physiologic trafficking. While more challenging to design, protein tags can alternatively be inserted within a protein if special attention is paid to its structural significance. In the case of Gα, an internal tag works well between the helical domain and GTPase domain. This was first demonstrated in Gα$_s$ using the hemagglutinin epitope (HA) and later with eGFP(Levis and Bourne 1992; Yu and Rasenick 2002). Characterization of both tagged Gα$_s$ constructs has demonstrated

coupling to adenylyl cyclase and trafficking among membrane and cytosolic domains.

Experiments in this chapter explore the development of a highly reproducible FRAP assay using GFP-Gα_s that can be used to study Gα_s raft-association in live cells. In particular we wanted to generate a stable, clonal cell line expressing GFP-Gα_s so that artifacts from a transient transfection would not interfere. Surprisingly, eGFP-Gα_s expression longer than a few days proved extremely toxic to cells and further experiments below strongly suggest eGFP-Gα_s forms toxic aggregates. In response, a novel monomeric GFP-Gα_s was generated and C6 glioma clones stably expressing mGFP-Gα_s were isolated.

3.2 Results

3.2.1 Fluorescence microscopy reveals eGFP-Gα_s displays variable cellular localization among different cells

Similar to many peripheral membrane proteins, Gα_s is found in various cellular compartments, including the cytosol, plasma membrane and intramembranes. This has been previously been characterized by cell fractionation and immunocytochemistry. Changes in localization are largely attributed to GPCR-mediated activation, coupling to its primary effector, adenylyl cyclase, and down regulation through internalization with lipid rafts(Thiyagarajan et al. 2002; Allen et al. 2005). To better understand this regulation, a GFP fusion protein was previously generated, eGFP-Gα_s, and characterization showed normal trafficking between cellular compartments and coupling to GPCRs and adenylyl cyclase.

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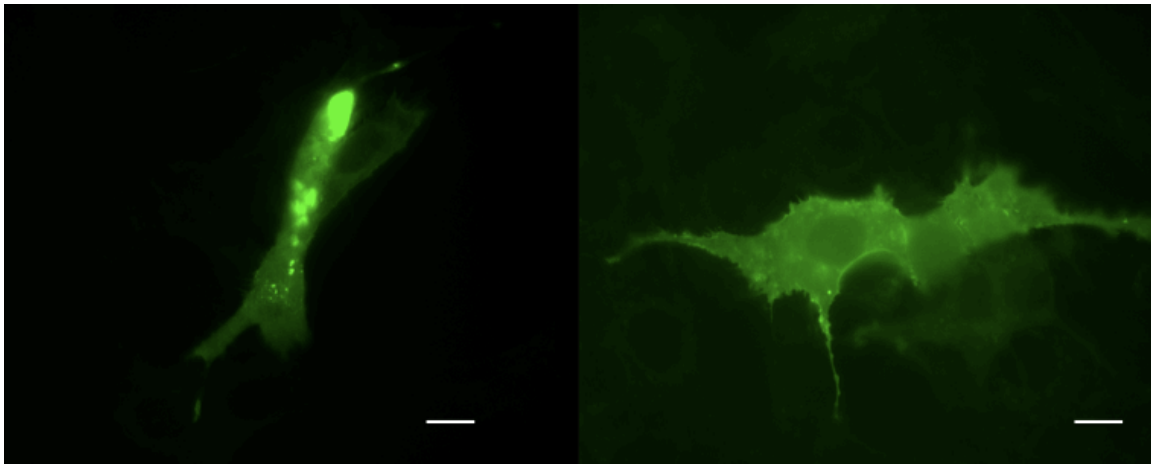
Figure 2. Variable pattern of eGFP-Gα_s distribution is seen in C6 glioma.
C6 glioma transfected with eGFP-Gα_s were visualized after three days. Cells displayed two patterns of fluorescence; representative images of both are shown above. On the left, GFP is largely localized in large bright clusters compared to the image on the right where GFP distribution closely matches Gα_s immunostaining. Scale bars for both images equal 10 μm.

Nonetheless, a subpopulation of transfected cells showed aberrant localization relative to endogenous Gα_s. Specifically, regions of exceptionally bright fluorescence were observed in some GFP-Gα_s expressing cells (**Figure 2**). This difference became more prevalent three days after transfection, where few cells remained with normal Gα_s distribution of fluorescence. Furthermore, neither G418 selection nor Fluorescence-Activated Cell Sorting (FACS) were able to isolate cells with stable GFP fluorescence. This is in contrast to other work showing stable overexpression of HA-tagged Gα_s was not deleterious to cell survival. In addition, many tumors demonstrate Gα_s activating mutations suggesting Gα_s overexpression is not particularly lethal.

3.2.2 eGFP-Gα_s has increased localization in detergent resistant fractions relative to endogenous Gα_s

Transiently transfected cells were fractionated after three days of eGFP-Gα_s expression and localization was compared to that of endogenous Gα_s. Five compartments were examined: whole cell lysate, cytosol, TTX-100 membranes, TTX-114 membranes and the detergent-insoluble remainder. Unlike endogenous Gα_s, which are found in similar concentrations in all membrane compartments, eGFP-Gα_s was found almost exclusively in the detergent-insoluble remainder suggesting the formation of insoluble aggregates (
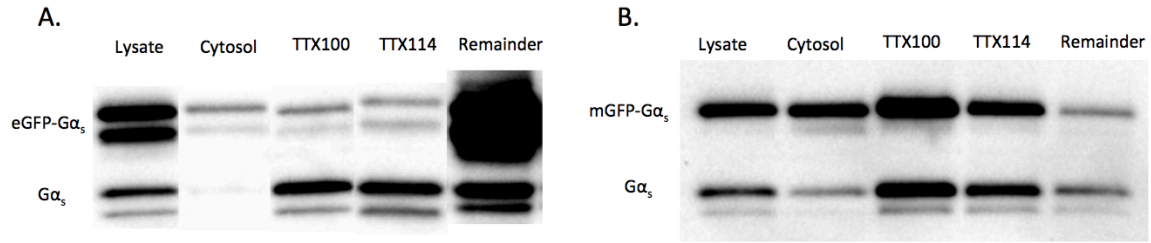
Figure 3 A).
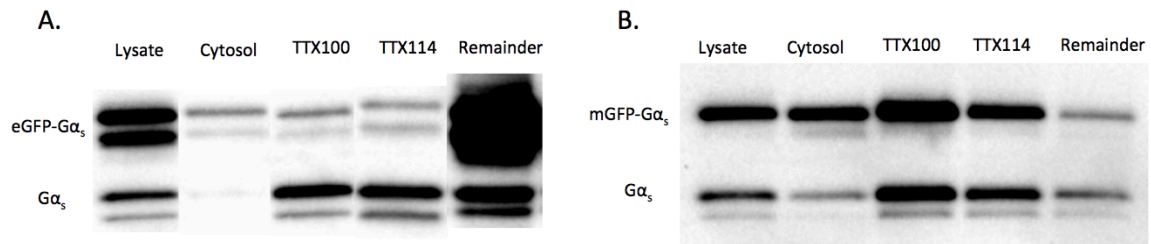
Figure 3. Cellular fractionation of cells transfected with GFP-Gα$_s$ reveals its compartmentalization relative to Gα$_s$.
C6 glioma transfected with eGFP-Gα$_s$ or mGFP-Gα$_s$ were cultured for three days post transfection. Lysed membranes were then sequentially fractionated with TTX-100 and TTX-114 detergents. In addition, the insoluble remainder was also collected. The five fractions were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted for Gα$_s$ content. A) eGFP-Gα$_s$ accumulates in detergent resistant membranes whereas B) mGFP-Gα$_s$ localizes similar to endogenous Gα$_s$.

3.2.3 Fluorescence Recovery After Photobleaching shows some eGFP-Gα_s accumulates as immobile aggregates

Next, cells transfected with eGFP-Gα_s were imaged and different cellular regions were measured using FRAP (**Figure 4**A). Not surprisingly, membrane regions recovered significantly slower than most cytosolic regions. Suspected cytosolic aggregates however demonstrated a significantly less mobility (**Figure 4**B).
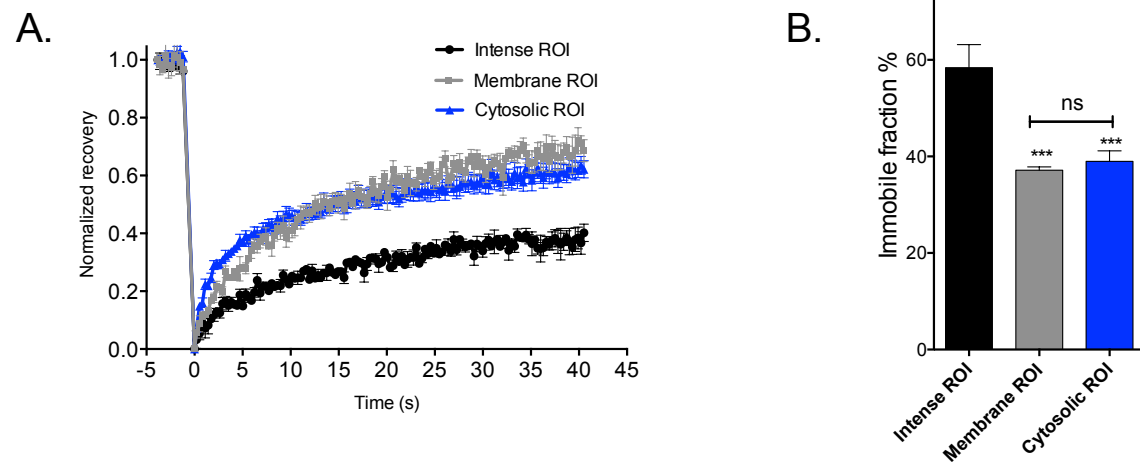
Figure 4. Cellular distribution of eGFP-Gα$_s$ affects diffusion.
C6 Cells transiently transfected with eGFP-Gα$_s$ and the indicated cellular regions were selectively photobleached. A) Membrane regions recovered slower than most cytosolic regions, except for intense clusters. B) The immobile fraction of intense ROI was significantly increased relative to membrane and other cytosolic ROI.

3.2.4 Monomeric eGFP-Gα$_s$ maintains physiologic cellular distribution

Given the evidence that lipid raft targeted GFPs can dimerize, we mutated eGFP-Gα$_s$ to incorporate the A206K mutation. Transfected cells displayed a significantly greater homogeneity in fluorescence pattern, including the absence of high intensity clusters. This pattern was not only maintained three days post-transfection, but indefinitely after the G418 selection. Furthermore, clones isolated through FACS maintained mGFP-Gα$_s$ expression without G418 (Figure 5). Both stable expressing and transiently transfected mGFP-Gα$_s$ fractionated in identical cellular compartments relative to endogenous Gα$_s$ (
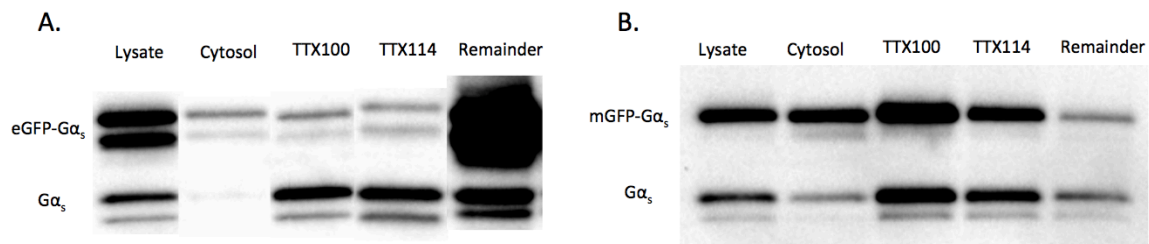
Figure 3 B). FRAP of mGFP-Gα$_s$ was nearly identical to regions of eGFP-Gα$_s$ without intense GFP clusters (**Figure 4**).
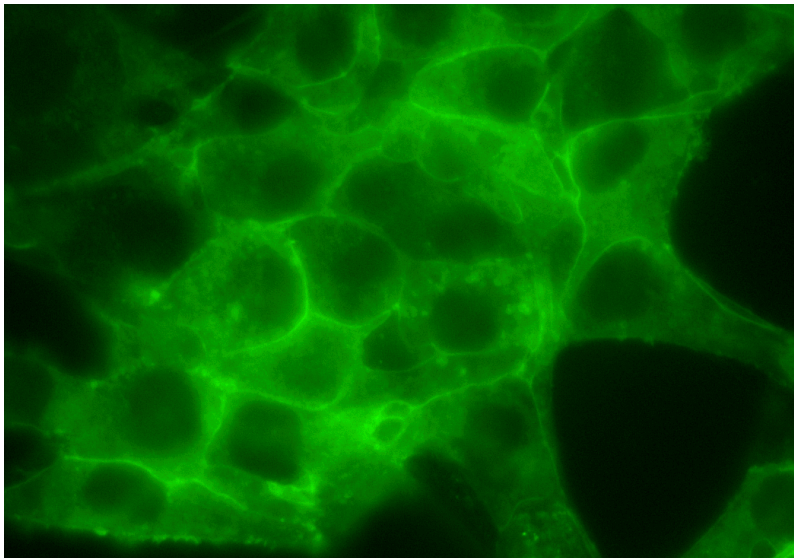
Figure 5 Clones C6 glioma expressing mGFP-Gα$_s$.
C6 glioma were transfected with mGFP-Gα$_s$ and after 24 hours of recovery, treated with G418 for two weeks. Then individual cells were isolated with FACS and clonal cell lines were cultured. Unlike dimeric GFP-Gα$_s$, the monomeric variant did not accumulate toxic aggregates of GFP and therefore stable expressing clones were easily isolated using a combination of G418 selection and FACS. Scale bar equals 10 μm.

3.3 Discussion

3.3.1 GFP-Gα$_s$ is sensitive to oligomerization

Previous characterization of GFP-Gα$_s$ after transient transfection reveals similar properties to endogenous Gα$_s$, including its ability to activate adenylyl cyclase and its distribution within the cell(Yu and Rasenick 2002; Allen et al. 2005). Therefore it was surprising that our group was unable to produce stably expressing clones of cell lines with GFP-Gα$_s$. Furthermore, it was noted that GFP-Gα$_s$ expressing cells developed large clusters of fluorophores and became apoptotic over time (**Figure 2**). Since some raft-targeted FPs have been shown to oligomerize we tested whether the A206K mutation in GFP would benefit stable transfection of GFP-Gα$_s$.

Our results demonstrate the variant of GFP fused to Gα$_s$ has a profound effect of the fusion protein's ability to traffic within cells. Fused with the dimeric GFP, Gα$_s$ eventually accumulates in detergent resistant membrane fractions that recover significantly slower after photobleaching(
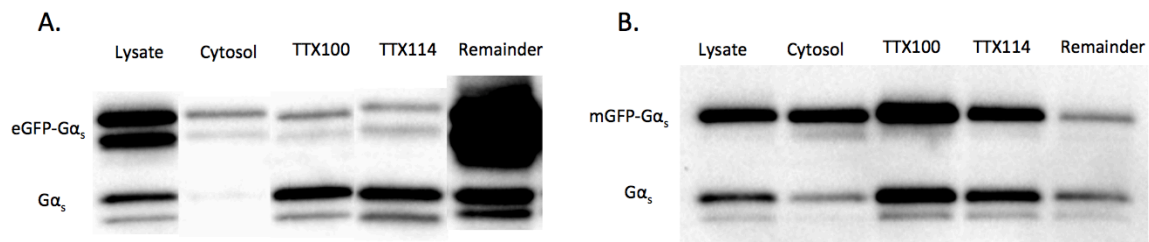
Figure 3A). In contrast, monomeric GFP-Gα$_s$ displays trafficking similar to the native protein (

48

A. | Lysate | Cytosol | TTX100 | TTX114 | Remainder

eGFP-Gα_s

Gα_s

B. | Lysate | Cytosol | TTX100 | TTX114 | Remainder

mGFP-Gα_s

Gα_s
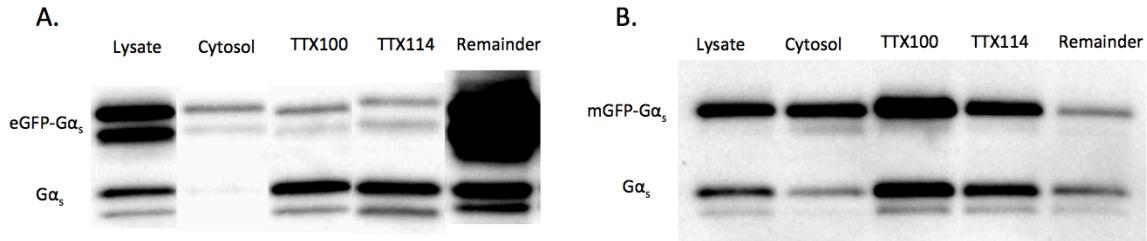
Figure 3B). These results are likely at least in part because of Gα_s association with lipid rafts. Unlike native eGFP which are diffuse in the cytosol, eGFP-Gα_s are highly concentrated and constrained in membrane nanodomains (Figure 6). While eGFP has a relative high dissociation constant for binding with other eGFP compared to unrelated fluorescent proteins like dsRed, oligiomerization becomes relevant when protein is enriched in a confined domain.

Previous reports have demonstrated differences between oligomeric and monomeric bare FPs, these data are unique in highlighting differences between FPs fused with functional proteins. It is especially interesting that the effects are relative subtle, and are only pronounced after long-term protein expression. Together, these experiments reinforce the importance of FP selection in designing novel fusion proteins.

A. eGFP-Gα$_s$
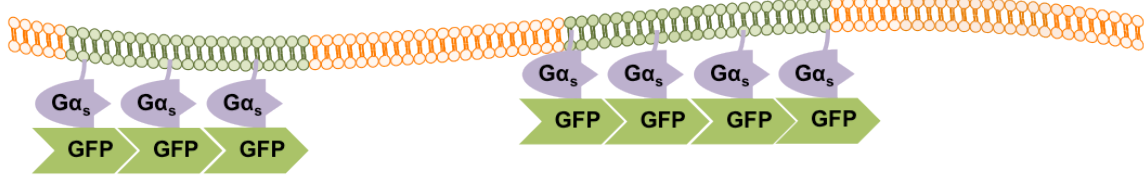
B. Monomeric GFP-Gα$_s$
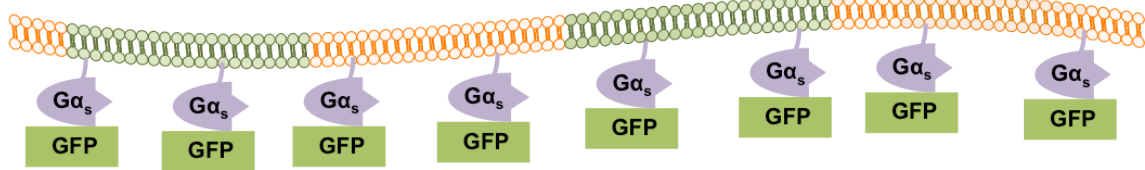
Figure 6. A model for eGFP-Gα$_s$ aggregation in lipid rafts.
The A206K mutation in GFP significantly decreases the self-association of GFP. In the model above, this eGFP-Gα$_s$ is highly localized to lipid rafts where it GFP oligomerizes, preventing normal trafficking. On the other hand, monomeric GFP-Gα$_s$ can freely move in and out of rafts similar to endogenous Gα$_s$.

Chapter 4: To determine the effects of antidepressant treatment and lipid raft disruption on GFP- Gα$_s$ dynamics using Fluorescence Recovery After Photobleaching

Parts of this chapter were previously published as:
Czysz. Schappi J. and Rasenick M. Lateral diffusion of Ga$_s$ in the plasma membrane is decreased after chronic but not acute antidepressant treatment: Role of lipid raft and non-raft membrane microdomains. Neuropsychopharmacology. Sept 2014.

4.1 Introduction to fluorescent recovery after photobleaching

4.1.1 Fluorescent Proteins to study protein diffusion

Fluorescence photobleaching and recovery is among the most common approaches for studying protein and lipid lateral diffusion(Edidin 1994). The technique works exactly how it sounds - a region of fluorescence is rapidly photobleached and then fluorescence intensity is measured in the region over time. If the fluorophores is mobile, diffusion will result in a recovery of fluorescence. Data can then be reported as either the half-time to recovery or as a diffusion coefficient (distance over time). Furthermore, if the fluorescent population is not homogenous, some fluorophores may be immobile and never recover. Therefore, one can also calculate an immobility fraction. Historically, fluorescent recovery after photobleaching (FRAP) was used almost exclusively to study lipid diffusion in highly artificial model systems(Koppel et al. 1976). In the past two decades, however, the development of fluorescent proteins has helped FRAP gain traction studying protein diffusion too(Reits and Neefjes 2001).

Studying diffusion of FPs in live cells has proven to be quite a bit different than lipids in model membranes. By nature, all parameters in model systems are easily manipulated, but cells are significantly more complex. This makes it all the more challenging to isolate factors that influence FP diffusion rates.

The most common application of fluorescent protein FRAP involves investigating the role of lipid rafts in regulating protein signaling. In a typical experiment, a fluorescent fusion protein will be photobleached with and without some method of lipid raft disruption. Changes in diffusion are then attributed to the protein's relationship with lipid rafts. While insightful, these manipulations are limited by the methods used to disrupt rafts. Specifically, cholesterol depletion through methyl-β-cyclodextrin (MβCD) chelation is widely used since rafts are described as cholesterol-rich. Nonetheless, this approach is somewhat crude and diffusion of raft and non-raft associated proteins are equally perturbed by MβCD(Kenworthy et al. 2004). Diffusion was much better correlated with the type of membrane anchor (transmembrane, acyl group, GPI) rather than raft association. Furthermore, despite rafts being thought of as highly immobile, rigid structures, both protein and lipid diffusion decrease after MβCD treatment(Hao, Mukherjee, and Maxfield 2001). While these data do not dismiss the possibility that rafts regulate protein diffusion, they certainly imply that raft manipulation is not a straight forward technique.

4.1.2 FRAP to study Gα_s regulation by lipid rafts

Organization and accessibility of G-proteins to receptors and effectors are thought

to be important means of their regulation(Allen, Halverson-Tamboli, and Rasenick

2006). Indeed, previous work suggests that $G\alpha_s$ signaling is dampened when $G\alpha_s$ is

localized to lipid rafts(Chen and Rasenick 1995a). Chronic antidepressant

treatment alters this association, decreasing $G\alpha_s$ raft content and increasing cAMP

signaling(Chen and Rasenick 1995b; Ozawa and Rasenick 1989; Menkes and

Aghajanian 1983). Currently it is unclear by what mechanism these drugs affect G-

protein signaling since the presence of the SERT transporter is not necessary(L.

Zhang and Rasenick 2010).

A better understanding of this mechanism requires investigating the nature of G-

protein association with lipid rafts and membranes in general. The concept of lipid

rafts remains controversial, and their study in relationship to G-protein signaling is

mostly limited to highly non-physiologic cold detergent extractions. While this is

the traditional means to study raft association, there has been some progress

studying raft association using microscopy under physiologic conditions. These

include new high-resolution techniques like PALM and STORM, but also older

techniques like Fluorescence Recovery After Photobleaching (FRAP). The latter

does not actually visualize protein clustering in nanodomains, but instead measures

protein diffusion over a much larger area. The speed of diffusion acts as a proxy for

protein anchoring to rafts or other scaffolds.

To investigate this, we have measured GFP-Gα_s FRAP under a variety of conditions known to alter its signaling and raft association. We report that changes in FRAP correlate well with treatments that alter Gα_s raft association and cAMP signaling, but do not correlate with treatments that just alter Gα_s raft association.

4.2 Results

A.

B.
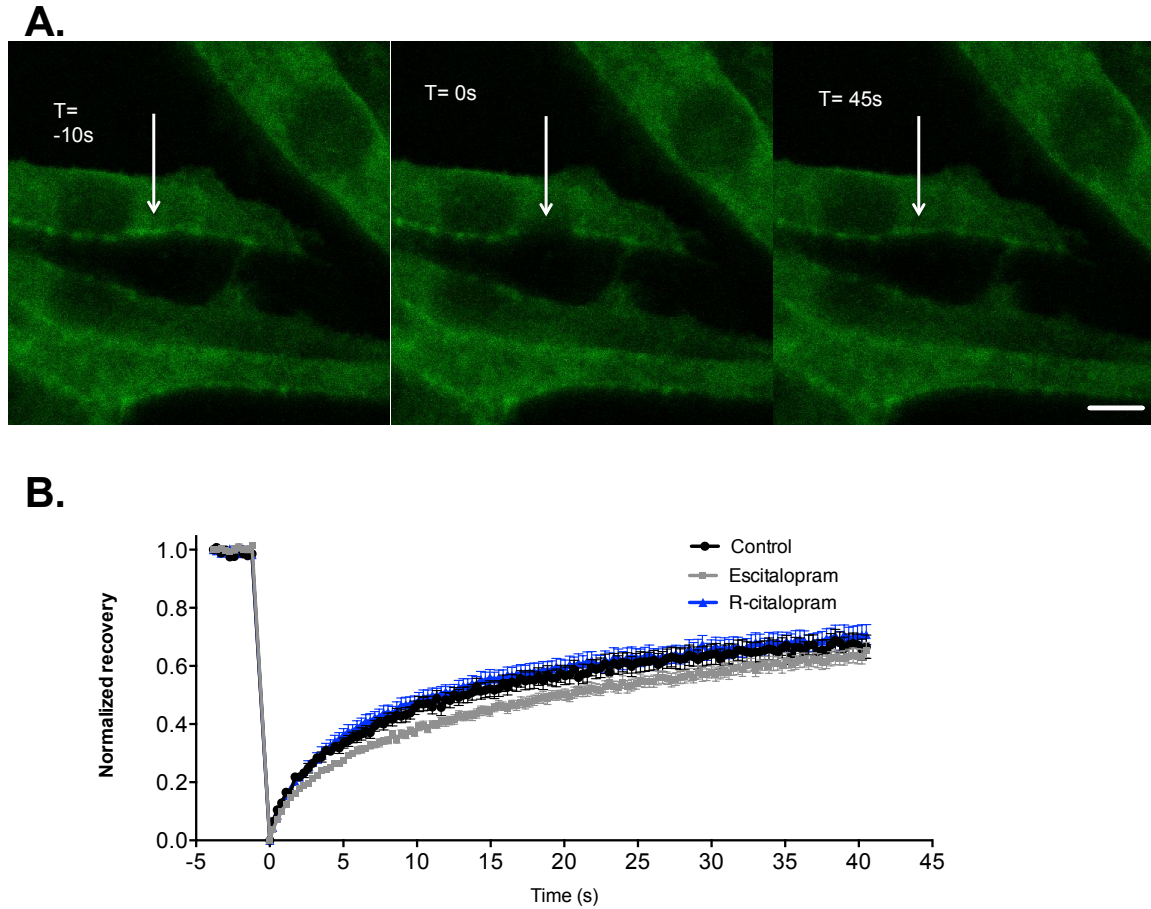
Figure 7. Fluorescence Recovery After Photobleaching (FRAP) provides reproducible measurements to study the mobility of GFP-Gα$_s$ in C6 glioma.
C6 glioma cells stably expressing GFP-Gα$_s$ were cultured in phenol-red free DMEM and membrane regions were rapidly photobleached. A) A representative photobleaching and recovery of GFP-Gα$_s$. B) Averaged recovery curves of control and 10 μM escitalopram (3 days) treated cells.

A. Control
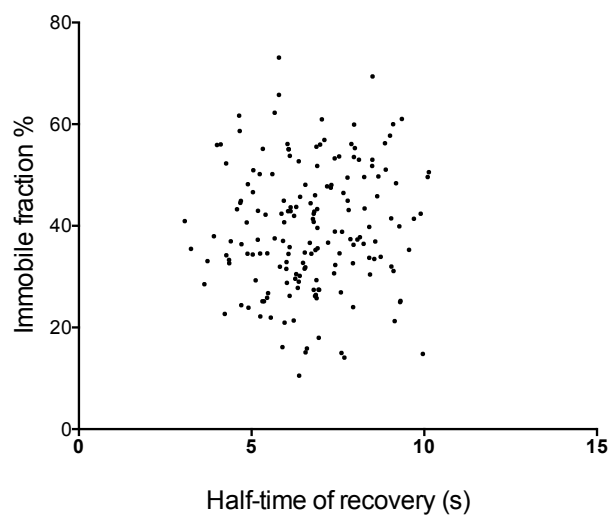
B. Escitalopram
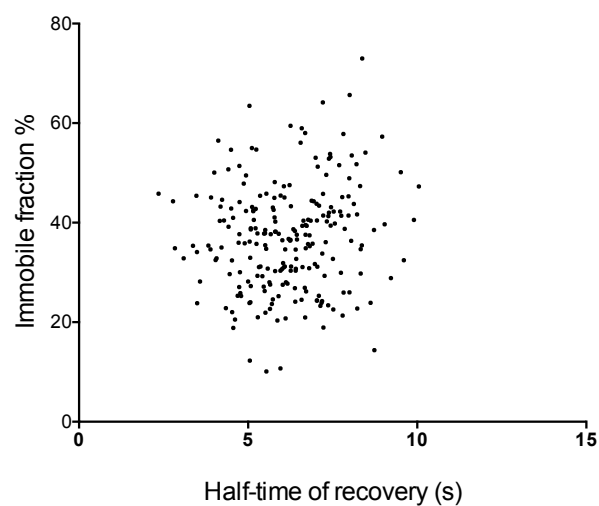
Figure 8. Immobile fraction and half-time to recovery are independent measures of GFP-Gα_s diffusion.

C6 glioma cells stably expressing GFP-Gα_s were cultured in phenol-red free DMEM after three days of 10 μM escitalopram treatment and membrane regions were rapidly photobleached. Half-time to recovery and immobile fraction were calculated using a one-phase association fit of the intensity as it recovered from photobleaching. While both values were significantly different from each between A) control and B) escitalopram, they did were not associated with each other.
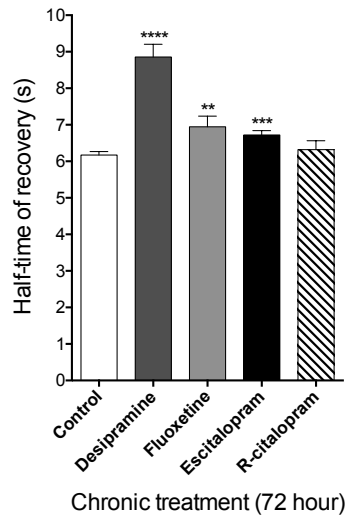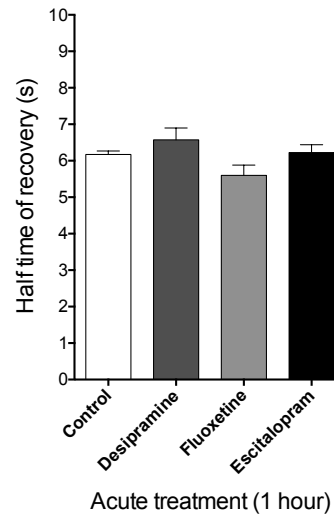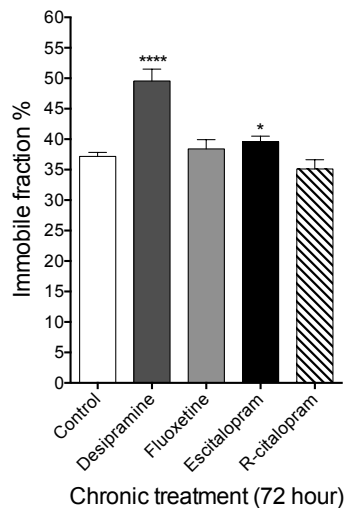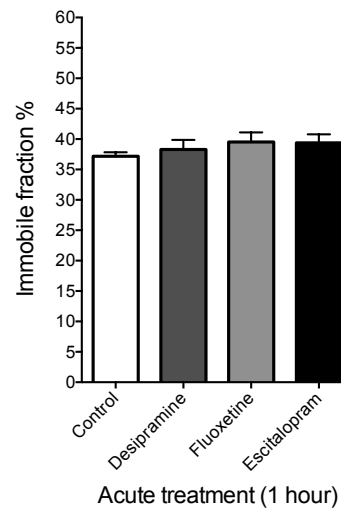
Figure 9. GFP-Gα_s recovery after photobleaching is slower after chronic but not acute antidepressant treatment.
GFP-Gα_s was stably expressed in a C6 glioma cell line and FRAP was used to assess the mobility of Gα_s after antidepressant treatment. Half-time to recovery of GFP-Gα_s is increased after (a) chronic (3 day) but not (b) acute (1 hour) escitalopram, desipramine and fluoxetine treatments. The immobile fraction of GFP-Gα_s was also increased with (c) chronic but not (d) acute desipramine and escitalopram treatment. Chronic R-citalopram had no effect on either half-time of recovery or immobile fraction. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.

4.2.1 GFP-Gα_s diffusion is altered in response to chronic antidepressant treatment

Gα_s raft association and Gα_s-AC coupling are sensitive to chronic treatment (3 days) of a variety of antidepressants, including SSRIs, TCAs and MAOIs. To test whether diffusion of Gα_s may also be affected, we treated a stably transfected GFP-Gα_s C6 glioma cell line with escitalopram, desipramine or fluoxetine and then performed Fluorescence Recovery After Photobleaching. Relative to control, 3 day antidepressant treated cells all had a signature decrease in half-time to maximal

A.

Chronic treatment (72 hour)

B.

Acute treatment (1 hour)

C.

Chronic treatment (72 hour)

D.

Acute treatment (1 hour)
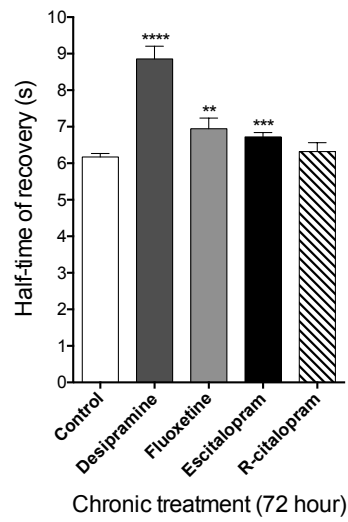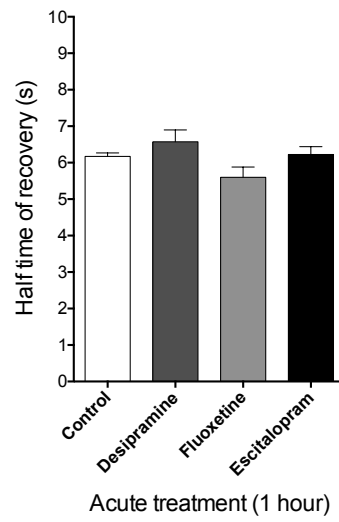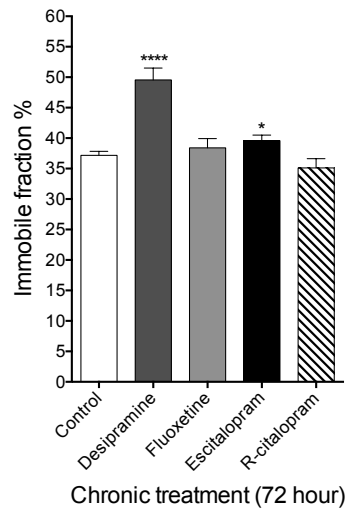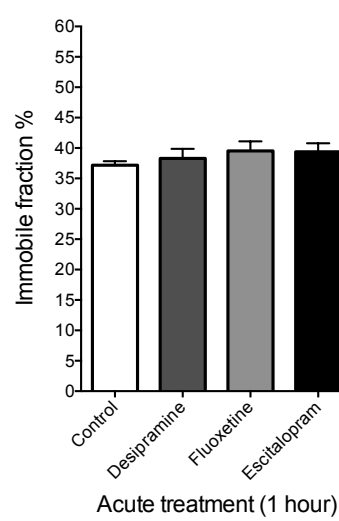
recovery (

Figure 9 A) as well as a decrease in total extent of recovery (

A.

Chronic treatment (72 hour)

B.

Acute treatment (1 hour)

C.

Chronic treatment (72 hour)

D.

Acute treatment (1 hour)

Figure 9 B).

60
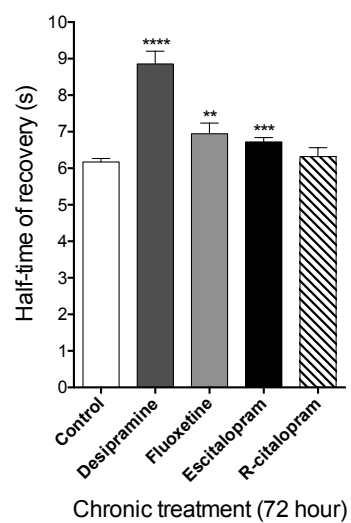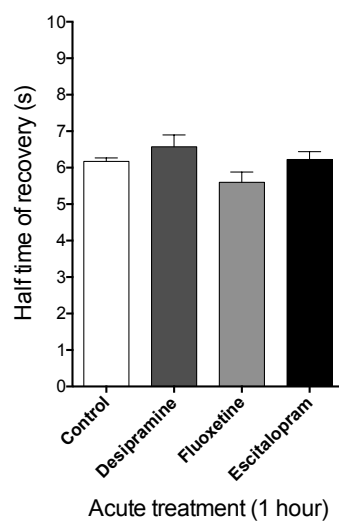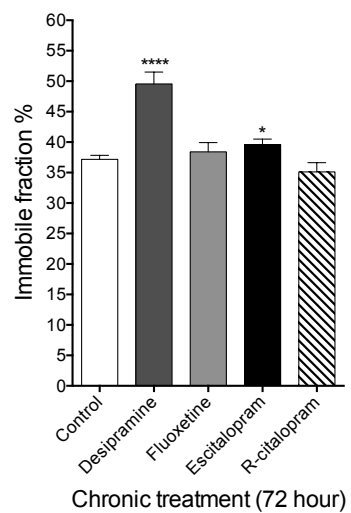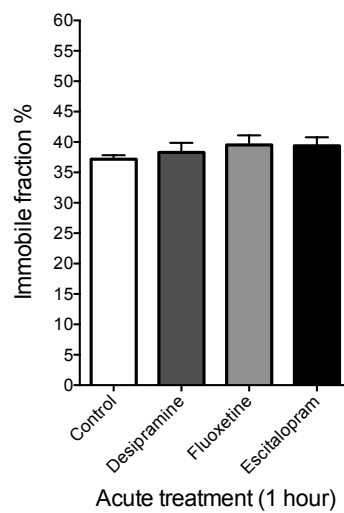
In contrast, FRAP measurements were unchanged in cells treated for only one hour (

A.
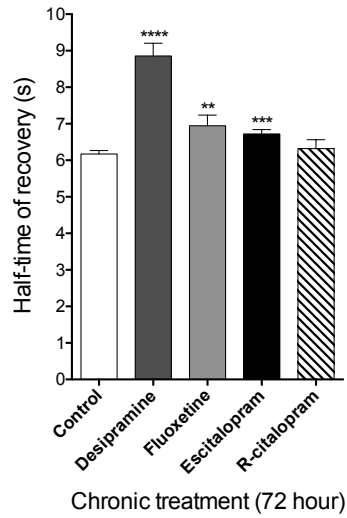
B.
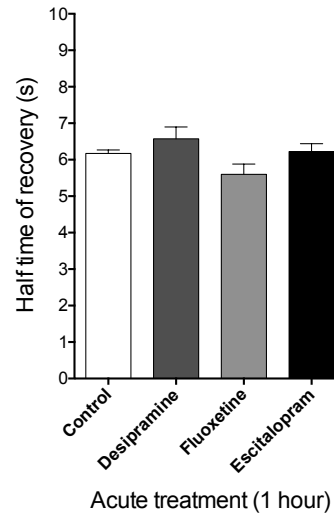
C.
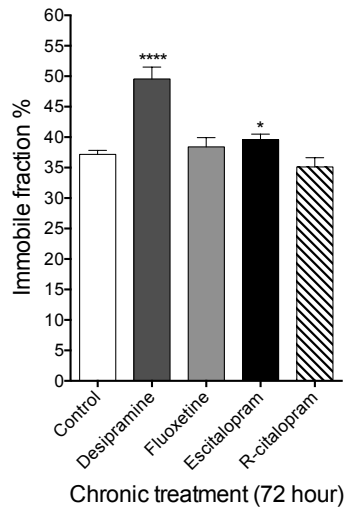
D.
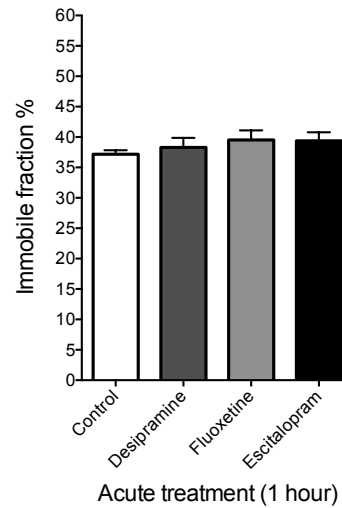
Figure 9 B,C). Additional time points at 24 and 48 hours reveals a minimum of 48

hours to see a significant change in half-time (Figure 10 B) to recovery.

4.2.2 R-citalopram does not alter GFP-Gα$_s$ diffusion

While the canonical target of SSRIs is the SERT transporter, augmentation of cAMP

signaling is still seen in cells that lack the SERT transporter, such as C6 glioma.

Nonetheless, this effect is highly specific to the drug's structure since only the S

enantiomer of citalopram regulates cAMP signaling. Consistent with this, chronic

treatment with R-citalopram does not affect GFP-Gα_s recovery after photobleaching

(
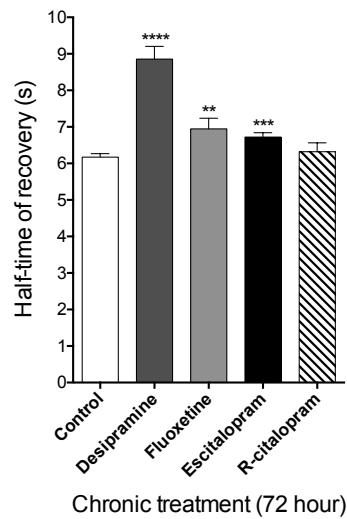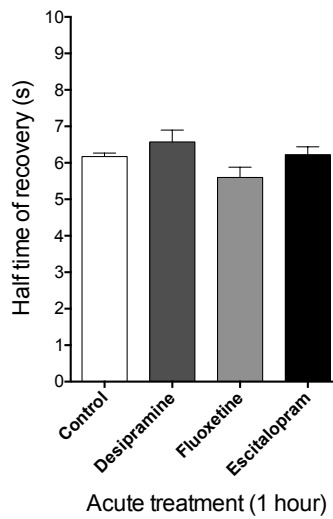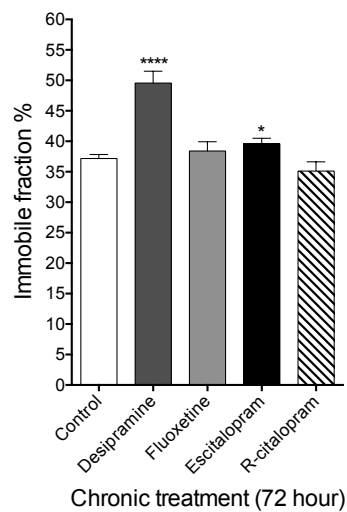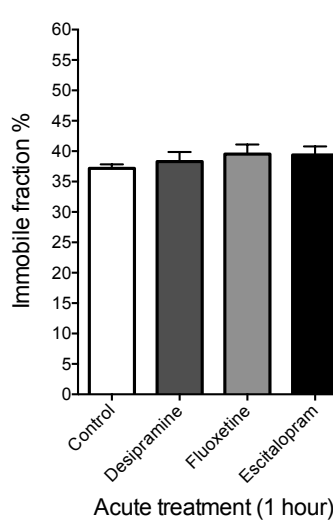
Figure 9 A, C).

Treatment	t $_{1/2}$	sem	n	p
Control	6.175	0.09543	226	-
Escitalopram	6.7209	0.1184	171	0.0003
R-citalopram	6.321	0.2445	66	0.5066
Despiramine	8.855	0.3471	32	<0.0001
Fluoxetine	6.945	0.294	31	0.0063
Bupropion	8.039	0.4411	24	0.0001
Phenelzine	7.044	0.3081	23	0.0062
Impramine	7.362	0.3729	24	0.0002
Venlafaxine	6.961	0.3714	27	0.0103
Sertaline	10.22	0.4713	24	0.0001
Tianeptine	7.066	0.3105	17	0.0135

Table 1. All tested classes of antidepressants affect GFP-Gα$_s$ half-time to recovery.
FRAP experiments were performed as above but with various additional antidepressants. All classes of antidepressant increased the half-time to recovery although the magnitude varied slightly between drugs.

Treatment	Immobile fraction %	sem	n	p
Control	37.17	0.6837	226	-
Escitalopram	39.58	0.9168	181	0.0316
R-citalopram	35.11	1.525	66	0.1829
Despiramine	49.57	1.935	32	0.0001
Fluoxetine	38.14	1.514	31	0.5364
Bupropion	46.53	3.141	24	0.0001
Phenelzine	39.29	2.672	23	0.3764
Impramine	39.4	2.693	24	0.3446
Venlafaxine	37.47	2.227	27	0.8922
Sertaline	49.52	2.003	24	0.0001
Tianeptine	41.5	3.359	17	0.1175

Table 2. Not all antidepressants affect the extent of GFP-Gα$_s$ recovery.
FRAP experiments were performed as above but with various additional antidepressants. Unlike half-time to recovery, the immobile fraction is not significantly different in all treatment conditions.

4.2.3 Multiple classes of antidepressants decrease GFP-Gα_s diffusion

Antidepressants belonging to the MAOI, TCAs and SSRI families have all previously been shown to augment cAMP signaling. Consistent with this, chronic treatment with numerous drugs from these families show a significant increase in $t_{1/2}$ and trend higher immobile fractions (Table 1). In addition, bupropion, venlafaxine and tianeptine, all additional classes of antidepressant all demonstrated similar effects.

Figure 10. Escitalopram's effect on GFP-Gα_s diffusion is both time and dose dependent.
A) C6 cells stably expressing GFP-Gα_s were cultured for 3 days at various doses of escitalopram before imaging. FRAP was performed on 3-6 cells per dish and the (a) half time to recovery was calculated using a one-phase association fit. B) C6 cells stably expressing GFP-Gα_s were cultured for 3 days and escitalopram treatment (10 μM) was initiated in the final 1, 24, 48 or 72 hours of culture before imaging. FRAP was performed on 3-6 cells per dish and the half time of recovery was calculated. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars represent SEM.

4.2.4 Altered GFP-Gα$_s$ diffusion is dose-dependent

Previous study has shown that antidepressant induced redistribution of Gα$_s$ from lipid rafts is dose-dependent. To support the idea that FRAP is measuring the same effect, we measured changes in GFP-Gα$_s$ after chronic treatment with a range of escitalopram concentrations. The t$_{1/2}$ showed a similar trend among doses to changes in Gα$_s$ raft content (Figure 10A). A time course study also revealed at least 48 hours of drug treatment is necessary for an effect (Figure 10B).

4.2.5 Lipid raft disruption also decreases GFP-Gα$_s$ diffusion

Previous studies show that both cholesterol chelation and microtubule disruption liberate Gα$_s$ from lipid rafts. Therefore we speculated that rafts constrain Gα$_s$ diffusion and therefore changes in raft localization could be measured by FRAP. Indeed, data from FRAP experiments show a consistent effect with both raft disruption and antidepressant treatment (both manipulations cause Gα$_s$ to leave lipid rafts), but surprisingly this effect is a decrease in diffusion and extent of recovery (
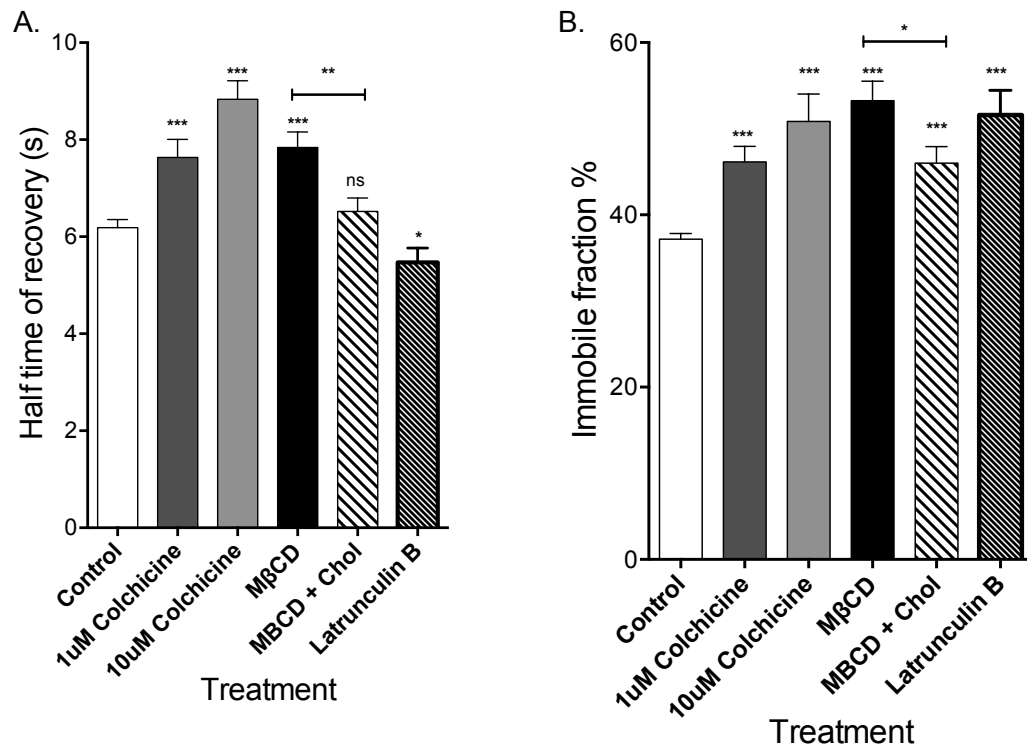
Figure 11).
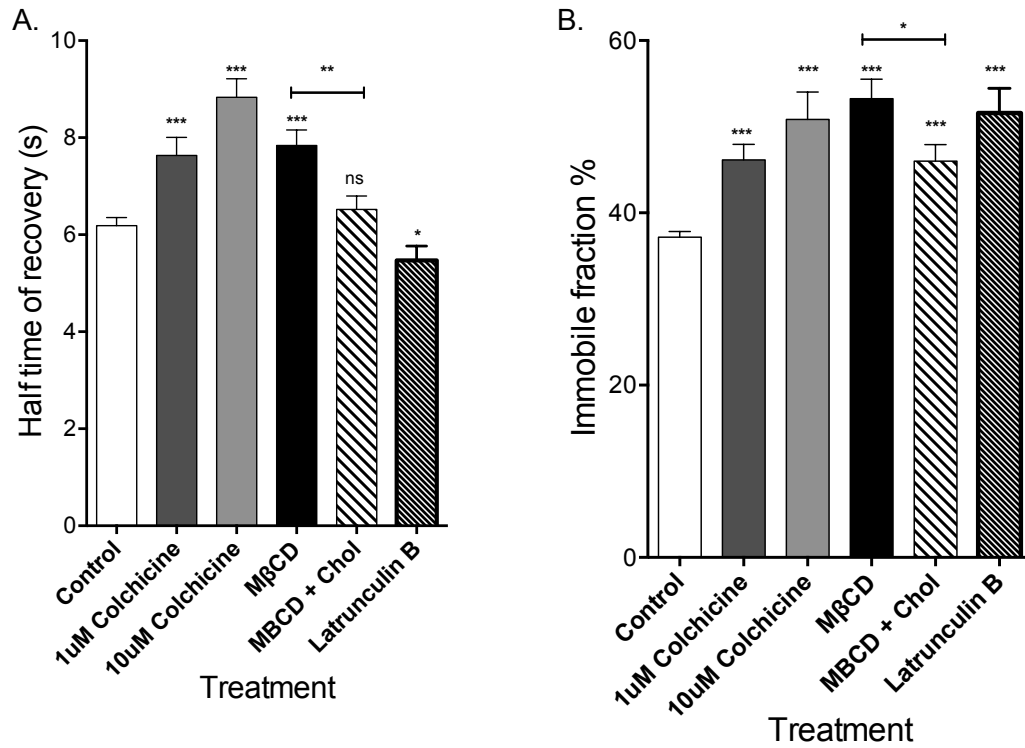
Figure 11. Lipid raft disruption alters GFP-Gα_s FRAP.
Lipid raft disruption by cholesterol chelation or colchicine treatment increased both the A) $t_{1/2}$ and B) immobile fraction of GFP-Gα_s after FRAP. The effect of cholesterol chelation was partially reversed by reintroducing cholesterol after chelation. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.

4.2.6 Protein mobility is dependent on the protein's cellular scaffolds

GFP-$G\alpha_s$ diffusion was also compared to several other fluorescent proteins. GFP-$G\alpha_{i1}$ has similar diffusivity to GFP-$G\alpha_s$. GFP-**β**-adrenergic receptor and RFP-caveolin both had significantly slower $t_{1/2}$ and larger immobile fractions. Conversely, pure GFP has a very fast diffusion and a very small immobile fraction (Figure 12). Likewise, a palmitoylation deficient GFP-$G\alpha_s$, which is also primarily cytosolic, also has a relatively fast $t_{1/2}$ and small immobile fractions (Figure 13).
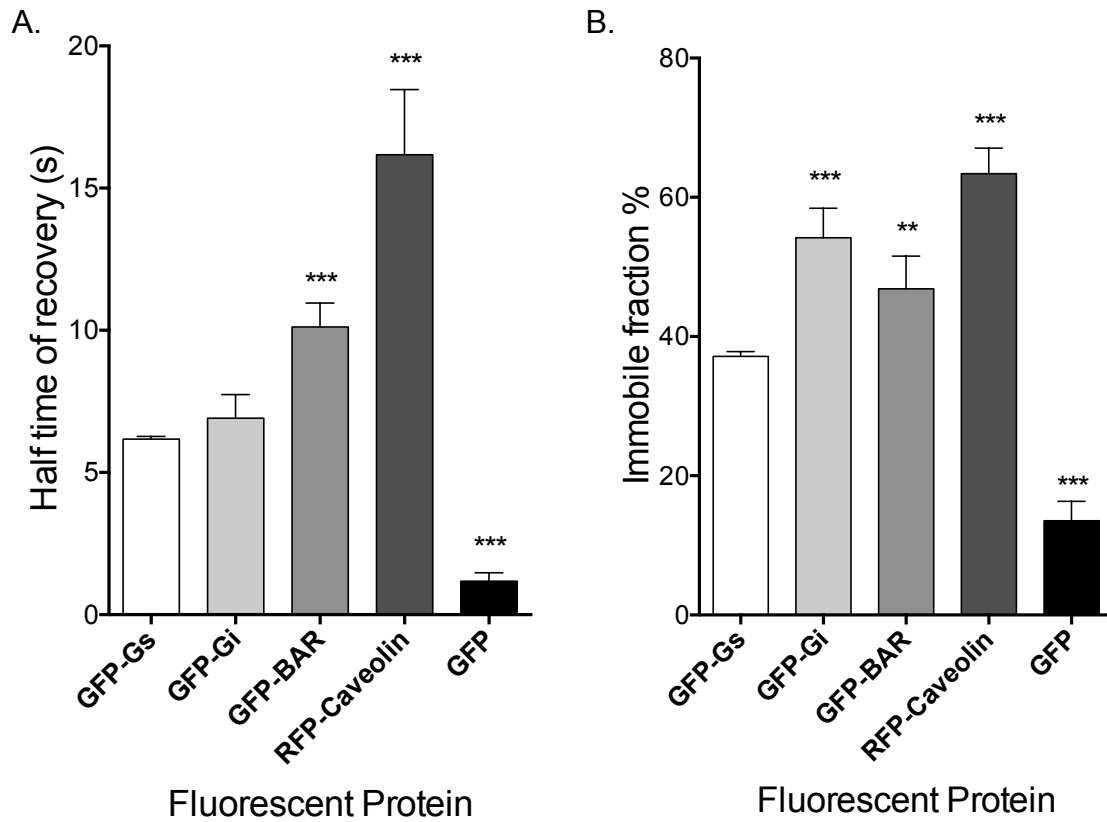
Figure 12. Diffusion of fluorescent proteins is dependent of their cellular scaffolds.
C6 glioma were transiently transfected with various FP fusion proteins and FRAP was performed 24 hours after transfection. A) Half-time of recovery was faster for peripheral membrane and cytosolic proteins relative to transmembrane proteins. B) Immobile fraction varied between constructs, but was not correlated to membrane association. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, $*p < 0.05$, $** p < 0.01$, $*** p < 0.001$). Error bars represent SEM.
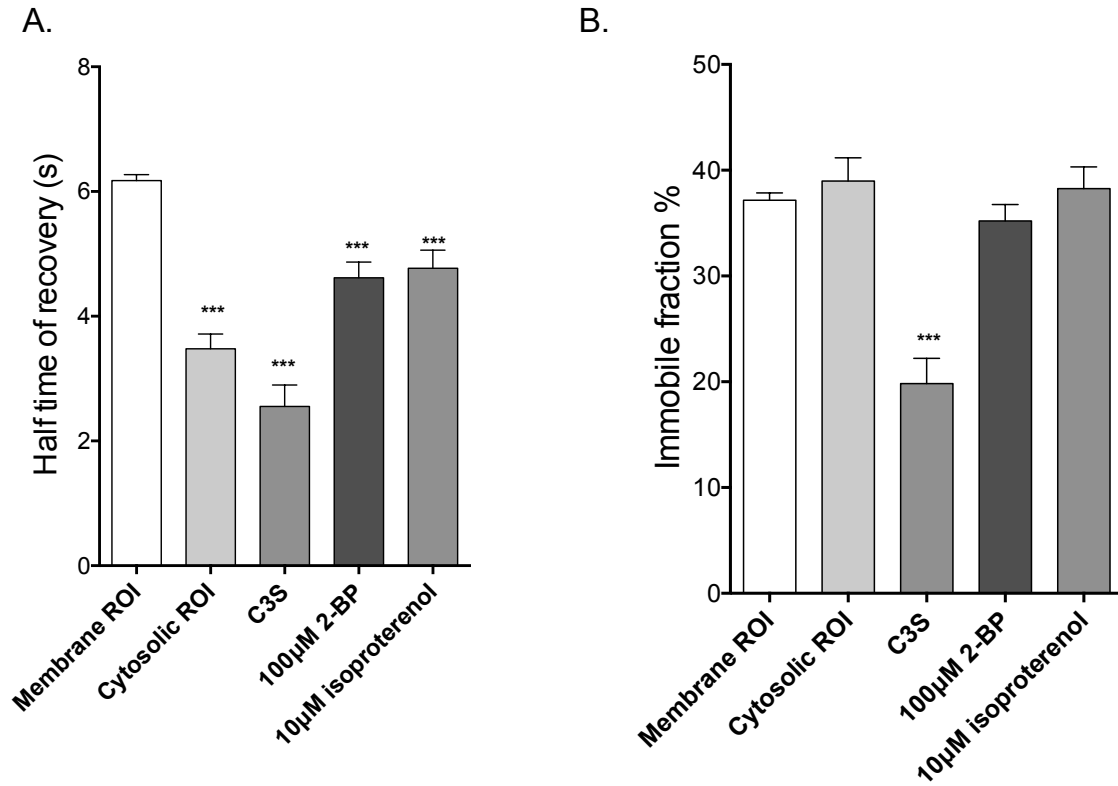
Figure 13. FRAP measurements depend of the protein's cellular scaffold.
FRAP was performed on cells expressing GFP-Gα$_s$ under a variety of conditions that alter Gα$_s$
membrane association. A) Conditions generating a cytolic GFP-Gα$_s$ result in significantly faster half-
time to recovery, but do not alter B) immobile fraction. C) A model for correlating diffusion speed
with cellular scaffold. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc
multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001).
Error bars represent SEM.
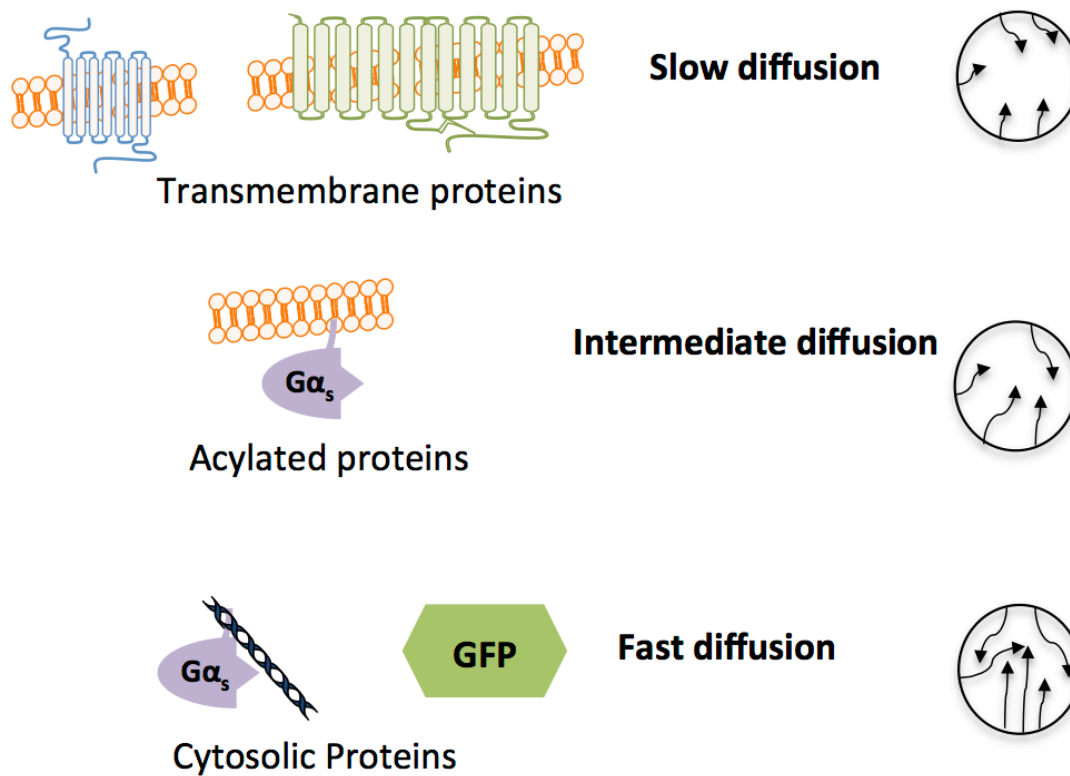
Figure 14. A model for protein diffusion.
Protein diffusion is mediated dependent on its cellular scaffolding. In particular, FRAP reveals that how a protein is anchored to plasma membranes is predictive of the speed at which it diffuses. Cytosolic proteins diffuse rapidly, especially GFP, which has no natural effectors. Acylated proteins associated with the plasma membrane diffuse slower, but still faster than transmembrane proteins.

Treatment	t $_{1/2}$	sem	n	p
Membrane ROI	6.175	0.09543	226	-
Cytosolic ROI	3.480	0.2361	27	0.0001
C3S	2.555	0.3426	16	0.0001
100μM 2-BP	4.616	0.2527	85	0.0001
10μM isoproterenol	4.771	0.2892	36	0.0001

Table 3. Cytosolic and isoproterenol stimulated GFP-Gα$_s$ diffuses faster than inactivated membrane GFP-Gα$_s$. See figure 13 for experimental details.

Treatment	Immobile fraction %	sem	n	p
Membrane ROI	37.17	0.6837	226	-
Cytosolic ROI	38.97	2.208	27	0.4281
C3S	19.82	2.403	14	0.0001
100μM 2-BP	35.21	1.543	85	0.1879
10μM isoproterenol	38.26	2.056	36	0.5787

Table 4. Mutant C3S is more mobile than wild-type GFP-Gα$_s$. See figure 13 for experimental details.

Treatment	t $_{1/2}$	sem	n	p
GFP-Gα$_s$	6.175	0.09543	226	-
GFP-Gα$_{i1}$	6.913	0.8260	7	0.1888
GFP-BAR	10.12	0.8410	15	0.0001
RFP-Caveolin	16.18	2.291	21	0.0001
GFP	1.182	0.2895	5	0.0001

Table 5. Fluorescent proteins diffuse at different speed depending on their membrane association. See figure 12 for experimental details.

Treatment	Immobile fraction %	sem	n	P
GFP-Gα$_s$	37.17	0.6837	226	-
GFP-Gα$_{i1}$	54.18	4.249	7	0.0001
GFP-BAR	46.84	4.723	15	0.0015
RFP-Caveolin	63.40	3.662	21	0.0001
GFP	13.54	2.793	5	0.0001

Table 6. Immobile fraction varies among fluorescent fusion proteins. See figure 12 for experimental details.

4.3 Discussion

Lipid rafts remain a difficult concept in cell signaling to investigate, and therefore different and complimentary approaches are necessary. Previous studies suggest that $G\alpha_s$ association with lipid rafts(Chen and Rasenick 1995b; Chen and Rasenick 1995a) may underlie antidepressant regulation of cAMP (Ozawa and Rasenick 1989; Ozawa and Rasenick 1991; Menkes and Aghajanian 1983). This work has relied on lipid raft preparations from lysed tissue and cells rather than intact living cells. Here we have studied $G\alpha_s$ diffusion under a variety of raft-altering conditions, including antidepressant treatment. Our findings show that treatment which translocate $G\alpha_s$ from raft to non-raft membrane domains also cause decreases in FRAP measurements that represent diffusion and mobility.

Regarding antidepressant treatment, changes in FRAP measurement closely match in dose and time-course antidepressant-induced changes in cAMP production and $G\alpha_s$ raft localization(Table 1. All tested classes of antidepressants affect GFP-$G\alpha_s$ half-time to recovery.

FRAP experiments were performed as above but with various additional antidepressants. All classes of antidepressant increased the half-time to recovery although the magnitude varied slightly between drugs.). Antidepressant induced changes in $G\alpha_s$ signaling require days, not hours, which is also reflected in the FRAP assay (Figure 10. Escitalopram's effect on GFP-$G\alpha_s$ diffusion is both time and dose dependent.

A) C6 cells stably expressing GFP-$G\alpha_s$ were cultured for 3 days at various doses of escitalopram

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before imaging. FRAP was performed on 3-6 cells per dish and the (a) half time to recovery was calculated using a one-phase association fit. B) C6 cells stably expressing GFP-Gα_s were cultured for 3 days and escitalopram treatment (10 μM) was initiated in the final 1, 24, 48 or 72 hours of culture before imaging. FRAP was performed on 3-6 cells per dish and the half time of recovery was calculated. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).   Error bars represent SEM.B). Furthermore we believe this is not a general effect on adding lipophilic drugs to the membrane, since only the S-enantiomer of citalopram demonstrates this effect unlike R-citalopram

A.
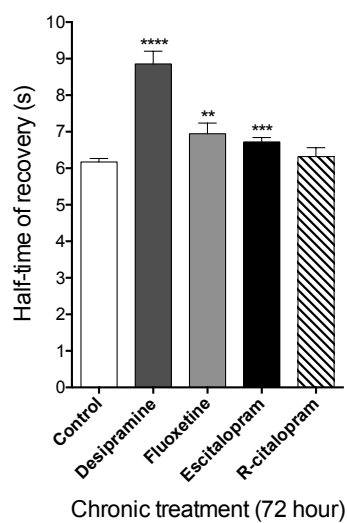
B.
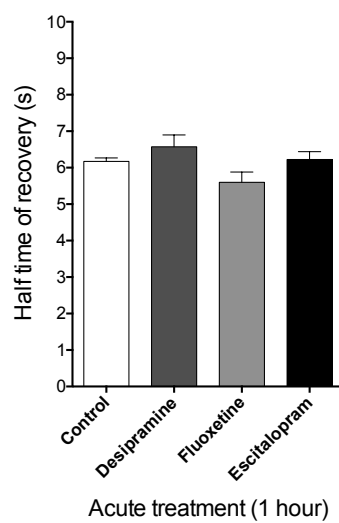
C.
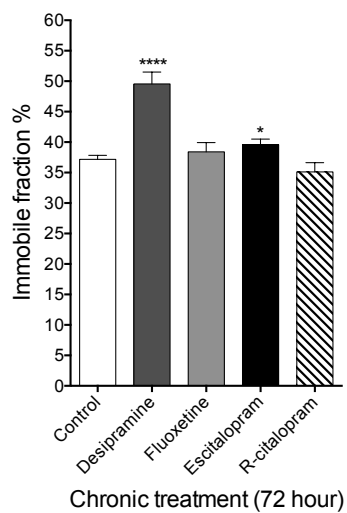
D.
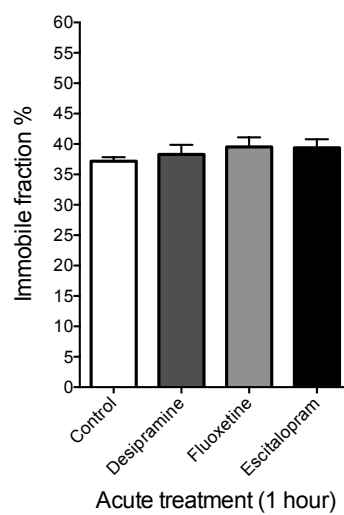
Figure 9A). Again, this matches the enantiomeric specificity of escitalopram on cAMP

production and $G\alpha_s$ raft localization(L. Zhang and Rasenick 2010).

We also explored the FRAP assay responds to other modulators of $G\alpha_s$ signaling.

Lipid raft disruption by MβCD has been previously shown to increase $G\alpha_s$-adenylyl

cyclase coupling and also induces a slower and less mobile recovery of GFP- $G\alpha_s$

after photobleaching(Donati and Rasenick 2005). The same is true for colchicine treatment, which is thought to liberate tubulin-$G\alpha_s$ binding(Rasenick 1986; Rasenick and Wang 1988; Rasenick et al. 2004; Donati and Rasenick 2005).
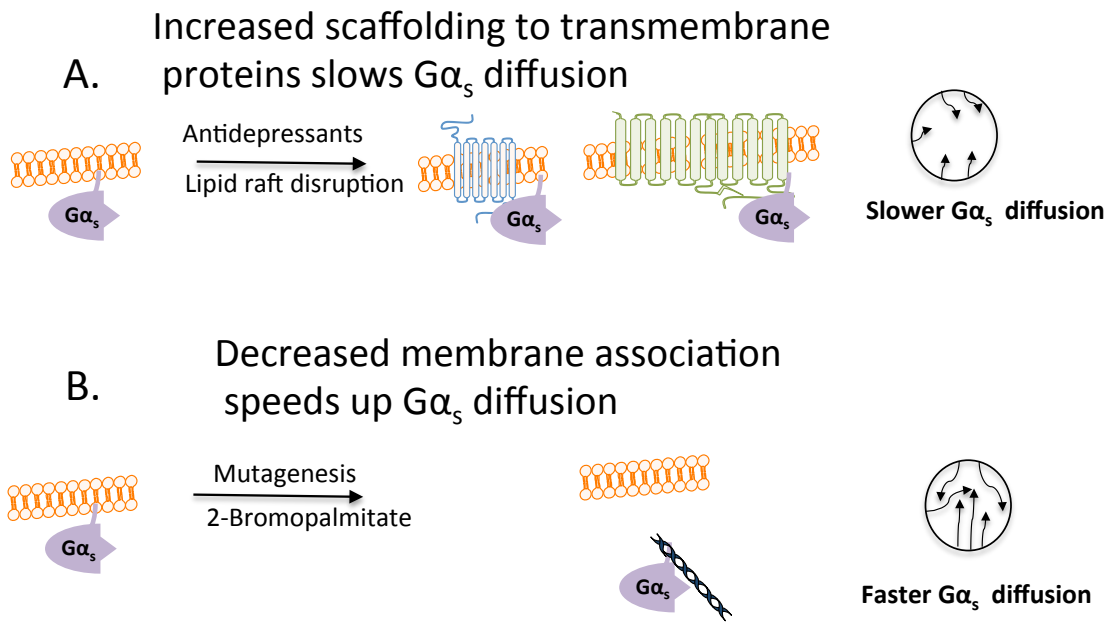
Figure 15. A model for changes in GFP-Gα$_s$ diffusion.
A) Antidepressant treatment and lipid raft disruption increase GFP-Gα$_s$ coupling to transmembrane scaffolds, which decreases GFP-Gα$_s$ diffusion. B) In contrast, cytosolic GFP-Gα$_s$ diffuses relatively fast.

Together these data indicate a strong correlation between lower diffusion speed and mobility with decreased $G\alpha_s$ raft association and increased cAMP production. Therefore it may be tempting to speculate difference in diffusion in raft and non-raft domains may be responsible changes in GFP-$G\alpha_s$ recovery, but this conclusion runs counter to the concept that rafts are rigid, highly ordered domains where diffusion would be slow. Instead we suspect altered proteins scaffolding may be playing a larger role. Other groups have shown that cholesterol chelation through MβCD treatment restricts diffusion of a variety of raft and non-raft membrane-associated-FPs. Furthermore, they demonstrated diffusion better correlates with type of membrane anchor, rather than raft localization(Lenne et al. 2006). Our results are consistent with these, as FRAP measurements of integral membrane proteins GFP-βAR and RFP-caveolin were significantly slower than peripheral membrane proteins like GFP-$G\alpha_s$, GFP-$G\alpha_i$, (Figure 12, Figure 14).

Therefore we suggest that antidepressant treatment and raft disruption decrease GFP-$G\alpha_s$ diffusion by increasing $G\alpha_s$ association with transmembrane proteins such as GPCRs and adenylyl cyclase (

A. Increased scaffolding to transmembrane proteins slows Gα$_s$ diffusion

Antidepressants
Lipid raft disruption

Gα$_s$

Gα$_s$

Gα$_s$

Slower Gα$_s$ diffusion

B. Decreased membrane association speeds up Gα$_s$ diffusion

Mutagenesis
2-Bromopalmitate

Gα$_s$

Gα$_s$

Faster Gα$_s$ diffusion
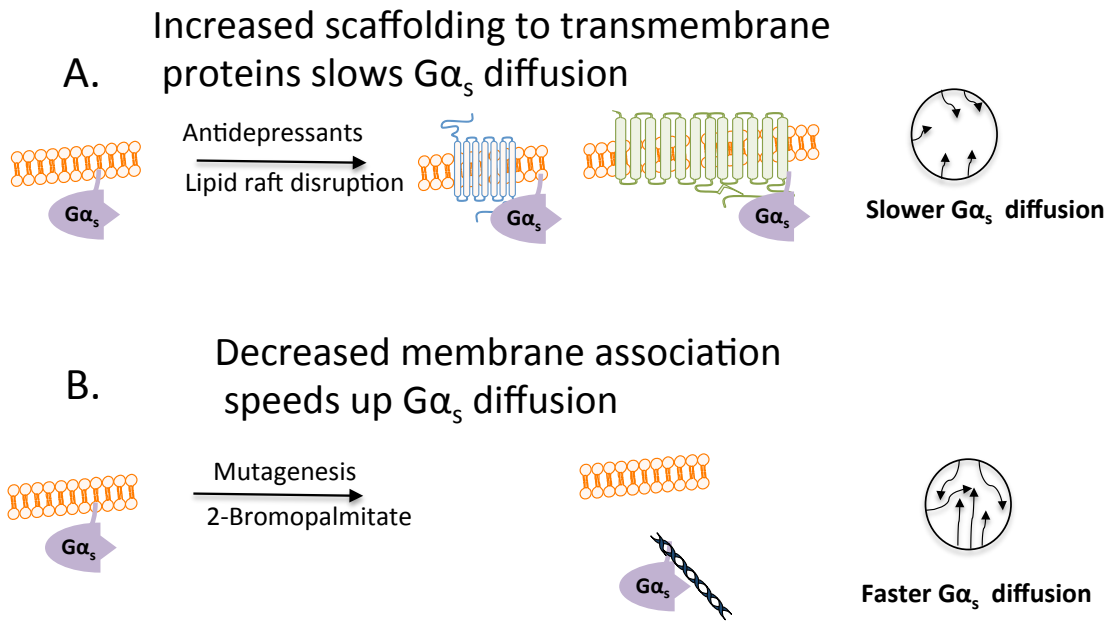
Figure 15). Consistent with this, diffusion of GFP-Gα$_s$ appearing in the cytosol

recovers much faster than that in the membrane. This was also confirmed using a

palmitoylation deficient GFP-Gα$_s$, which is exclusively found in the cytosol, and with

cells treated with 2-bromopalmitate, a palmitoylation inhibitor (Figure 13). These

cytosolic Gα$_s$ have significantly less scaffolds than their membrane-associated

counterparts, which is why we suspect they are able to diffuse are greater speeds.

Not surprisingly, they still diffuse much slower than un-fused GFP, since cytosolic

Gα$_s$ still has some associations, such as tubulin (Figure 14).

A commonly cited function of traditional lipid rafts is to organize and scaffold

signaling pathways in close proximity to foster efficient signaling. Gα$_s$ signaling is

thought to act the opposite, experiencing more potent transduction out of rafts. In

this sense, it is not surprisingly raft-associated Gα$_s$ would diffuse faster than non-

raft Gα$_s$ and further suggests that it is a rash to generalize the roles of raft and non-

raft membrane domains. Rafts are possibily little more than a descriptive concept

that generalizes a variety of membrane and protein scaffolds.

Chapter 5: To determine the effects omega-3 fatty acids on Gα$_s$ signaling

Parts of this chapter were previously published as:
Czysz, Andrew H, and Rasenick, Mark M. 2013. "G-Protein Signaling, Lipid Rafts and the Possible Sites of Action for the Antidepressant Effects of N-3 Polyunsaturated Fatty Acids.." *CNS & Neurological Disorders Drug Targets* 12 (4): 466–73.

5.1 Introduction to omega-3 fatty acids in depression

5.1.1 PUFAs and depression

Dietary fish oil has become an increasingly attractive part of antidepressant therapy because about half of patients treated with prescription antidepressants fail to response or discontinue therapy due to side effects. N-3 PUFA as a putative depression therapy may originate from reports suggesting that dietary n-3 PUFA deficiency is linked to depression (Hibbeln 1998). Frasure-Smith and colleagues(Frasure-Smith, Lespérance, and Julien 2004) showed that depressed patients had lower concentrations of total omega-3 and docosahexanoic acid (DHA), higher ratios of arachidonic acid (AA) to DHA, higher ecosapentanoic acid (EPA), and higher n-6:n-3 ratios than controls, and this has been confirmed by a recent meta-analysis(Lin, Huang, and Su 2010). In a small sample (n = 24), depressed subjects had lower RBC membrane levels of omega-3 fatty acids than healthy controls, and the severity of depression correlated with both levels and dietary intake of omega-3 fatty acids(Edwards et al. 1998). The omega-3 fatty acid composition of RBC membrane phospholipids and in particular DHA content, was significantly depleted among depressed subjects compared with control subjects in a similar study (Peet, Murphy, and Shay 1998). Still, there is significant controversy

as to the effectiveness of fish oil in depression. In one study, patients with major

depressive disorder were found to have a reduction of total omega-3 fatty acids as

well as alpha-linoleic acid (ALA) and EPA in serum cholesteryl esters, compared

with adults with minor depression or healthy controls (n = 74) (Maes et al. 1996).

These same authors did not observe increases in membrane n-3 PUFA following

SSRI treatment. This amalgamation of data helps strengthen the case that further

study is required.

5.1.2 n-3 PUFA, G protein signaling and lipid rafts

The localization of G proteins to particular membrane domains such as caveolae (Li

et al. 1995) and lipid rafts has spawned interest in these cholesterol and

sphingolipid-rich detergent-resistant membrane domains and how they affect G-

protein targeting and function(Allen, Halverson-Tamboli, and Rasenick 2006). Lipid

rafts have variable effects on signaling, as they promote $G\alpha_q$ signaling(Bhatnagar et

al. 2004)and inhibit $G\alpha_s$ signaling(Allen et al. 2005). There is a long experimental

history of agents that increase "membrane fluidity" increasing agonist- and $G\alpha_s$-

mediated adenylyl cyclase(Rimon, Hanski, and Braun 1978; Rasenick, Stein, and

Bitensky 1981). $G\alpha_s$ activates adenylyl cyclase more efficiently outside of lipid rafts

and chronic treatment with antidepressants facilitates G protein exodus from those

rafts (Donati and Rasenick 2005; Allen et al. 2009; L. Zhang and Rasenick 2010).

This may be because n-3 PUFA associates with lipid rafts and modifies their

structure, releasing some raft-associated proteins into non-raft membrane fractions

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(Schley, Brindley, and Field 2007). N-3 PUFA treatment facilitated coupling between the estrogen GPCR, GPER1, $G\alpha_s$ and adenylyl cyclase(Cao et al. 2012). DHA, but not EPA modified raft clustering and size (Shaikh et al. 2009) but both n-3 PUFAs targeted raft lipids (Rockett et al. 2011). On the other hand, Wassal and Stillwell (Wassall and Stillwell 2008) suggested that n-3 PUFA (DHA in this case) partition in non-raft domains where they alter the interactions among proteins in those domains. Curiously, n-3 PUFA have been shown to inhibit palmitoyl transferases, which suggest the existence of another mechanism whereby they alter raft localization of $G\alpha_s$ (Webb, Hermida-Matsumoto, and Resh 2000).

While it is highly possible that n-3 PUFA have multiple sites of action, the focus below is on how n-3 PUFA modify G protein signaling and how those sites relate to both depression and antidepressant action. Much of the focus herein will be on specialized membrane domains (lipid rafts) and the effects that agents modifying those rafts have on elements of G protein signaling cascades. The relevance of specific alterations of G protein signaling for both depression and antidepressant action will be discussed, as will the ability for n-3 PUFA to act either as an antidepressant or in concert with antidepressants.

5.1.3 Introduction to PUFAs

Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and related n-3 PUFAs are major constituents of neural membranes and therefore potential mediators of

neuronal signaling pathways. A number of studies have addressed the possibility

that dietary PUFAs are relevant to psychiatric disease, particularly depression.

Research has focused both on dietary deficiencies in these fatty acids contributing to

pathology and the potential for supplementation to ameliorate malaise, either alone

or acting synergistically with conventional therapies. The former is derived from

longitudinal studies and the latter is very attractive because fish oil

supplementation has a very low side effect profile and likely includes somatic

benefits. This interest is perhaps highest for depression, where dozens of clinical

trials have attempted to clarify whether fatty acids can affect disease outcome. To

date, meta-analyses of these studies have proven inconclusive, largely because of

inconsistencies between study design, particularly dosage and choice of fatty acid

compound.

The lack of clear outcome of these clinical studies and the increasing therapeutic

potential of fatty acids in other systems has lead to the investigation of putative

mechanisms of n-3 PUFA action at the cellular level. From this, a diverse but related

collection of signaling pathways have been identified that are affected by n-3 PUFAs.

Among these, we believe one of the most prominent and relevant to depression is

the effect n-3 PUFAs have on G-protein Coupled Receptor (GPCR) signaling.

G-protein coupled receptors are a family of about 1,000 transmembrane proteins

that respond to a variety of hormones and neurotransmitters as well as odorant and

tastant molecules. It is estimated that 50% of current pharmaceuticals target these

receptors. Most intriguing for the possibility of modification by PUFA is the evidence that these receptors and their attendant signaling cascades are influenced by their distribution among membrane microdomains. These microdomains, referred to herein as lipid rafts, selectively influence GPCR signaling, potentiating some signaling pathways and inhibiting others (Allen, Halverson-Tamboli, and Rasenick 2006).

5.1.4 Membrane fluidity and fatty acylation

Membranes are not homogeneous proteolipid sheets, but are considerably varied. In addition to heterogeneous distribution of protein, membrane lipids are divided into cholesterol-rich, cytoskeletal-associated domains, commonly referred to as lipid rafts and non-raft regions with a different lipid composition(Lingwood and Simons 2010). It is noteworthy that DHA, depending upon the cell type, can comprise as much as 50% of the plasma membrane lipid. Even in membranes where DHA is a lesser component, it appears to influence membrane function. While cholesterol-rich lipid rafts represent a highly ordered structure, DHA is often considered the "anti-cholesterol." Highly disordered DHA domains have been called the antithesis of lipid raft domains, as well as the ultimate non-raft membrane domain (Wassall and Stillwell 2009; Wassall and Stillwell 2008). Regardless of diet, membrane DHA content varies tremendously throughout the body. The most inclusive membranes include synaptosomes, sperm membranes and the retinal rod outer segment, where DHA accounts for 50 mol% of the lipid species.

Supplementation, however, can augment some membrane's DHA content 2 to 10 fold (Wassall and Stillwell 2009).

Controversy exists over the site of DHA incorporation in membranes. Computational models suggest DHA and cholesterol do not interact, but analysis of actual raft and non-raft samples show DHA is present in both (Wassall and Stillwell 2009). This discrepancy is likely the result of our limitation in resolving lipid raft domains. One group proposes that most of the DHA may be partitioning in discreet regions within rafts to avoid cholesterol interactions (Shaikh et al. 2009). Computational analysis of DHA, stearic acid and cholesterol binding with rhodopsin predicts interactions with all of these, but only DHA is suggested to associate in an arrangement that increase rhodopsin's kinetics. This is consistent with in vitro experiments that show DHA increases phototransduction whereas cholesterol inhibits it (Grossfield, Feller, and Pitman 2006).

The idea of DHA being anti-cholesterol extends into the clinic as well where it is suggested to benefit hypertriglyceridemia. The cardiovascular benefit of n-3 PUFAs has even lead to an unusual move for a dietary supplement: FDA approval and marketing as a prescription drug for a specific blend called Lovaza. Given similar indications, n-3 PUFAs are often discussed alongside statins, even though they are chemically distinct compounds. Interestingly, however, statins are believed to worsen symptoms of depression and anxiety whereas n-3 PUFAs alleviate them

(Farooqui et al. 2007).   This suggests that their effects on the brain may result from effects outside of cholesterol regulation and membrane fluidity.

Compared to n-3 PUFAs, the signaling disruption of statins is better understood. These drugs do not simply prevent cholesterol synthesis, but inhibit the synthesis of many cholesterol precursors. The most significant is geranyl-geranyl pyrophosphate, a necessary lipid that, through prenylation, covalently attaches to numerous proteins to mediate membrane association. G-proteins, especially Gγ, are reliant on prenylation to anchor to the membrane and therefore signal through their receptors(Marrari et al. 2007). N-3 PUFAs, on the other hand, may interfere with another lipid modification, fatty acylation. While this process may also be important in global membrane anchoring, it is generally considered to be more instrumental in targeting proteins to lipid raft membrane regions. Instead of a geranyl-geranyl pyrophosphate moiety, fatty acylation usually occurs with a medium chain saturated fatty acid such as palmitic acid. It is unclear however, if sites of fatty acylation are completely faithful to palmitic acid, or whether they can be interchangeable with similar length fatty acids (such as DHA or EPA). At least one group suggests that palmitoylation of Fyn can be inhibited by DHA and EPA through acylation with these fatty acids instead. They also showed that this swap decreased Fyn localization to rafts, perhaps because n-3 PUFAs are less hydrophobic than palmitic acid (Webb, Hermida-Matsumoto, and Resh 2000).

If n-3 PUFAs are regulating fatty acylation in the brain, this effect may be responsible for many of the benefits of n-3 PUFA supplementation. Synaptic proteins are heavily palmitoylated, and changes in their acylation state are thought to be essential to proper trafficking and function (Y. Fukata and Fukata 2010). Many neuropathologies such as Huntington's (Singaraja et al. 2011; Ehrnhoefer, Sutton, and Hayden 2011) and schizophrenia (Mukai et al. 2008; Mukai et al. 2004) show alterations in protein acylation. In fact, almost all monoamine activated GPCRs have putative or demonstrated palmitoylation sites (Chini and Parenti 2004; Escribá et al. 2007). Serotonergic receptors are among the best studied (Renner et al. 2007).

Both increases and decreases in acylation may correlate with disease because, akin to phosphorylation, acylation levels may require fine-tuning. Unlike phosphorylation, palmitoylation cannot be easily examined because fatty acids are much less antigenic and difficult to radiolabel with strong emitters. Therefore the significance of palmitoylation in signaling is mostly derived from mutant proteins where acylation is blocked. As a result, acylation by other fatty acids such as n-3 PUFAs are also prevented. Fortunately, advances in mass spectroscopy and the identification of acyl-transferase proteins may soon help offer insight into their role.

5.1.5 GPCRs and fatty acids

The enormous diversity of GPCRs throughout biology justifies the current gaps in our understanding of them despite decades of research and many substantial findings. The range of GPCR ligands was recently expanded to include fatty acids. Long-hydrophobic carbon chains were initially controversial as GPCR ligands because they were structurally very dissimilar from most described ligands and due to their ability to associate with the membrane very directly.

Traditionally the focus of fatty acids and GPCRs has been on the ability of fatty acids to be metabolized into ligands for GPCRs. This is the basis for the dueling-roles of n-6 and n-3 fatty acids, as their various metabolites are implicated in opposing biologic roles. For example, both the n-6 archadonic acid and the n-3 eicosapentaenoic acid are metabolized into various eicosanoids, a well studied class of GPCR ligands in the immune and cardiovascular systems (Schmitz and Ecker 2008). Many of the effects on inflammation are reviewed elsewhere (Chapkin, Kim, and Lupton 2009).   Pariante and colleagues have suggested that anti-inflammatory properties of n-3 PUFA are the mediators of the observed antidepressant effects (K. Su et al. 2010). While this is highly possible, we suggest multiple roles for these compounds, and focus on membrane properties thereof.

Recently, however, research has also begun to focus on the role of these fatty acids themselves, rather than their metabolites, on GPCR activation. In the past decade, several previously orphaned GPCRs have been identified as receptors for lipid messengers (Briscoe et al. 2003). The first characterized, GPR40, possesses

promiscuity for many lengths and saturation of fatty acids, whereas others, like

GPR120, are more selective. Presently, it is believed that all of these receptors are

coupled to the $G\alpha_q$ pathway, which is described below. It has also been

hypothesized that particular fatty acids interact differently with the same receptor.

Still, the physiologic significance of these free fatty acid receptors is not well

established. The leading clue is their distribution pattern in the body. Most are

expressed in at least one type of immune cell, whereas GPR41 and GPR43 are also

highly expressed in adipose tissue, GPR40 is also found in brain and pancreas tissue,

and GPR120 is also found in adipose, lung, gut and brain tissues (Briscoe et al.

2003).

Most physiologic characterization has been spent investigating the relationship of

these receptors in metabolic disorders. Oh et al. hypothesize that GPR120 plays a

significant role in both insulin resistance and inflammation. They demonstrated

that n-3 PUFAs increase insulin sensitivity in obese mice only when GPR120 is

present(D. Y. Oh et al. 2010). Other efforts to study free fatty acid receptors in

metabolic disorders are reviewed here (Talukdar and Olefsky 2011; Hara, Hirasawa,

and Ichimura 2011).

As an extension of this research, much of the work on these receptors in the nervous

system relate to the gut-brain axis as these compounds, which are generated in the

GI system, freely penetrate the blood-brain barrier, have signaling properties in

both brain and gut.   The ability of GPR40 and GPR120 to help mediate taste

perception lead some researchers to ponder if these are valuable targets for fighting

obesity(Cartoni, Yasumatsu, and Ohkuri 2010). It is even possible that these

receptors are important in neurogenesis and neurodevelopment (Boneva et al.

2011). One preliminary line of evidence is that immunostaining of newborn

neurons is especially high for GPR40 versus mature neurons (Yamashima 2008).

Other ideas about free fatty receptor signaling in the brain are purely speculative.

Below, we describe many second-messenger signaling pathways that are affected by

fatty acids which may be acting in concert with these receptors.

5.1.6 Regulation of cAMP through lipid rafts in depression

The localization of G proteins to specific membrane domains such as caveolae (Li et

al. 1995) and lipid rafts has generated interest in these cholesterol and sphingolipid-

rich detergent-resistant membrane domains and how they affect G protein

targeting and function (Allen, Halverson-Tamboli, and Rasenick 2006). More recent

data suggest that lipid rafts represent areas where $G\alpha_q$ signaling is promoted

(Bhatnagar et al. 2004) and where signaling through $G\alpha_s$ is inactivated (Allen,

Halverson-Tamboli, and Rasenick 2006; Allen et al. 2005; Allen et al. 2009). There is

a long experimental history of agents that increase "membrane fluidity," increasing

agonist- and $G\alpha_s$ -mediated adenylyl cyclase. Levitski and his colleagues (Rimon,

Hanski, and Braun 1978) examined this thoroughly in turkey erythrocytes and this

was also observed in synaptic membranes (Rasenick, Stein, and Bitensky 1981). $G\alpha_s$ activates adenylyl cyclase more efficiently outside of lipid rafts and chronic treatment with antidepressants facilitates G-protein exodus from those rafts (Donati and Rasenick 2005; Allen et al. 2009). Treatment of lymphocytes with DHA displaced phospholipase D (PLD) from lipid rafts, increasing PLD activity by facilitating association with its non-raft small G-protein activator (Diaz et al. 2002).

A number of studies suggest that chronic antidepressant treatment increased physical coupling between $G\alpha_s$ and adenylyl cyclase. This was investigated using immunoprecipitation of $G\alpha_s$ adenylyl cyclase complexes with anti- $G\alpha_s$ antibodies (Chen and Rasenick 1995a). This study also provided independent verification that there was no increase in $G\alpha_s$ content after antidepressant treatment. The total amount of adenylyl cyclase immunoprecipitated by anti-$G\alpha_s$ increased after antidepressant treatment, consistent with the idea that antidepressant treatment increases coupling between $G\alpha_s$ and adenylyl cyclase (Chen and Rasenick 1995a). This is consistent with the observation that chronic treatment with antidepressants results in long-term increases in cellular cAMP (Hill et al. 2007).

More recent data demonstrated that $G\alpha_s$ (and not other G proteins) became more detergent-soluble after antidepressant treatment and decreased its localization in lipid rafts (Toki, Donati, and Rasenick 1999; Donati, Thukral, and Rasenick 2001; Donati and Rasenick 2005; L. Zhang and Rasenick 2010). This has been observed after chronic treatment with fluoxetine, desipramine, escitalopram and phenelzine.

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Eisensamer et al showed that some antidepressant and some antipsychotic drugs (no tested antipsychotic affects $G\alpha_s$ raft localization of $G\alpha_s$ -adenylyl cyclase coupling) concentrated in lipid rafts during the course of chronic treatment (Eisensamer et al. 2005) . Taken together, these data suggested that chronic antidepressant treatment moved $G\alpha_s$ to a region of the plasma membrane where it was less complexed with cytoskeletal elements and more available to activate adenylyl cyclase. This was consistent with the increased "cAMP tone" that investigators looking at products of genes activated by cAMP response elements have observed subsequent to antidepressant treatment (Hill et al. 2007).

Note, however, that while both lipid raft disruption and chronic antidepressant treatment increase $G\alpha_s$-adenylyl cyclase coupling, antidepressant treatment is selective both in the signaling proteins that it affects (only $G\alpha_s$) and the cell types in which it works (Menkes and Aghajanian 1983; Donati and Rasenick 2005). HEK 293 cells show concentration of antidepressants in lipid rafts but do not show increased $G\alpha s$-adenylyl cyclase coupling unless those HEK-293 cells are expressing type VI adenylyl cyclase. It is noteworthy that the acute effects of cytoskeletal-disrupting drugs at increasing the coupling between $G\alpha_s$ and adenylyl cyclase are also not additive with chronic antidepressant treatment (Donati and Rasenick 2005). There may be an intimate relationship between $G\alpha_s$, adenylyl cyclase, and microtubules in lipid rafts (Head et al. 2006).

Postmortem studies reveal increased lipid raft localization of $G\alpha_s$ in subjects who committed suicide compared to matched control subjects (Donati et al. 2008), and this is consistent with an attenuation of $G\alpha_s$-AC coupling. It appears that human peripheral tissue may show similar effects. A study of about 1,500 subjects shows that AlF4- stimulated adenylyl cyclase activity is significantly lower in platelets from depressed subjects than from non-depressed counterparts (Hines and Tabakoff 2005). Similarly, a decreased activation of platelet PGE2-activated adenylyl cyclase resolved in as early as one week in those subjects showing an antidepressant response at 6 weeks (Mooney et al. 1985).

As mentioned above, clinical trials of n-3 PUFA, alone or in combination with conventional antidepressants, have had mixed results. A recent meta-analysis by Mischoulon et al suggests a slight advantage of n-3 PUFA over placebo, something which many classic antidepressants often struggle to achieve (Freeman et al. 2010). Furthermore, recent data by Rapaport and colleagues demonstrate a clear advantage of n-3 PUFA supplementation to SSRI therapy (Gertsik et al. 2012). This suggests the possibility that n-3 PUFA may have multiple sites of action. Two possible sites include direct changes in lipid composition and combination between n-3 PUFAs and palmitic acid for the acylation of lipid raft associated proteins.

A number of authors have suggested the involvement of BDNF in both depression and antidepressant action. Pandey and colleagues have measured both BDNF and trkB in depression and antidepressant response (Pandey et al. 2010). trkB the

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receptor for BDNF is lipid raft associated. N-3 PUFA treatment has been suggested to increase BDNF and the structural changes associated with synaptic plasticity in rats (Venna et al. 2009).

5.1.7 Summary

The role of n-3 PUFA as antidepressants remains unclear, but there is a clear importance of continued investigation into the role of fish oil, both as a modulator of G-protein signaling and as a therapeutic partner for depression. Below, the role of fatty acids in Gα raft-localization is investigated.

5.2 Results

5.2.1 Chronic treatment with DHA and EPA move Gα$_s$ out of lipid rafts

N-3 PUFAs are widely suggested to possess antidepressant properties, but a cellular mechanism is unknown. It is suggested however that PUFAs may regulate lipid raft organization. Since antidepressants decrease Gα$_s$ localization to rafts, PUFAs were hypothesized to act in a similar manner. C6 glioma were chosen as a model system since they display a robust response to antidepressants and grow fast enough to extract sufficient lipid raft fractions. Cells were treated with either antidepressants, DHA or EPA for three days and lipid rafts were extracted using sucrose gradient

sedimentation. Western blotting revealed a significant and comparable decrease in

$G\alpha_s$ localization in lipid rafts with both antidepressant and n-3 PUFA treatment, but

total $G\alpha_s$ content remained unchanged (Figure 16).
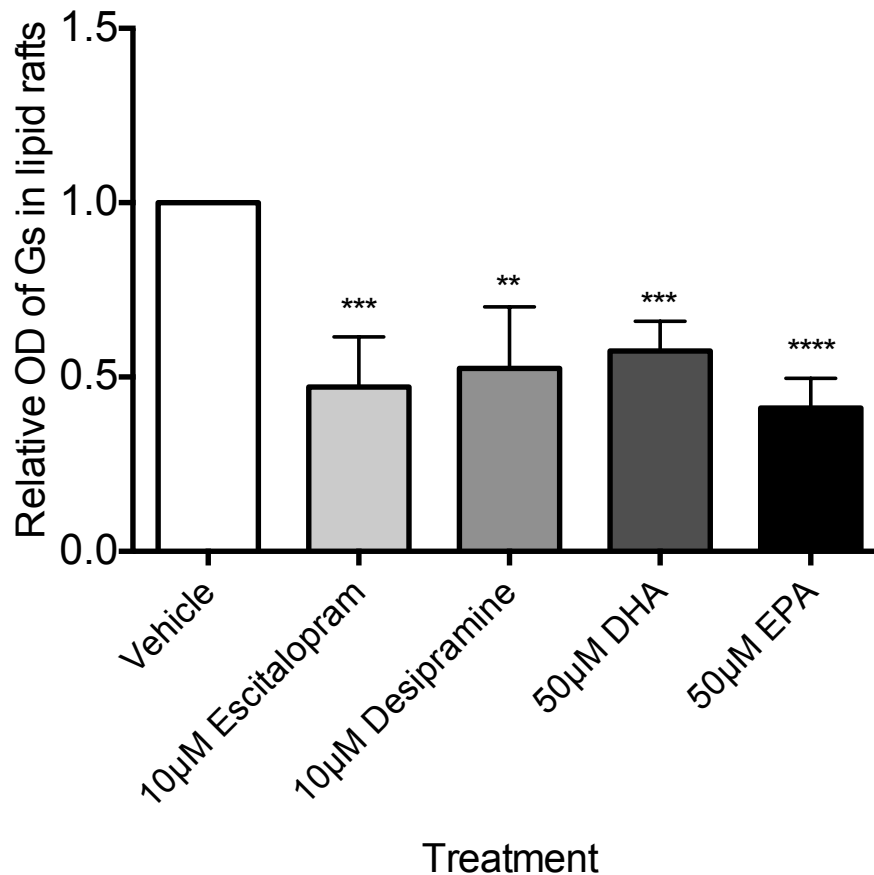
Figure 16. Omega-3 fatty acids DHA and EPA decrease $G\alpha_s$ raft localization similar to antidepressants.
C6 glioma cells were treated for 3 days with either antidepressants or n-3 PUFAs. The detergent-insoluble lipid rafts were obtained by sucrose density gradient fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for $G\alpha_s$ content. The figure shows the percentage of change in $G\alpha_s$ protein above control in the lipid raft membrane fractions from four independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.

Since antidepressants alter $G\alpha_s$ localization in a dose-dependent manner and only after chronic treatment, we next examined whether n-3 PUFAs were similar. We demonstrated that acute DHA treatment does not significantly alter $G\alpha_s$ raft association, but that 48- and 72-hour treatment does (Figure 18). Unlike antidepressants, DHA treatment had opposite effects at different concentrations(L. Zhang and Rasenick 2010). Concentrations between 100nm-10µm increased accumulation in lipid rafts, whereas higher concentrations decreased $G\alpha_s$ raft association.
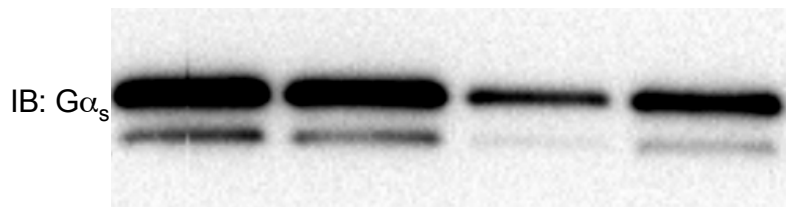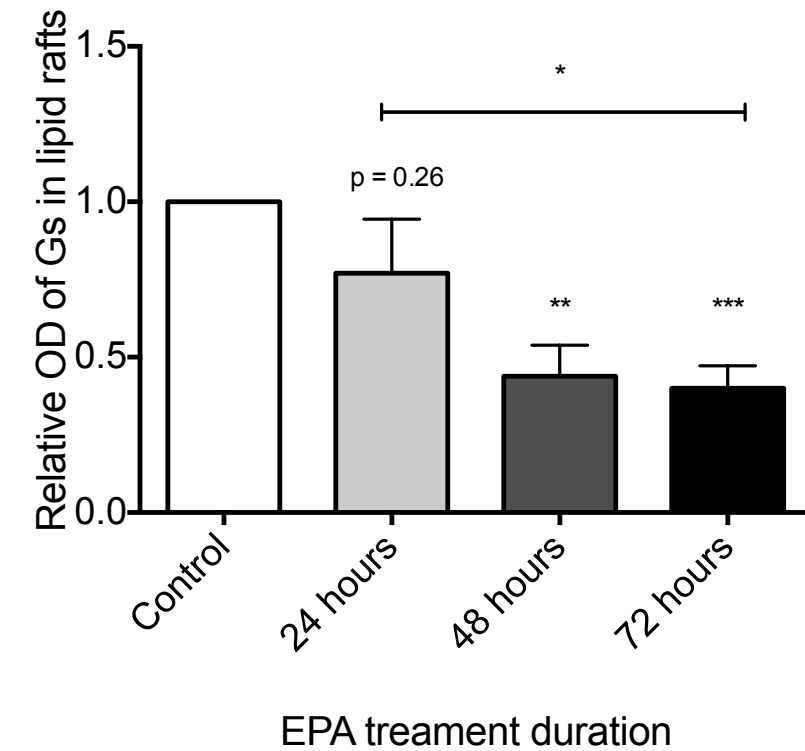
Figure 17. EPA moves Gα$_s$ out of lipid rafts after chronic, but not acute treatment.
C6 glioma cells were treated for the indicated time with the n-3 PUFA EPA (50 μm). The detergent-insoluble lipid rafts were obtained by sucrose density gradient fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for Gα$_s$ content. The figure shows the percentage of change in Gα$_s$ protein above control in the lipid raft membrane fractions from three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.

Figure 18. DHA regulation of Gα$_s$ raft content is dose-dependent.
C6 glioma cells were treated for three days with the indicated concentration of n-3 PUFA DHA. The detergent-insoluble lipid rafts were obtained by sucrose density gradient fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for Gα$_s$ content. The figure shows the percentage of change in Gα$_s$ protein above control in the lipid raft membrane fractions from three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.

5.2.2 Omega-6 PUFAs also move Gα$_s$ out of lipid rafts, but saturated fatty acids do not

Next we studied other fatty acids to determine whether n-3 PUFAs were uniquely regulating Gα$_s$ or if similar fatty acids also altered raft association. Similar to n-3 PUFAs, linoleic and arachidonic acid, both n-6 PUFAs, elicited a movement of Gα$_s$ out of lipid rafts akin to antidepressants. In contrast, two saturated fatty acids - palmitic and stearic acid - did not (Figure 19). Furthermore, none of the fatty acid treatments altered Gα$_s$ content in the whole cell lysate (Figure 20).
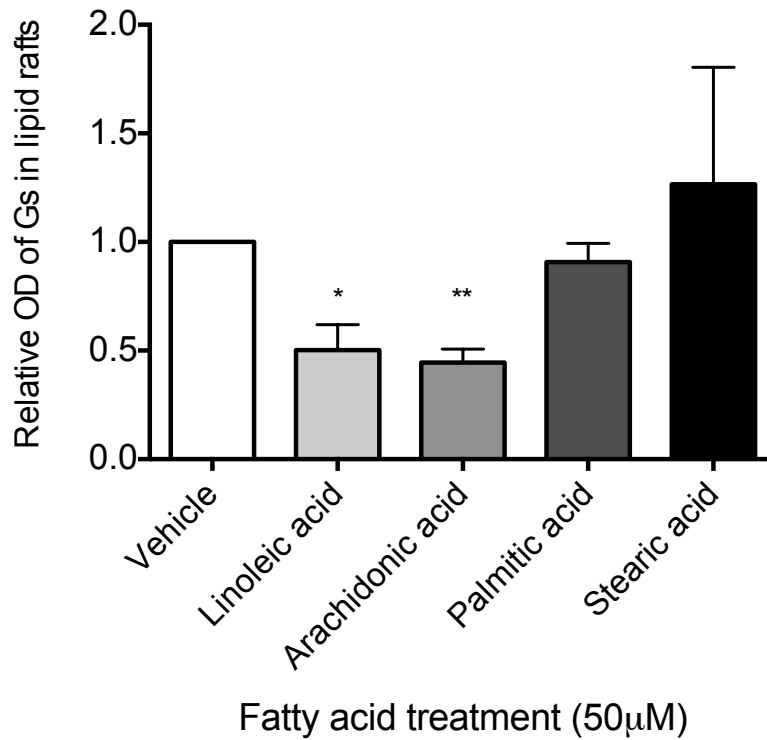
Figure 19. Omega-6 PUFAs also remove Gα_s from lipid rafts, but saturated fatty acids do not.
C6 glioma cells were treated for three days with either n-6 polyunsaturated fatty acids or saturated fatty acids. The detergent-insoluble lipid rafts were obtained by sucrose density gradient fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for Gα_s content. The figure shows the percentage of change in Gα_s protein above control in the lipid raft membrane fractions from four independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001). Error bars represent SEM.
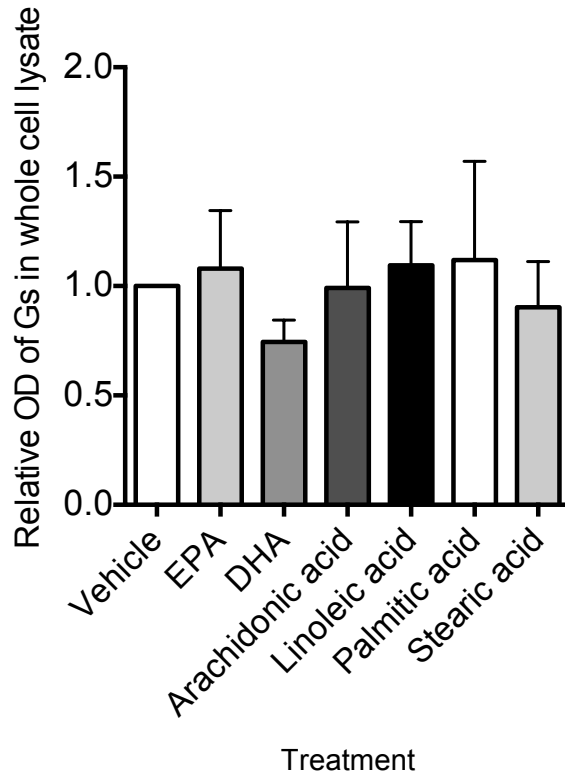
Figure 20. Gα_s content in whole cell lysate is not altered by fatty acid treatment.
C6 glioma cells were treated for three days with the indicated fatty acid and whole cell lysates were analyzed for Gα_s content by immunoblotting. The figure shows the percentage of change in Gα_s protein above control in the whole cell lysate fractions from three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001).   Error bars represent SEM.

5.2.3 Omega-3 and antidepressants have an additive effect on $G\alpha_s$ raft redistribution

Omega-3 supplementation, particular EPA, to antidepressant therapy is actively being investigated in both clinical trials(Rush and Suppes 1999) and basic science experiments(Venna et al. 2009). This may be especially beneficial if PUFA and antidepressants regulate signaling through different mechanisms. To investigate, we treated cells with both EPA and escitalopram. Co-treatment elicited a stronger effect than either compound alone (Figure 21).
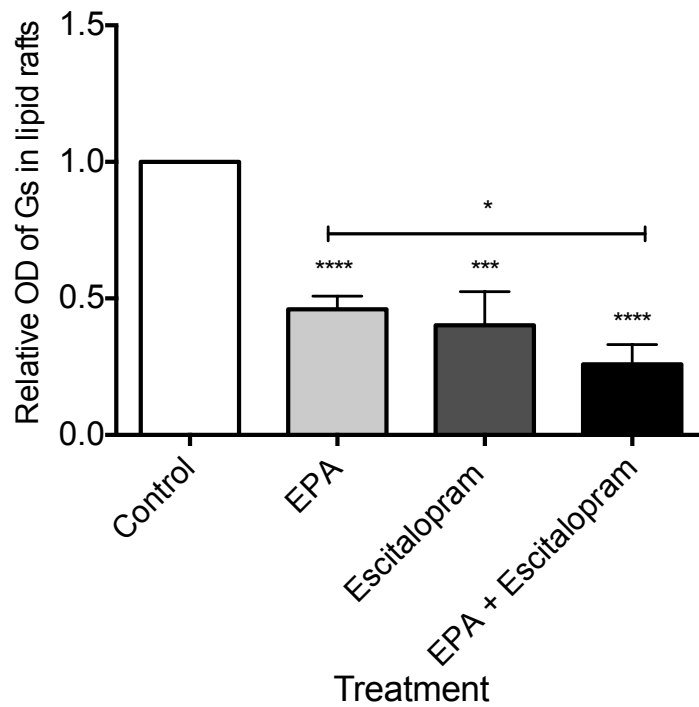
Figure 21. EPA and escitalopram have an additive effect on translocating Gα_s out of lipid rafts.
C6 glioma cells were treated for three days with EPA, escitalopram or both. The detergent-insoluble lipid rafts were obtained by sucrose density gradient fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for Gα_s content. The figure shows the percentage of change in Gα_s protein above control in the lipid raft membrane fractions from four independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).   Error bars represent SEM.
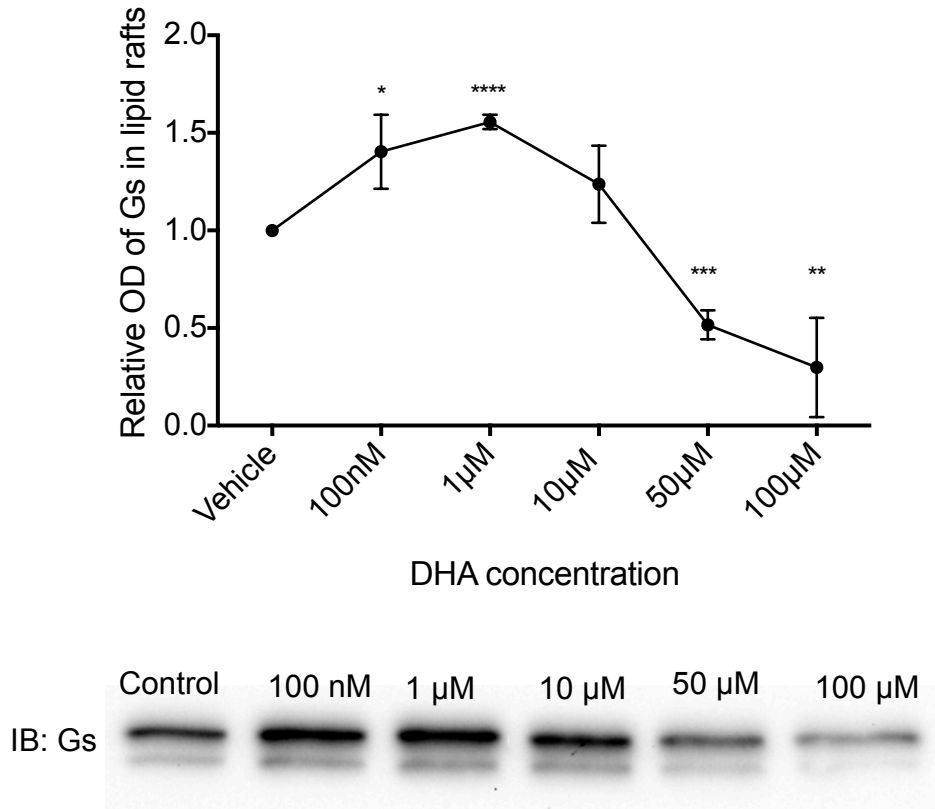
5.2.4 DHA and EPA do not effect mobility of GFP-Gα$_s$ in FRAP assays

Previous results demonstrated FRAP is a useful tool for assaying regulation of Gα$_s$ signaling. In particular, decreased mobility in a FRAP assay of GFP-Gα$_s$ accurately predicts increased Gα$_s$-adenylyl cyclase coupling, which is also correlated with decreased lipid raft association. Surprisingly however, results here demonstrate PUFA treatment has no effect on GFP-Gα$_s$ mobility.
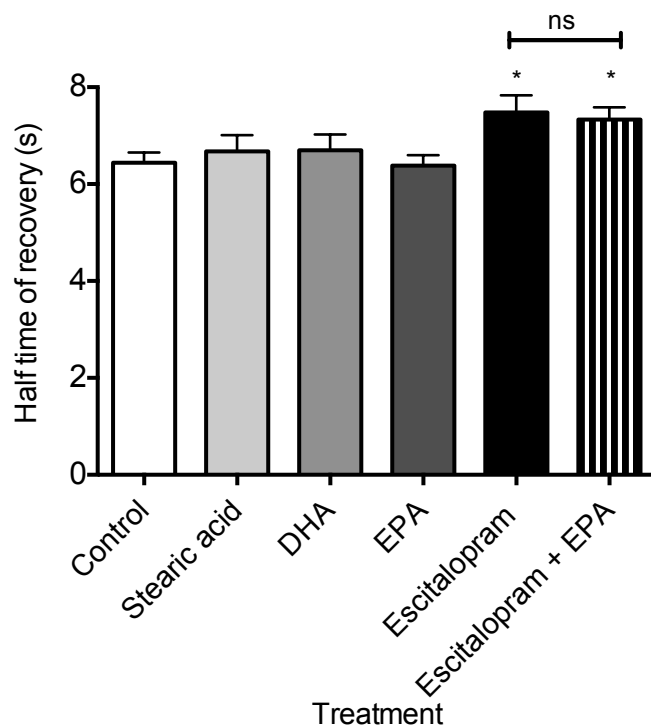
Figure 22. Fatty acid treatment does not affect GFP-Gα_s diffusion in FRAP assay.
C6 glioma cells stably expressing GFP-Gα_s were treated for three days with the indicated combination of antidepressants or fatty acids. On the fourth day, treatment was washed out and media was switched to phenol-red free DMEM for one hour. Membrane regions were rapidly photobleached and recovery was measured for 45 seconds. One-phase association curves were plotted and the half-time to recovery was compared between conditions. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, * $p < 0.05$).   Error bars represent SEM.

5.2.6 Statins alter $G\alpha_s$ cellular distribution

Like PUFAs, statins are a popular class of drugs used in the management of cardiovascular disease, but may also be important in the brain. Currently however, despite their wide-use and popularity, their effects in systems beyond the heart are less clear. Statins work by inhibition of HMG-CoA reductase, an enzyme far upstream from the final cholesterol product and therefore statins also inhibit production of several cholesterol precursors. In particular is farnesyl pyrophosphate, a lipid that is covalently attached to several proteins and facilitates their membrane association. This includes the $G\gamma$ subunit of the heterotrimeric G-protein complex. Given $G\gamma$'s role in recruiting $G\alpha_s$ to the plasma membrane(Evanko et al. 2001) and the importance of cholesterol in G-protein regulation by lipid rafts, statin regulation of $G\alpha_s$ was investigated.
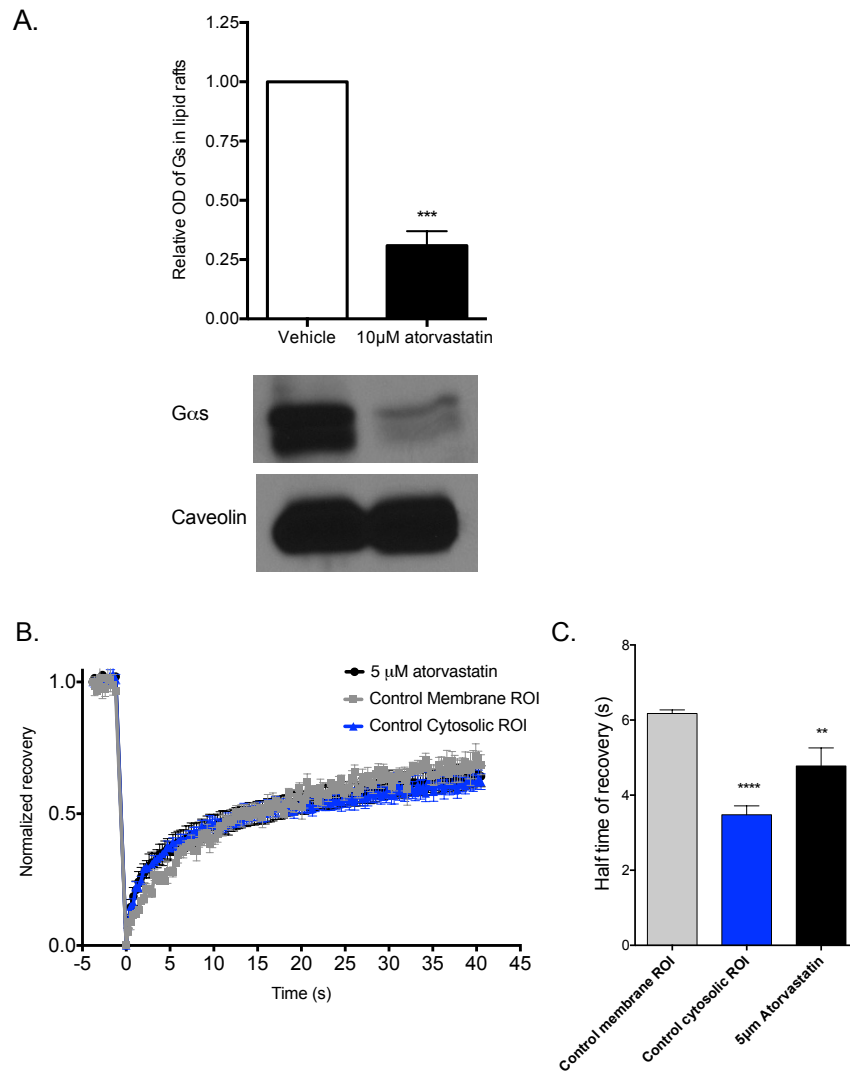
Figure 23. Atorvastatin alters Gα$_s$ cellular localization.
A) C6 glioma cells were treated for 24 hours with 5 μm atorvastatin. Gα$_s$ content in lipid rafts were analyzed as described above. B) C6 glioma cells stably expressing GFP-Gα$_s$ were treated for 5 μm atorvastatin for 24 hours. After, treatment was washed out and media was switched to phenol-red free DMEM for one hour. Indicated regions were rapidly photobleached and recovery was measured for 45 seconds. One-phase association curves were plotted and C) the half-time to recovery was compared between conditions. Data were analyzed by one-way ANOVA followed by Tukey test for post hoc multiple comparisons of means (control versus treatment, *p < 0.05, ** p < 0.01, *** p < 0.001).   Error bars represent SEM.

First, C6 glioma cells were treated with 5μM atorvastatin for 24 hours and then lipid rafts were extracted by sucrose density sedimentation. Similar to cholesterol depletion by MβCD, atorvastatin treatment decreased $G\alpha_s$ association with lipid rafts. Fluorescence microscopy of GFP-$G\alpha_s$ revealed this movement out of lipid rafts was primarily to the cytoplasm, unlike MβCD treatment where $G\alpha_s$ remains associated with the membrane. Finally, FRAP measurements of GFP-$G\alpha_s$ in statin-treated cells showed a relatively fast recovery, characteristic of cytoplasmic of $G\alpha_s$. Together, these data demonstrate a significant reorganization of G-protein signaling by statins.

5.3 Discussion

Numerous aspects of G-protein signaling are affected by PUFAs. The most well characterized mechanism is their indirect action as a ligand for GPCRs, but here we demonstrate a clear effect on the G-protein itself. Most significantly, polyunsaturated fatty acids decrease localization of $G\alpha_s$ to lipid rafts without altering total $G\alpha_s$ content (Figure 16,Figure 20). Curiously, this is altered localization is very similar to the regulation of $G\alpha_s$ by antidepressants. Indeed both PUFAs and antidepressants diminish $G\alpha_s$ raft content only with chronic treatment.

Nonetheless, PUFAs and antidepressants likely regulate $G\alpha_s$ signaling through different mechanisms. This is first highlighted by data demonstrating

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antidepressant and PUFA co-treatment have an additive effect (Figure 21).

Furthermore FRAP measurements after antidepressant treatment produce a

signature decrease in GFP-Gα_s diffusion whereas GFP-Gα_s in PUFA treated cells

diffuse normally (Figure 22).

The mechanism of this PUFA regulation of Gα_s could act through either direct

membrane effects or altered Gα_s acylation. In the former case, PUFAs may be

altering the structure and composition of rafts, causing a disruption similar to

cholesterol chelation (which also decrease Gα_s raft localization). Unlike PUFA

treatment however, cholesterol removal however produces changes in GFP-Gα_s

FRAP.

Altered Gα_s acylation on the other hand may result in altered raft localization

without changes in GFP-Gα_s diffusion. In this scenario fatty acid treatment would be

affecting cysteine acylation on the Gα_s amino terminus. Traditionally, this cysteine

is covalently modified by palmitic acid by the acyl transferases DHHC3 and

DHHC7(Tsutsumi et al. 2009). This has been robustly confirmed by both tritium

labeled palmitic acid and mass spectroscopy. Nonetheless, the fidelity of acyl

transferases for palmitic acid relative to other fatty acids is questionable. Indeed,

there is limited evidence other saturated fatty acids may covalently modify Gα_s.

Furthermore, PUFAs DHA and arachidonic acid have been shown to incorporate into

the similar peripheral membrane proteins Fyn and Lck(Webb, Hermida-Matsumoto,

and Resh 2000). In that study, PUFA incorporation also diminished Fyn and Lck

localization to lipid rafts. Together, these data strongly suggest the likelihood that PUFA fatty acylation, or pufalyation regulates $G\alpha_s$ raft association too.

Our model suggests a more complex capacity for *S*-acylation to regulate signaling than just canonical palmitoylation. Raft targeting via the saturated palmitic acid is logical since rafts tend to contain mostly saturated lipids, but raft vs non-raft organization may not be binary. Instead, there may be a spectrum of dynamic lipid domains based on lipid unsaturated and chain length
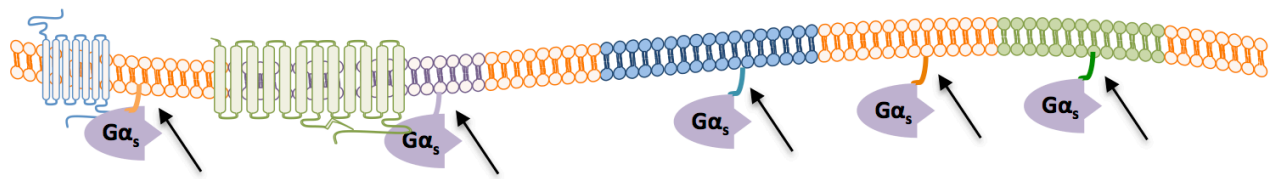
Acylation of $G\alpha_s$ by different fatty acids may target
$G\alpha_s$ to different membrane domains

Figure 24). Acylation by a medley of fatty acids may allow efficient protein targeting to specific lipid domains. Depending on the protein and lipid combination this may or may not have meaningful effects on signaling.

Acylation of Gα$_s$ by different fatty acids may target
Gα$_s$ to different membrane domains

Figure 24. A model for PUFA regulation of Gα$_s$ signaling.
Chronic treatment with n-3 and n-6 PUFA move Gα$_s$ out of lipid rafts similar to antidepressants. This effect is not seen with the otherwise similar but saturated fatty acids, stearic and palmitic acid. In this model, supplemented fatty acids compete with palmitic acid incorporation into Gα$_s$. The type of fatty acid incorporated directs Gα$_s$ to membrane regions with a similar phospholipids. Therefore PUFAs will direct Gα$_s$ to membrane regions rich in unsaturated fatty acids (non-rafts) whereas saturated fatty acids like stearic acid will promote localization to lipid rafts.

Chapter 6: To analyze the structural effects of antidepressants in model membranes.

6.1 Introduction to model membranes

6.1.1 Model membrane systems and applications

Model membranes are a valuable tool for studying lipid interactions that are either too difficult to control in live cells or too small to observe with imaging techniques compatible with life. In particular, this incudes the study of phase-separation of lipids into liquid-ordered and liquid-disorder phases. Ordered phases typically contain well-packed saturated fatty acids, whereas disordered phases are more often correlated with unsaturated tail groups. In model membranes, these domains occur on both nano- and micro-scales, but in biologic systems they are only hypothesized to occur in sizes between 1-100nm. Therefore, models are useful because they can exaggerate domain size to be compatible with light microscopy (>500nm). In addition, model systems can be studied using Small Angle Neutron Scattering, which unlike light microscopy allows nanometer structural resolution.

Common model membrane systems consist of only a few select lipids organized as vesicles, bilayers or monolayers. This simplicity affords them their biggest utility over natural membranes, as it allows researchers to determine the contributions of individual lipid species. In particular, it allows membranes to reliably be spiked with reporters of membrane properties.

In a light microscopy system, these reporters are fluorescent-tagged lipids. Here, dyes like rhodamine or fluorescein are covalently linked to either the head or tail group of a particular lipid species. This addition certainly alters the native properties of the lipid, and so tagged lipids are typically kept low molar concentrations (0.5-3%). Even still, rigorous controls are important to ensure minimal influence.

Fluorescent-labeled lipid mixtures can then be studied using a variety of traditional microscopy protocols. The most basic is simple imaging of the fluorescent species to observe whether mixing occurs. If some lipid species are separated, phases larger than 500nm can be visualized and quantified based on size, shape and frequency. Furthermore, fluorescent recovery after photobleaching (FRAP) or fluorescent loss in photobleaching (FLIP) can then be used to assess diffusion. If lipid species are separate but too small to resolve with light microscopy, förster resonance energy transfer (FRET) can still be used to interpret lipid interactions.

Regardless of the approach, systems using fluorescent-labeled lipids are inherently imperfect because of the bulky tag. For this reason, small angle neutron scattering is an attractive complement. In this system, signal is created by differential neutron scattering rather than by light. Therefore, instead of tagging lipids with bulky dyes, they are labeled with deuterium, which scatters neutrons different than hydrogen, but is otherwise does not change their chemical identity. In addition, SANS allows

nanometer structural resolution, making it a very useful though it is a relatively expensive approach.

6.1.2 Cholesterol's influence on domain formation in model membranes

Cholesterol or closely related sterols are a necessary component of complex biologic membranes and have numerous interesting properties in model systems. In fact, its presence in eukaryotic, but not most prokaryotic species suggests it may have played a vital role in the development of more-elaborate forms of life. Indeed, cholesterol is considered an important mediator of biologic lipid rafts. Compartmentalization of signaling through lipid rafts may be an important difference between eukaryotic and prokaryotic life(Mouritsen and Zuckermann 2004).

6.1.3 Effects on lipid-soluble drugs in model systems

Membrane effects of hydrophobic drugs have long been speculated, but until recently this influence has been difficult to study. Since psychoactive drugs must be hydrophobic to pass the blood-brain barrier, compounds like mood stabilizers, antidepressants and anesthetics have been an area of focused research. In particular, the lipid hypothesis of general anesthetic action states that these compounds act through fluidization of membrane phases. Most striking is the

Meyer-Overton correlation – the more lipid soluble an anesthetic, the greater the potency.

Although popular for the past fifty years, the lipid hypothesis of general anesthetic action has lost favor to a modern lipid hypothesis that also accounts for protein interactions. The old theory is criticized because stereoisomers of general anesthetics demonstrate different potency and body temperature increases should mimic the membrane fluidization caused by anesthetics, but do not result in anesthesia(Franks and Lieb 1994). Therefore the modern lipid hypothesis has gained favor by suggesting protein interactions must play a role too. In this model, transmembrane proteins are sensitive to anesthetics because these compounds alter membrane pressure exerted on the hydrophobic, membrane embedded regions of the protein(Cantor 1997). In fact, key amino acids in the $GABA_A$ receptor have been demonstrated to play a role in at least some anesthetics(Weir 2006).

Antidepressants are another class of drug that may exert some effects because of direct membrane interactions. Historically however, these effects have been underscored since protein targets of these drugs are well accepted. As with anesthetics, protein and lipid targets may be working together. In fact, slow accumulation of antidepressants in membranes, could explain why some of their effects require chronic treatment. Indeed, the known protein targets of antidepressants fail to explain all of their biologic properties(L. Zhang and Rasenick 2010). NMR experiments in model membranes with the tricyclic antidepressant

desipiramine demonstrate it has a disordering effect on membranes (Bermejo et al. 1975). This could explain why chronic but not acute antidepressant treatment alters G-protein association with lipid rafts(Donati and Rasenick 2005).

6.1.4 Introduction to Small Angle Neutron Scattering

The small angle neutron scattering (SANS) technique derives its name from the use of small angles and/or the use of long wavelengths to achieve small scattering-vectors defined by

$$q = (4\pi/\lambda)\sin\varphi \approx 4\pi\varphi/\lambda \text{ (1)}$$

where φ is the scattering angle and λ is the wavelength of the neutron.

Experiments work using a narrowly collimated incident neutron beam that is scattered off by the sample and collected by the detector. The intensity on the detector is corrected for efficiencies of each detection, the quartz cell containing the sample, a blocked beam (to account for detector dark current, stray neutrons etc.) along with beam flux measurement in transmission mode for absolute scale calibration (Hammouda 2008). SANS tutorials are available at http://www.ncnr.nist.gov/SANS.

The scattering data are then plotted as intensity (absolute intensity) as a function of q, the momentum change due to scattering, which is related to the scattering angle, as expressed above (equation 1).

Mathematically, in the case of a dilute system of scattering, it is expressed as

follows:

$$I(q) = \left[\int_r (\rho(\boldsymbol{r}) - \rho_{\mathrm{solv}}) \exp\{i(\boldsymbol{q} \cdot \boldsymbol{r})\} \mathrm{d}\boldsymbol{r} \right]^2$$

(2)

The integral is called the form factor, which corresponds to the shape of the

scattering object(Masui, Urakami, and Imai 2008).

As can be seen from the equation above (2), the I(q) depends on the contrast

between the scattering length densities (SLDs) of the solvent relative to the SLD of

the scattering object. The scattering length density is a quantity that describes the

chemical make-up of the object/solvent and the relative scattering power of its

constituting atoms. Thus SANS is very sensitive to isotopic difference between

chemically identical molecules. This is what makes neutrons, versus x-rays,

extremely powerful, as it can differentiate clearly between deuterated versus non

deuterated molecules.

For these SANS experiments we used this dependence on contrast to enhance the

appearance of domains on vesicles. As shown in equation 1, if the SLD of the object

is the same as the solvent then the intensity becomes zero (or background). In our

case the objects were small unilamellar vesicles (SUV). If all lipids, cholesterol and

drugs fully mix (as is the case at 37C), the vesicle can be thought as having a single,

uniform SLD. By using a solvent that matches this SLD, the scattering disappears.

The lipids, cholesterol and drugs redistribute into distinct phases or regions upon

lowering the temperature. The vesicle thus becomes "patchy". One phase

corresponds to a DOPC-rich phase, identified as the fluid part of the membrane, and the other as the DPPC-rich phase, corresponding to the ordered (in the absence of cholesterol) or liquid ordered region of the membrane.

As a result of this segregation into fluid and ordered/liquid-ordered regions scattering emerges since the SLD of the vesicle can not be described with one single SLD. Thus the emergence of scattering is the indication that phase separation occurs.

In this study, the effects of antidepressants in model membranes are examined using SANS and fluorescence microscopy. The model system of DOPC, DPPC and cholesterol is used for numerous practical reasons. First, deuterated DPPC is available at a relatively low cheap relative to other deuterated lipids. Perhaps as a result, dDPPC containing mixtures are a commonly studied in SANS and thus other work is available for reference. These experiments aim to determine whether antidepressant effect domain formation in model systems and if these changes are similar those caused by cholesterol.

6.2 Results

6.2.1 Microscopy reveals influence of antidepressants on model monolayers

Escitalopram incorporation into model membrane monolayers was first investigated using fluorescence microscopy. DOPC,DPPC and cholesterol mixtures

were used as a base since they separate into different phases at scales visible with microscopy. Then samples were spiked with escitalopram either through the aqueous phase or premixed with the lipids. Samples were also spiked with 1% rhodamine-labeled DPPE and 3% NBD-labeled DPPE to visualize domains. Despite having the same lipid group, NBD and rhodamine labeled DPPE partition differently.

Monolayers showed a striking difference in domain size with the addition of escitalopram (Figure 25). Rhodamine-labeled domains were significantly smaller in the presence of escitalopram. NBD-labeled domains dissolved completely, suggesting escitalopram caused NBD-DPPE to mix homogenously with the membrane. These significant differences between the subtly different NBD-DPPE and rhodamine-DPPE highlight how proteins too may be very sensitive to small alternations in lipid structure.

Lipid Composition		Rhodamine	NBD
DPPC	1		
DOPC	1		
DPPC	1		
DOPC	1		
Cholesterol	2		
DPPC	1		
DOPC	1		
Cholesterol	2		
Escitalopram	1		
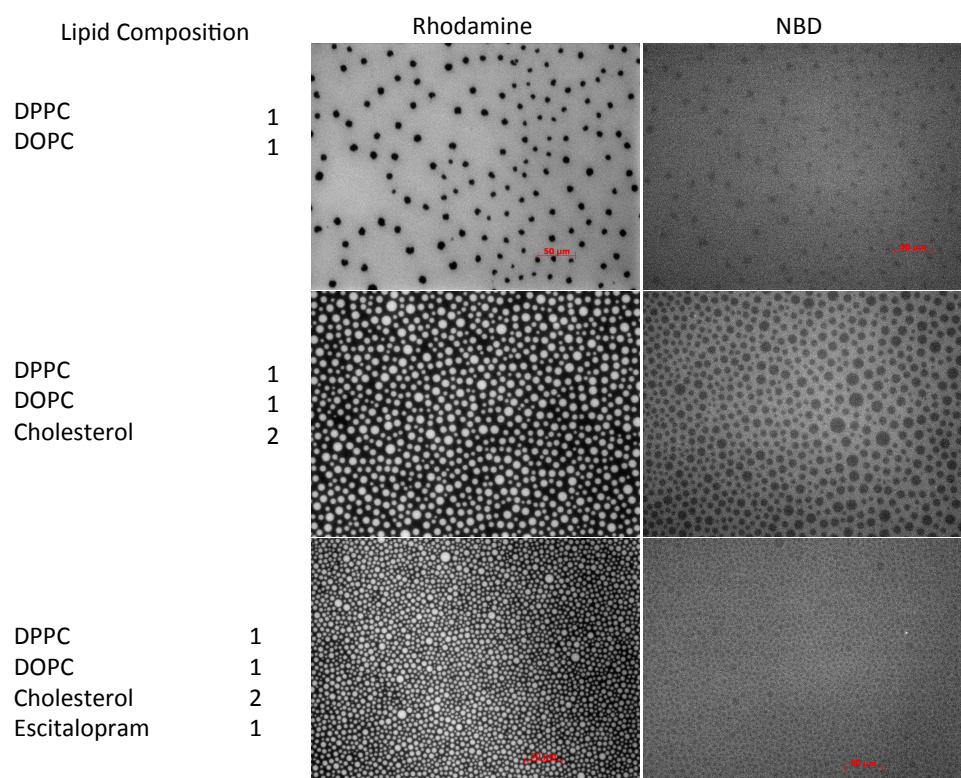
Figure 25. Cholesterol and Escitalopram alter lipid structure in model monolayers. The indicated mixtures of lipids and drugs spiked with 1% rhodamine and 3% NBD-tagged DPPE were dissolved in chloroform and deposited on the air-water interface of a Langmuir trough at 37°C. The trough's barriers were adjusted to a stable pressure of 10mN and chloroform was allowed to evaporate for 30 minutes. After the water was cooled to 15°C and phase separation of the fluorescent lipids was observed.

6.2.2 SANS demonstrates antidepressants alter lipid organization similar to cholesterol in model 30nm membrane vesicles.

First we characterized lipid phase separation using SANS. In a simple DOPC:DPPC 1:1 mixture, a high peak intensity is demonstrated at a q ($Å^{-1}$) value corresponding to the vesicle size (50nm). This intensity is related to the extent that the vesicle does not contrast match the scattering length density of the solvent, as well as any difference in scattering length density within the vesicle itself. At 37°C the intensity is very low, indicating that the mixed sample is well contrasted matched to the solvent. Therefore, when cooled to 18°C, the increase in peak intensity is likely attributed to the separation of DPPC, which is deuterated, from the hydrogenated DOPC (Figure 26).

The same experiment was then performed with mixtures spiked with various combinations of cholesterol and drugs. At 37°C, all mixtures had a comparable intensity to dDPPC:DOPC (1:1). In contrast, at 18°C, the samples with cholesterol or drug had significantly reduced peak intensity relative to dDPPC:DOPC (1:1). This effect was dependent on the amount of cholesterol added, since using a ratio of 2:2:2 dDPPC:DOPC:cholesterol produced a even lower peak that the 2:2:1.5 dDDPC:DOPC:cholesterol mixture(Figure 26).

Escitalopram spiked samples also exhibited a drastic reduction in peak intensity, but slightly less than that caused by cholesterol (
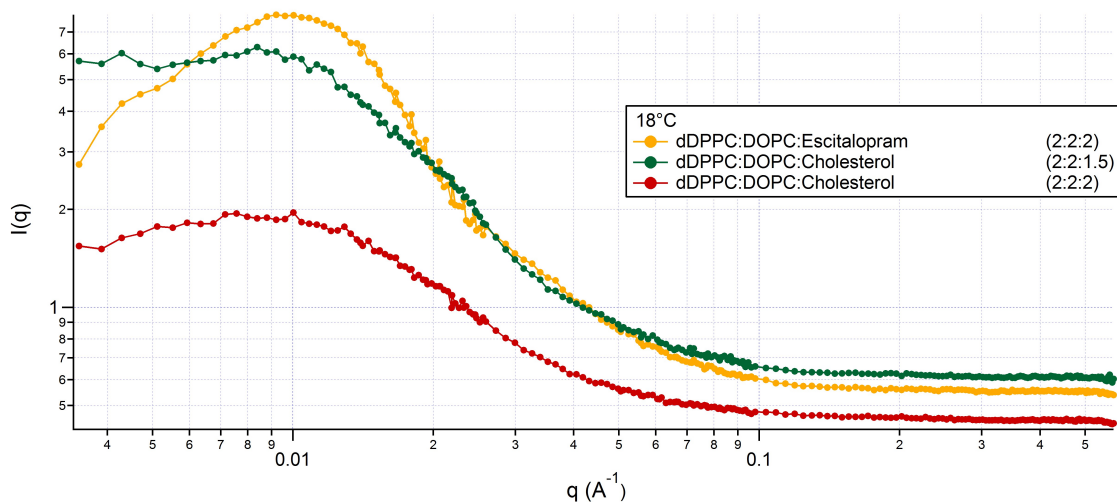
Figure 28). Another antidepressant, imipramine however, produced a peak intensity that closely resembling cholesterol
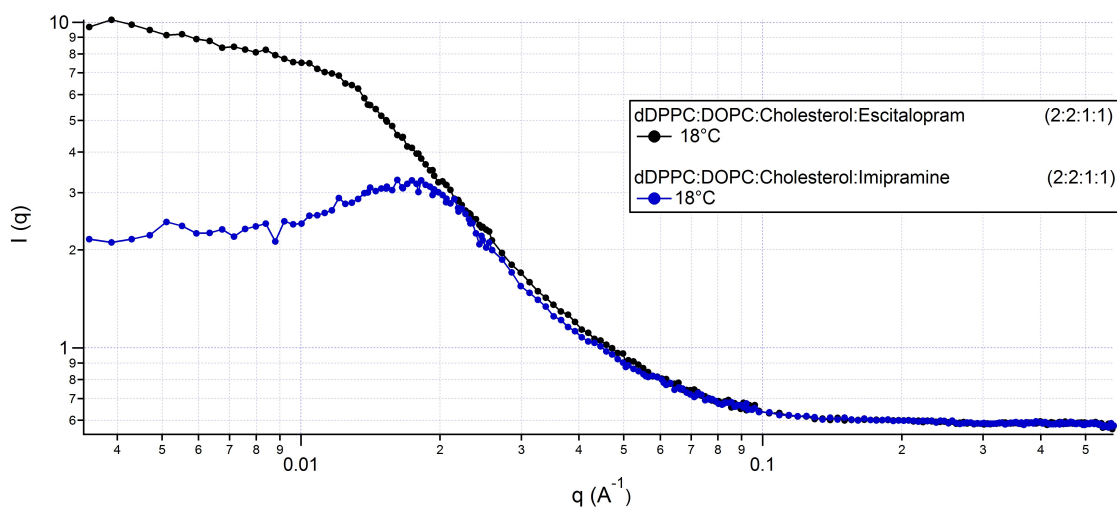
Figure 29). SANS also demonstrates escitalopram and R-citalopram have similar influence on membrane structure (Figure 30). This is in contrast to the differential pharmacologic profile of these compounds.
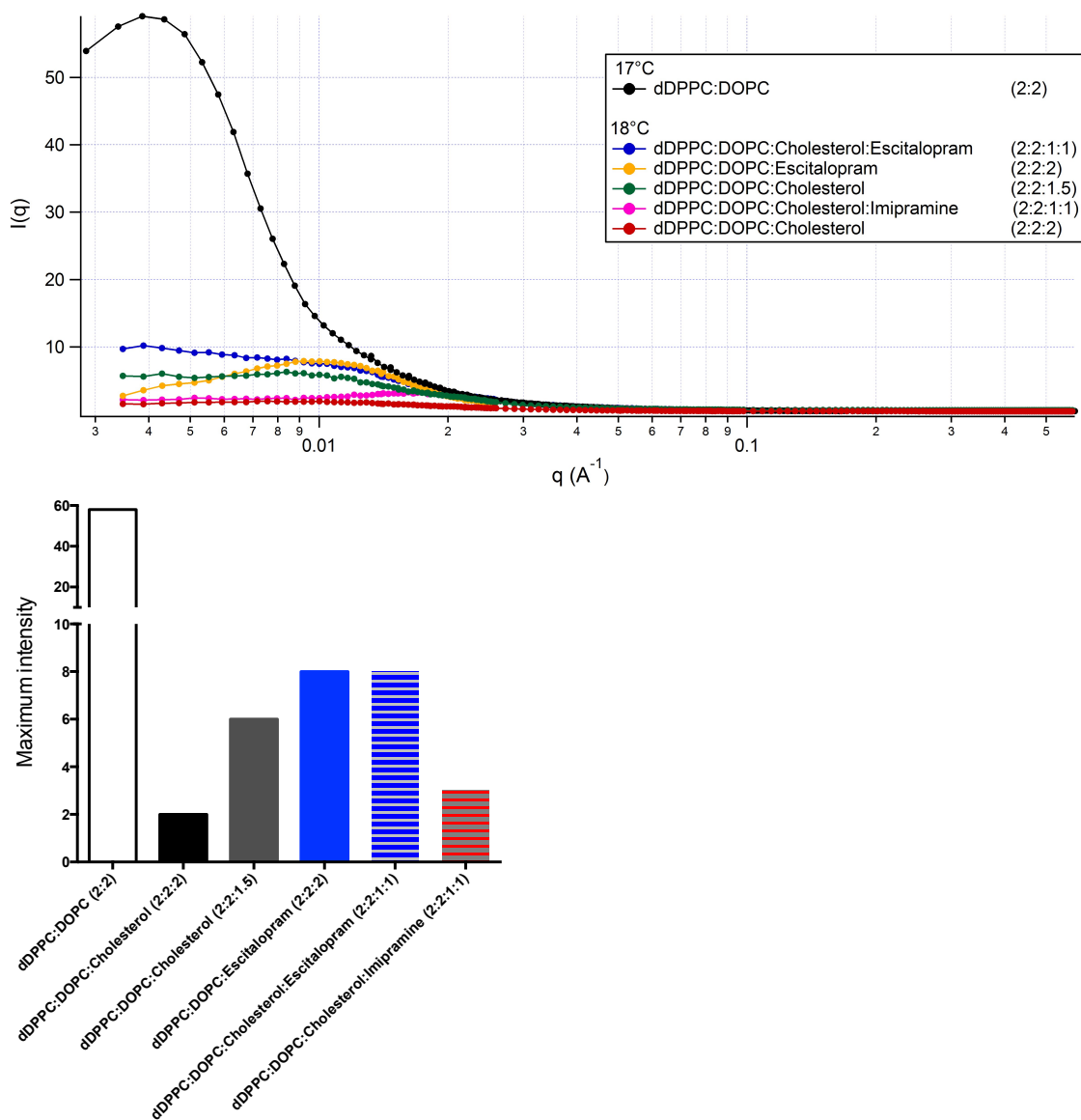
Figure 26. Cholesterol, escitalopram and imipramine alter the miscibility of dDPPC with DOPC.
The indicated mixtures of lipids and drugs were prepared as 30nm vesicles. The samples were then contrast matched at 37°C and SANS data was collected at 17°C. (top) Cholesterol, escitalopram and imipramine are all miscible with dDPPC and DOPC, but peak intensity indicates they partition differently (bottom).
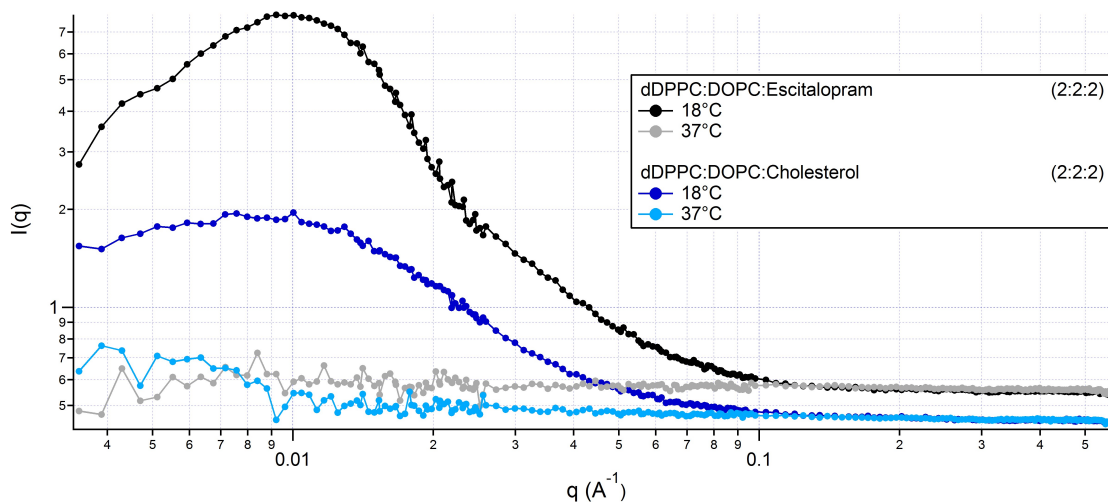
Figure 27. Membranes containing escitalopram or cholesterol phase separate at 18°C but not at 37°.
Equal molar ratios of dDPPC:DOPC were spiked with either cholesterol or escitalopram and prepared in constrast-matched water as 30nm vesicles. SANS analysis reveals both mixtures produce an intensity peak at 18°C, but not 37°C, suggesting the formation of domains.
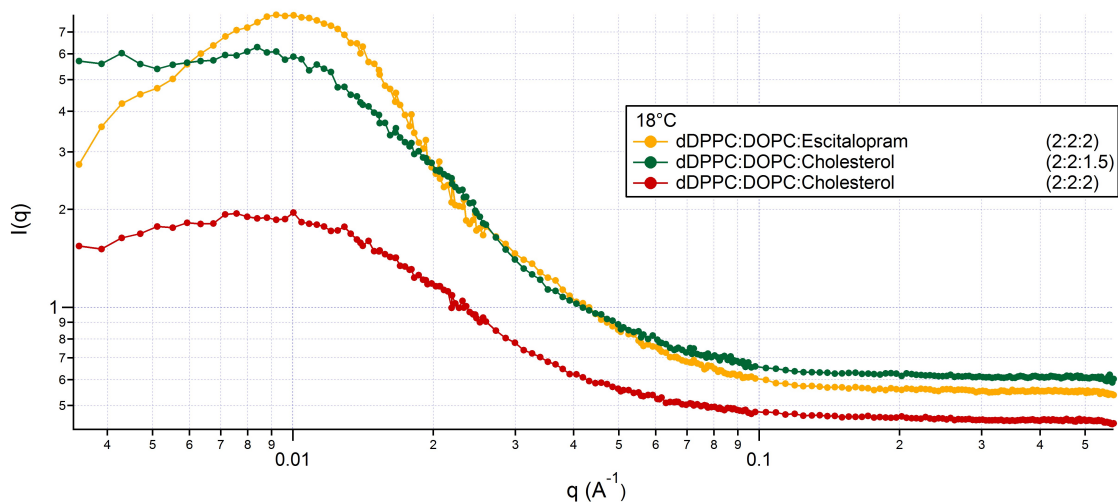
Figure 28. Membranes containing escitalopram or cholesterol phase separate at 18°C but not at 37°.
30nm vesicles in contrast matched water were prepared with different amounts of cholesterol. The 2:2:1.5 dDPPC:DOPC:Cholesterol mixture produced an intensity peak greater than the 2:2:2 dDPPC:DOPC:Cholesterol vesicles, but nearly identical to a 2:2:2: dDPPC:DOPC:Escitalopram mixture.
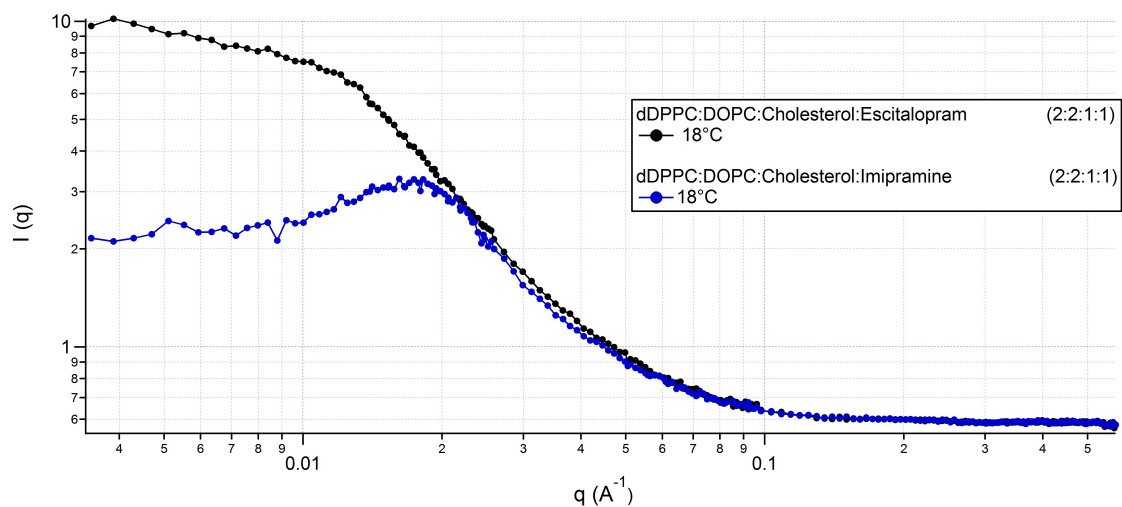
Figure 29. Escitalopram and imipramine demonstrate partition differently into lipid membranes.
30nm vesicles were prepared with the indicated ratio of dDPPC:DOPC:Cholesterol and the either drugs escitalopram or imipramine. Peak intensities from SANS analysis suggest different partitioning between dDPPC and DOPC.
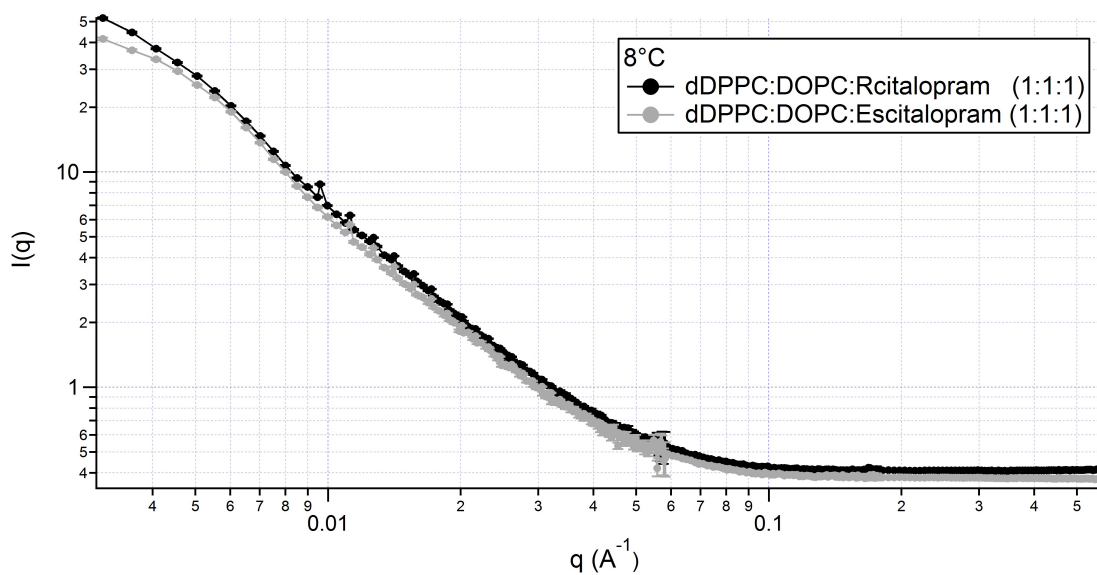
Figure 30. Escitalopram and R-citalopram spiked dDPPC:DOPC vesicles demonstrate similar scattering in SANS.
30nm vesicles were prepared with an equal molar ratio of dDPPC:DOPC:citalopram. Peak intensities from SANS analysis suggest similar partitioning of hydrogenated and deuterated species with both enantiomers of citalopram.

6.3 Discussion

Data from both SANS and fluorescence microscopy demonstrate antidepressants have a significant impact on lipid phase separation in model systems. SANS analysis suggests that the antidepressants escitalopram and imipramine both influence DPPC/DOPC miscibility similar to cholesterol. Based on their structures, this is not surprising since these drugs and cholesterol are all hydrophobic, aromatic compounds with similar molecular weight. By the same notion, it is also not surprising that escitalopram and R-citalopram demonstrated similar effects on these membranes.

The significance the intensity signal from SANS is somewhat open to interpretation. The value is derived from any deviation in the deuterium/hydrogen distribution in the vesicle relative to the deuterium/hydrogen content in the solvent. Since the samples are matched to a solvent that has equal parts D_2O/H_2O relative to that of the deuterium/hydrogen content of that sample, intensity is close to zero when the sample is well mixed (as it is at 37°C). In the case of the two lipid mixture (dDDPC:DOPC), a large intensity is simple to interpret – the two lipids separate, resulting in regions that are either high in dDPPC (and hence deuterium) or high in DOPC (hence deuterium).

The lower signal seen when cholesterol or drugs are added is more challenging to analyze. Small intensity suggests that the lipid mixture's deuterium/hydrogen

distribution is relative uniform. Therefore, the addition of cholesterol or drugs is

increasing the miscibility of DPPC with DOPC.   It is note-worthy that the intensity is

closer to zero after the addition of cholesterol or drugs, at 18°C it still remains above

the intensity seen at 37°C. This temperature-dependent effect of the intensity

implies that phase-separation is occurring.

Future modeling will better illustrate the exact changes that cholesterol and

antidepressants have on dDPPC/DOPC miscibility and lipid raft formation.

Nonetheless, these current data strongly suggest that antidepressants can

profoundly lipid organization. Whether this effect may also impact protein signaling

in cells remains unclear.   In particular, G-protein coupled receptors have been

shown to directly interact with cholesterol and other lipids through their

hydrophobic, transmembrane amino acids. Therefore it is difficult to exclude the

possibility that these interactions could be altered by antidepressants and alter $G\alpha_s$

scaffolding to these proteins.

Chapter 7: General Discussion, Significance and Conclusions

7.1 Findings and interpretations

Regulation of $G\alpha_s$ signaling occurs through a diverse array of mechanisms, including both protein and lipid scaffolds. Findings reported in this dissertation suggest lipid rafts represent a crossroad of these two types of scaffolds. Lipids, like cholesterol, may have specific interactions with $G\alpha_s$, modifying its ability to diffuse and signal throughout a membrane. Indeed, other work suggests that the N-terminus of $G\alpha_s$ has direct interactions with membranes through a string of polybasic amino acids as well as at least one palmitoylation site(Thiyagarajan et al. 2002; Crouthamel et al. 2008; Tsutsumi et al. 2009). Effector proteins like microtubules, GPCRs and adenylyl cyclase also scaffold $G\alpha_s$ to membranes and are further regulated themselves by lipids.

Data reported here indicate that the lipid raft localization of eGFP-$G\alpha_s$ creates a unique problem relative to eGFP. Expression levels of eGFP-$G\alpha_s$ and eGFP are similar within a cell, but the pattern of distribution varies considerably. eGFP-$G\alpha_s$ is highly concentrated in small, constrained lipid rafts whereas eGFP can diffuse freely throughout the vast cytoplasm. As a result, the otherwise weak tendency of eGFP to oligomerize becomes very significant for eGFP-$G\alpha_s$ and over time large aggregates create a strong selective pressure against cells expressing eGFP-$G\alpha_s$. In contrast, a less-oligomeric variant, monomeric GFP-$G\alpha_s$, maintains normal cellular trafficking throughout dozens of cell generations.

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Stable expression of this new monomeric GFP-Gα$_s$ was then used to create a highly reproducible FRAP assay to study Gα$_s$ diffusion. In particular, it was observed that speed and extent of GFP-Gα$_s$ recovery are related to its cellular localization. Cytosolic GFP-Gα$_s$ was found to move faster than the wild-type plasma membrane anchored version. Diffusion was also explored in relation to GFP-Gα$_s$ association with lipid rafts. Antidepressant treatment and lipid raft disruption both translocate Gα$_s$ out of lipid rafts and increase its coupling with adenylyl cyclase. Here it is demonstrated that these treatments also significantly reduce Gα$_s$ diffusion. Transmembrane proteins like β-adrenergic receptor diffuse slowly relative to peripheral membrane associated proteins like Gα$_s$ suggesting that these treatments cause slower Gα$_s$ diffusion due to increased association with transmembrane proteins.

Interestingly, treatment with fatty acids alter Gα$_s$ raft localization too, but did not change FRAP measurements. Regardless, chronic treatment with n-3 PUFAs (DHA or EPA) and n-6 PUFAs (linoleic acid or arachidonic acid) translocate Gα$_s$ out of lipid rafts similar to antidepressants. Furthermore, this effect is additive when co-treated with antidepressants, suggesting a different locus of action. Similar treatment with the unsaturated stearic acid however, increased Gα$_s$ raft association. Since none of these treatments altered FRAP, FRAP may be a better measurement of Gα$_s$-AC coupling than lipid raft association.

Finally, investigation of antidepressant incorporation into model membranes suggests membrane effects may at least partially mediate antidepressant regulation of $G\alpha_s$ signaling. Accumulation of escitalopram and imipramine into model membranes induced changes in DOPC/DPPC miscibility similar to the addition of cholesterol. Preliminary analysis of these data imply that these drugs may be increasing the fluidity between these lipid species, perhaps allowing greater accessibility of $G\alpha_s$ to important effectors.

7.2 Challenges and the future of studying lipid rafts with fluorescent proteins

Advances in light microscopy (nanoscopy) are bringing the direct visualization of membrane nanodomains and their associated proteins to a reality. Fluorescence photoactivation localization microscopy in particular holds promise to better elucidate nano-scale clustering of raft-associated proteins. Future experiments are likely to finally demonstrate the concept of lipid rafts is more than an artifact of cellular fractionation.

Unfortunately, the permeation of nanoscopy into mainstream biology will be a slow transition. Complex algorithms for calculating high-resolution fluorescent protein localization are still being optimizing and far from practical for users outside the field. In addition, the catalogue of photoactivatable fluorophores necessary for fPALM is relatively meager compared to traditional FPs. Aside from PA-GFP and a

few others most are poorly characterized or exhibit the problems like oligomerization that plagued initial FPs(Chudakov et al. 2010). Fortunately both of these roadblocks are not only surmountable, but actively being investigated.

7.3 Lipid rafts as an evolving concept

Since their infancy lipid rafts have been a controversial and hence dynamic concept. The basic tenant of preferential association of similar lipids has always been difficult to refute since it boils down to simple chemistry. Instead the devil has always been in the details. Phase separation of lipids between solid, liquid-ordered and liquid-disorder phases is highly dependent on the system. From a practical stand-point, this is best observed in highly artificial, simple model systems rather than intact biological ones. Until recently, translating results from model systems back to biologic ones has been challenging.

Advances in FP imaging coupled with a general increased focus on lipid rafts in molecular biology is finally offering a glimpse of how rafts exist in living cells. Data presented here supports the concept that lipids are diverse, dynamic entities. Classifying different membrane structures as raft and non-raft is probably over simplistic for non-model systems. In live membranes, resident proteins also influence lipid organization. Therefore the countless permutations proteins and lipids species allow the potential for an equally infinite population of membrane domains.

7.4 Future development of antidepressants and understanding depression

The slow progress in the treatment of depression over the past forty years represents a fundamental inadequacy in understanding the related molecular biology of the brain. While modestly successful, the monoamine hypothesis for depression has failed to detail a comprehensive understanding of the disease and treatments derived from this theory are insufficient for too many patients. This suggests that depression is merely an umbrella term for a collection of common symptoms rather than a disease with a specific etiology.

Fortunately, mental health research is poised to enter a new era of exciting advancements that will help isolate the particular pathologies that manifest as depression. Bioinformatic studies including genomics, proteomics, lipidomics and metabolomics will generate an unprecedented wealth of information that can be correlated with mental health. The development of valid biomarkers for psychiatric disorders will put the field on par with most other branches of medicine where clinical labs can rapidly assess disease. Furthermore, truly valid animal models will be feasible.

Recently, truly novel classes of antidepressants have begun to emerge in basic science research. Research has moved away from exclusively studying monoamine transporters, branching out into numerous cellular pathways and receptors

including NMDA receptors, delta-opioid receptors, mGluR receptors, phosphodiesterases, and various monoamine receptors.

7.5 Conclusions

Together, data in this dissertation highlight the significant influence lipids have on G-protein signaling. While this work is focused on better understanding the molecular mechanisms that underlie antidepressant action and the etiology of depression itself, an improved understanding of G-protein signaling is valuable to almost every niche of biology. FRAP experiments complemented by SANS data and lipid raft extractions present a diverse medley of perspective to our understanding of G-protein regulation.

APPENDIX

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