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Lateral Diffusion of $G\alpha_s$ in the Plasma Membrane Is Decreased after Chronic but not Acute Antidepressant Treatment: Role of Lipid Raft and Non-Raft Membrane Microdomains

Andrew H Czysz[1], Jeffrey M Schappi[2] and Mark M Rasenick*[,1,2,3,4]

[1]Graduate Program in Neuroscience, University of Illinois at Chicago, College of Medicine, Chicago, IL, USA; [2]Departments of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA; [3]Departments of Psychiatry, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA; [4]Jesse Brown Westside VA Medical Center, Chicago, IL, USA

GPCR signaling is modified both in major depressive disorder and by chronic antidepressant treatment. Endogenous $G\alpha_s$ redistributes from raft- to nonraft-membrane fractions after chronic antidepressant treatment. Modification of G protein anchoring may participate in this process. Regulation of $G\alpha_s$ signaling by antidepressants was studied using fluorescence recovery after photobleaching (FRAP) of GFP-$G\alpha_s$. Here we find that extended antidepressant treatment both increases the half-time of maximum recovery of GFP-$G\alpha_s$ and decreases the extent of recovery. Furthermore, this effect parallels the movement of $G\alpha_s$ out of lipid rafts as determined by cold detergent membrane extraction with respect to both dose and duration of drug treatment. This effect was observed for several classes of compounds with antidepressant activity, whereas closely related molecules lacking antidepressant activity (eg, *R*-citalopram) did not produce the effect. These results are consistent with previously observed antidepressant-induced translocation of $G\alpha_s$, but also suggest an alternate membrane attachment site for this G protein. Furthermore, FRAP analysis provides the possibility of a relatively high-throughput screening tool for compounds with putative antidepressant activity.
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INTRODUCTION

Most antidepressants in current clinical use have the ability to block uptake or catabolism of monoamine neurotransmitters. Unfortunately, these sites of action have failed to account for the slow onset of clinical antidepressant efficacy. One common downstream site of action for these drugs is the cAMP generating system, and cAMP has been implicated both in depression and antidepressant response (Fujita *et al*, 2012; Malberg and Blendy, 2005; O'Donnell and Xu, 2012). G protein coupled receptors (GPCRs) and their attendant G proteins and effectors, such as adenylyl cyclase, are the 'first responders' in cAMP generation. Organization and accessibility of G proteins to receptors and effectors are thought to be important means of their regulation (Allen *et al*, 2007). Indeed, previous work suggests that $G\alpha_s$ signaling is dampened when $G\alpha_s$ is localized to lipid rafts (Chen and Rasenick, 1995b). Three to five days of antidepressant treatment alters this association, decreasing $G\alpha_s$ raft content and increasing cAMP signaling (Allen *et al*, 2007; Chen and Rasenick, 1995a). Currently, it is unclear by what mechanism these drugs affect G protein signaling as the presence of serotonin transporters (SERTs) is not necessary for these actions (Zhang and Rasenick, 2010).

A better understanding of this mechanism requires investigation of the nature of G protein association with lipid rafts and other membrane structures. 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 or alkaline extractions. Although these are the traditional means to study raft association, there has been some progress studying raft association using microscopy under more physiologic conditions. These include super-resolution microscopy techniques like photo-activated localization microscopy and stochastic optical reconstruction microscopy, as well as older techniques such as fluorescence recovery after photobleaching (FRAP) that utilize confocal microscopy. The latter does not actually visualize protein clustering in microdomains, but instead measures protein diffusion over a larger area. The speed of diffusion, as measured by FRAP, is dependent on a number of factors, such as the size of the molecule in question, as well as such potentially limiting factors as protein–protein interaction, or interaction between protein and cytoskeleton (Lenne *et al*, 2006; Reits and Neefjes, 2001).

To investigate $G\alpha_s$ mobility subsequent to antidepressant treatment, we utilized a fluorescent GFP-$G\alpha_s$ fusion protein

*Correspondence: Dr MM Rasenick, Biomedical Neuroscience Training Program, Department of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Avenue M/C 901, Room E202, Chicago, IL 60612-7342, USA, Tel: +312 996 6641, Fax: +312 996 1414, E-mail raz@uic.edu
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(Yu and Rasenick, 2002). 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 antidepressant treatments that alter Gα_s raft association and cAMP signaling. Curiously, translocation of Gα_s from rafts retards Gα_s mobility, suggesting that the increased association between Gα_s and adenylyl cyclase evoked by these treatments results in alternate membrane anchoring of Gα_s. Regardless, the consistency of these effects and the specificity for compounds with antidepressant activity suggest a cellular platform for efficient screening of novel compounds with putative antidepressant activity.

MATERIALS AND METHODS

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 5% CO_2 atmosphere. The cells were treated with 10 μM drug for 3 days or as otherwise specified. The culture media and drug were changed daily. There was no change in the morphology of cells during the period of treatment.

Escitalopram and *R*-citalopram were gifts from Lundbeck, Copenhagen. Venlafaxine and sertraline were gifts from Pfizer. Desipramine hydrochloride, reserpine, tianeptine sodium salt, amphetamine sulfate, diazepam, haloperidol, olanzapine, and bupropion hydrochloride were purchased from Tocris Bioscience, Ellisville, MO. Chlorpromazine hydrochloride, phenelzine sulfate, imipramine hydrochloride, colchicine, MβCD, and 2-bromopalmitate were purchased from Sigma-Aldrich, St Louis, MO.

Expression Plasmids

A206K GFP-Gα_s was constructed with Stratagene QuikChange mutagenesis using previously described GFP-Gα_s as a template (Yu and Rasenick, 2002) and primers described elsewhere (Zacharias *et al*, 2002). This point mutation in GFP was utilized to create a monomeric GFP with improved membrane expression. 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. GFP-AC8 was a kind gift from Dermot Cooper, University of Cambridge, England.

Transfection and Generation of Stable Cell Lines

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

Fluorescence Recovery after Photobleaching

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 h before microscopy for chronic treatments. The media were 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 512×512 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 300 ms 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 using Zeiss Zen software.

Statistical Analysis

All of the experiments were performed at least three times. Data were analyzed for statistical significance using one-way ANOVA followed by Tukey's test for *post hoc* multiple comparisons of means. Values of $p < 0.05$ were taken to indicate significance.

RESULTS

GFP-Gα_s Diffusion Is Altered in Response to Extended Antidepressant Treatment

Gα_s raft association and Gα_s-adenylyl cyclase coupling are sensitive to treatment (3–5 days) with a variety of antidepressants, including SSRIs, tricyclic (TCAs), and monoamine oxidase inhibitors (Chen and Rasenick, 1995b; Toki *et al*, 1999). To test whether membrane diffusion of Gα_s is also affected, we treated C6 glioma cells, stably transfected with GFP-Gα_s, with escitalopram, desipramine, or fluoxetine. GFP-Gα_s membrane dynamics were then assayed by FRAP. Representative membrane photobleaching and recovery are demonstrated in Figures 1a and b. Relative to control, cells treated with antidepressant for 3 days all demonstrated a significant increase in half-time to maximal recovery (Figure 1c), as well as a decrease in total extent of recovery (Supplementary Figures 1A and 2), as shown by an increase in immobile fraction percentage.

In contrast, FRAP measurements were unchanged in cells treated for only 1 h with these compounds (Figure 1d, Supplementary Figure 1B). Additional treatments of 24 and 48 h reveal a minimum treatment period of 24 h to develop a significant change in half-time (Figure 2b) to recovery.

R-Citalopram does not Alter GFP-Gα_s Diffusion

Although the presumptive target of SSRIs is SERT, membrane redistribution of Gα_s and augmentation of cAMP

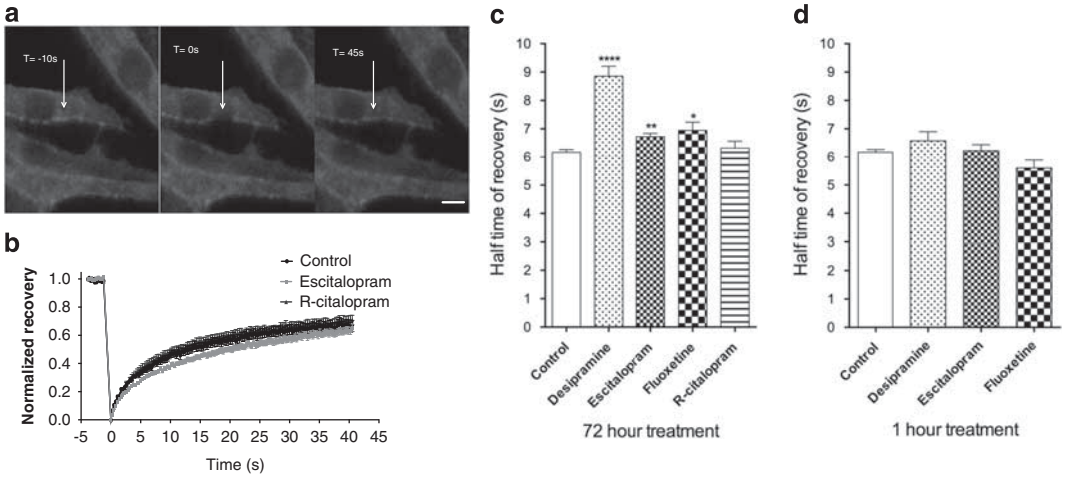

Figure 1 GFP-Gα_s recovery after photobleaching is slower after chronic but not acute antidepressant treatment. C6 glioma cells stably expressing GFP-Gα_s were cultured in phenol-red-free DMEM and membrane regions were photobleached. (a) Demonstration of representative photobleaching and recovery of GFP-Gα_s. Scale bar represents 10 mm. (b) Demonstration of time course of recovery after photobleaching of control and 10 μM escitalopram or R-citalopram (72 h) -treated cells. Half-time to recovery of GFP-Gα_s is increased after (c) chronic (72 h) but not (d) acute (1 h) escitalopram, desipramine, and fluoxetine treatments at 10 μM. Chronic (72 h) R-citalopram had no effect on half-time of recovery. Data were analyzed by one-way ANOVA followed by Tukey's test for *post hoc* multiple comparisons of means (control *vs* treatment, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$). Error bars represent SEM.

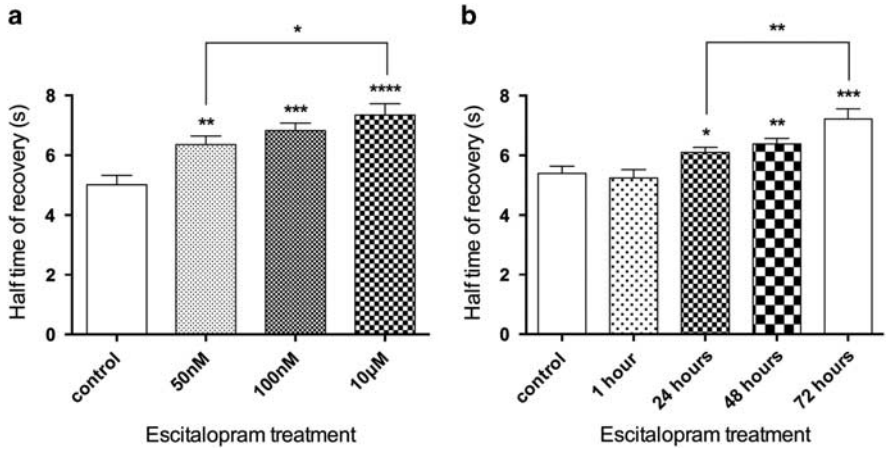

Figure 2 Escitalopram effect on GFP-Gα_s diffusion is both dose- and time-dependent. (a) C6 cells stably expressing GFP-Gα_s were cultured for 3 days at various doses of escitalopram before imaging. (b) C6 cells stably expressing GFP-Gα_s were cultured for 3 days with escitalopram treatment (10 μM) initiated in the final 1, 24, 48, or 72 h of culture before imaging. FRAP was performed on 3–6 cells per dish and the half-time to recovery was calculated using a one-phase association fit. Data were analyzed by one-way ANOVA followed by Tukey's 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.

signaling occurs in cells lacking SERT, such as C6 glioma (Zhang and Rasenick, 2010). Citalopram exists as a racemic mixture of *R*-and *S*-citalopram (escitalopram), with only the *S*-isomer escitalopram demonstrating clinical antidepressant efficacy (Sánchez *et al*, 2003). Although escitalopram treatment resulted in the redistribution of Gα_s from lipid rafts with an according increase in FRAP recovery half-time, treatment with *R*-citalopram did not affect GFP-Gα_s recovery after photobleaching (Figure 1c, Supplementary Figure 1A). This finding is also consistent with previous data demonstrating a lack of change in Gα_s membrane disposition following C6 glioma treatment with *R*-citalopram (Zhang and Rasenick, 2010), suggesting the existence of additional and stereoselective binding sites for escitalopram and other antidepressants.

Multiple Classes of Antidepressants Decrease GFP-Gα_s Diffusion: Other Psychotropic Drugs do not have this Effect

Antidepressants belonging to the monoamine oxidase inhibitor, TCA, and SSRI families have all previously been shown to cause a redistribution of Gα_s and augment cAMP signaling (Donati and Rasenick, 2003). Consistent with

Table 1 All Tested Classes of Antidepressants Affect GFP-Gα_s Half-Time to Recovery

Treatment	$t_{1/2}$	SEM	*n*	*P*
Control	6.06	0.095	303	—
Escitalopram	6.76	0.118	213	<0.001
R-citalopram	6.32	0.245	66	0.507
Desipramine	8.86	0.347	32	<0.0001
Fluoxetine	6.95	0.294	31	<0.01
Bupropion	8.04	0.441	24	<0.0001
Phenelzine	7.04	0.308	23	<0.01
Imipramine	7.36	0.373	24	<0.001
Venlafaxine	6.96	0.371	27	<0.05
Sertraline	10.2	0.471	24	<0.0001
Tianeptine	7.07	0.311	17	<0.05
D-amphetamine	5.81	0.245	59	0.276
Haloperidol	5.92	0.231	68	0.507
Olanzapine	5.83	0.212	84	0.273
Diazepam	5.71	0.352	28	0.655

FRAP experiments were performed as described but with various additional antidepressants. All classes of antidepressant increased the half-time to recovery, although the magnitude of this effect varied among drugs, rather than classes. *R*-citalopram had no effect on the membrane mobility of GFP-Gα_s. Psychotropics from a variety of classes including stimulants, antipsychotics, and anxiolytics did not alter GFP-Gα_s FRAP recovery time.

these data, chronic treatments with numerous drugs from these families show significant increases in FRAP recovery half-time, and trend higher immobile fractions (Table 1, Supplementary Table 1). In addition, venlafaxine, a serotonin/norepinephrine reuptake inhibitor, as well as atypical antidepressants (eg, bupropion, and tianeptine) all demonstrated similar effects in retarding membrane mobility of Gα_s as demonstrated by increasing half-time of fluorescence recovery.

Although all antidepressants tested increased the mobility of GFP-Gα_s, a number of other psychoactive drugs were without effect. Amphetamine (a monoamine transporter antagonist), the antipsychotics haloperidol and olanzapine, and benzodiazepine anxiolytic, diazepam, did not alter GFP-Gα_s FRAP (Table 1).

Altered GFP-Gα_s Diffusion Is Antidepressant Treatment Time- and dose-Dependent

Our laboratory study has previously demonstrated that antidepressant-induced redistribution of Gα_s from lipid rafts to nonraft membrane fractions is time- and dose-dependent (Zhang and Rasenick, 2010). To assess the effect of antidepressant dosage on GFP-Gα_s FRAP recovery time, we measured changes in GFP-Gα_s FRAP after chronic treatment with a range of escitalopram concentrations. The calculated half-time showed a trend similar to dose-dependent changes in Gα_s raft content (Figure 2a). Specifically, treatment with increasing concentrations of escitalopram increasingly slowed recovery. Concentrations of escitalopram greater than 10 μM did not demonstrate further slowed recovery, but did demonstrate significantly greater immobile fraction and rounded cell morphology (data not shown). These data agree with our past observations regarding Gα_s distribution following antidepressant treatment with respect to treatment time and dosage. Notably, the effect of antidepressant treatment on FRAP recovery is detectable at lower antidepressant concentrations than those used previously in studies of detergent-extracted membranes, presumably due to the increased sensitivity of the FRAP technique. A time-course study also revealed at least 24 h of drug treatment (10 μM) is necessary for an effect, with a progressive increase in FRAP recovery half-time from 24 to 72 h (Figure 2b), consistent with our past studies of Gα_s redistribution upon antidepressant treatment (Zhang and Rasenick, 2010). The observed effect is more likely related to duration of treatment rather than cumulative dose of drug. Small doses of escitalopram (50 nM) demonstrate effect at 3 days of treatment, but larger doses (10 μM) at 1 h do not. Although both dose and time course studies showed significant increases compared with controls at each dose and time point (expect for 1 h treatment), and demonstrated an increasing trend in each study, only the treatment extremes (ie, 50 nM *vs* 10 μM dose, and 24 *vs* 72 h treatment) separated statistically ($p < 0.05$).

Lipid Raft Disruption also Decreases GFP-Gα_s Diffusion

Similar to antidepressants, cholesterol chelation and microtubule disruption liberate Gα_s from lipid rafts (Allen *et al*, 2007; Head *et al*, 2006). In the former case, lipid raft integrity requires cholesterol; in the latter, it appears that tubulin structures are involved in the membrane/raft anchoring of Gα_s (Schappi *et al*, 2014). Therefore, we hypothesized that, if rafts constrain Gα_s diffusion, raft disruption or microtubule-disrupting agents would also increase half-time of GFP-Gα_s FRAP. Indeed, data from FRAP experiments show a consistent effect with both raft and microtubule-disrupting agents and antidepressant treatment (both manipulations cause Gα_s to translocate from lipid rafts (Allen *et al*, 2009; Head *et al*, 2006)), and as is the case with chronic antidepressant treatment, result in a decrease in the speed of recovery (Figure 3). Given that raft disruption increases the mobility of a number of membrane proteins, the retardation of Gα_s mobility is counterintuitive.

Antidepressant Translocation of G Proteins Is Specific to Gα_s

GFP-Gα_s diffusion as measured by FRAP was also compared with the diffusion of several other fluorescent proteins with varied plasma membrane attachment. GFP-Gα_{i1}, which utilizes palmoyl- and myristoyl-lipid anchors, demonstrates similar diffusion properties to the singly palmitoylated GFP-Gα_s. Although raft disruption and microtubule-disrupting drugs also retard Gα_{i1} mobility, it is noteworthy that chronic antidepressant treatment has no effect on GFP-Gα_{i1} FRAP (Figure 4a). The specificity of this effect for Gα_s is also consistent with our past data showing redistribution of Gα_s, but not Gα_{i1}, from detergent-extracted lipid rafts of antidepressant-treated C6 membranes or rat brain (where 3 weeks of antidepressant treatment are required; Toki *et al*, 1999).

Protein Mobility Is Dependent on Cellular Anchors

GFP-β-adrenergic receptor and GFP-adenylyl cyclase 8 (GFP-AC8), both large multi-pass transmembrane proteins, had significantly slower half-time and larger immobile fractions than GFP-Gα_s. Conversely, GFP, which is largely cytosolic, demonstrates very fast diffusion (Figure 4b). Likewise, a palmitoylation-deficient GFP-Gα_s, which is also primarily cytosolic, also has a relatively fast half-time and small immobile fractions. Treatment with competitive inhibitor of palmitoylation (2-bromopalmitate) and GPCR/G protein activation with isoproterenol and subsequent internalization, both of which increase cytosolic Gα_s, similarly speed FRAP recovery (Figure 4c).

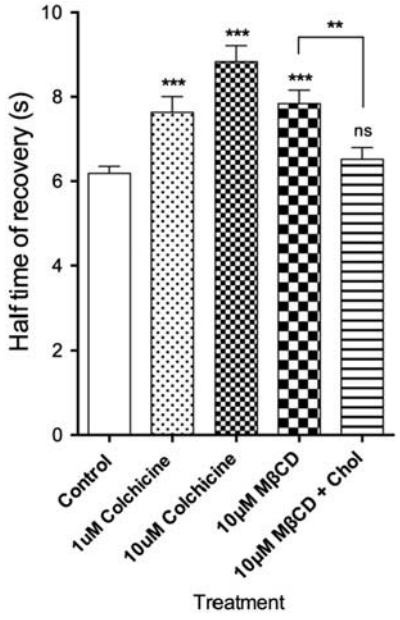

Figure 3 Lipid raft disruption alters GFP-Gα_s FRAP. Lipid raft disruption by cholesterol chelation with methyl-b-cyclodextrin, or by colchicine treatment, increased the recovery half-time of GFP-Gα_s after FRAP. The effect of cholesterol chelation was partially reversed by reintroducing cholesterol after chelation. Data were analyzed by one-way ANOVA followed by Tukey's test for *post hoc* multiple comparisons of means (control *versus* treatment, $*p<0.05$, $**p<0.01$, $***p<0.001$). Error bars represent SEM.

DISCUSSION

This work was undertaken in an attempt to determine some of the factors for the hysteresis between initiation of antidepressant treatment and antidepressant response. The work from this laboratory on lipid raft and G protein signaling, and the suggestion that antidepressants concentrate in lipid rafts (Eisensamer *et al*, 2005) combine to suggest that antidepressants translocate Gα_s from lipid rafts and, in doing so, alter the dynamic properties of that protein within the plasma membrane.

Lipid rafts remain a difficult concept to investigate, requiring multiple complementary approaches. Previous studies suggest that increased Gα_s association with adenylyl cyclase may underlie antidepressant regulation of cAMP (Chen and Rasenick, 1995a, 1995b; Menkes *et al*, 1983; Ozawa and Rasenick, 1991). Furthermore, translocation of Gα_s to non-raft membrane fractions following raft disruption results in increased coupling to adenylyl cyclase (Allen *et al*, 2009), and this is unique to Gα_s (Allen *et al*, 2009; Head *et al*, 2006; Rybin *et al*, 2000). Those earlier experiments relied on lipid raft preparations from lysed tissue and cells rather than intact, living cells. Here we have studied Gα_s diffusion under a variety of raft-altering conditions, including antidepressant treatment. Our

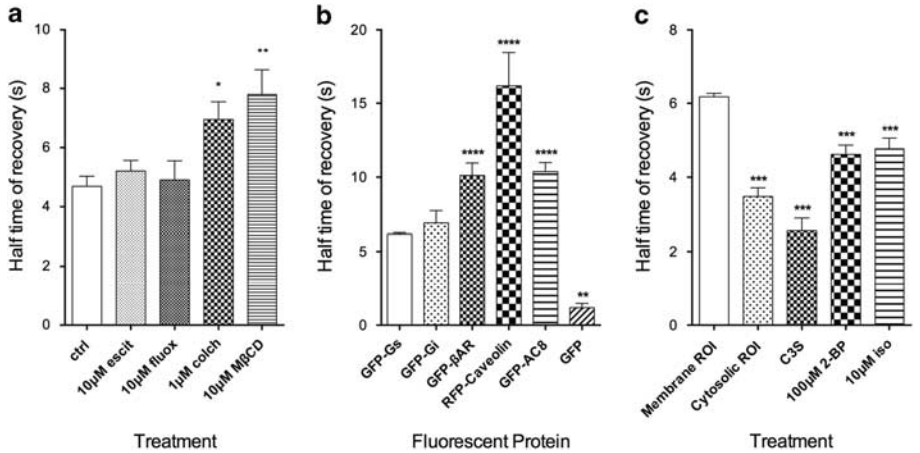

Figure 4 Diffusion of fluorescent proteins is dependent on their cellular scaffolds or relation with membrane environment. (a) GFP-Ga$_{i1}$ was stably expressed in a C6 glioma cell line and FRAP was used to assess the mobility of GFP-Ga$_{i1}$ after antidepressant treatment. Half-time to recovery of GFP-Ga$_{i1}$ is unaffected after chronic (3-day) escitalopram and fluoxetine treatments. Colchicine and methyl-b-cyclodextrin are presented as positive controls of cytoskeletal and membrane disruption on G protein distribution. (b) C6 glioma were transiently transfected with various fluorescent fusion proteins and FRAP was performed 24 h after transfection. Half-time of recovery was faster for peripheral membrane and cytosolic proteins relative to transmembrane proteins. (c) FRAP was performed on cells expressing GFP-Gα_s under a variety of conditions that alter Gα_s membrane association. Cytosolic GFP-Gα_s, whether 'normal' or resulting from a mutation (C3S) that blocks palmitoylation (and subsequently, membrane attachment) shows significantly faster half-time to recovery. Furthermore, agents that remove Gα_s from membrane, either by blocking palmitoylation (2-bromopalmitate) or by activation and subsequent internalization (isoproterenol) also enhance FRAP recovery. Data were analyzed by one-way ANOVA followed by Tukey's test for *post hoc* multiple comparisons of means (control *versus* treatment, $*p<0.05$, $**p<0.01$, $***p<0.001$). Error bars represent SEM.

findings show treatments that translocate $G\alpha_s$ from raft to non-raft membrane domains also retard mobility of $G\alpha_s$, as measured by FRAP.

Changes in FRAP measurement subsequent to antidepressant treatment closely match, in dose-dependence and time course, antidepressant-induced changes in cAMP production and $G\alpha_s$ raft localization (Table 1, Zhang and Rasenick, 2010). Antidepressant-induced changes in $G\alpha_s$ signaling require several weeks in animal models (Ozawa and Rasenick, 1991) or several days in cells (Donati and Rasenick, 2005), which is also reflected in the decreased GFP-$G\alpha_s$ mobility seen with FRAP (Figure 2b).

The initial results of these studies were contrary to expectations, as it was anticipated that the translocation of GFP-$G\alpha_s$ from lipid rafts would increase mobility of that protein. The opposite was seen. Adenylyl cyclase has 12 membrane spans and has been reported to have 'scaffolding' or 'anchoring' properties (Dessauer, 2009). The slow recovery seen with transmembrane proteins such as the β-adrenergic receptor and scaffolding proteins like caveolin-1 are consistent with this. Previous experiments have demonstrated increased co-immunoprecipitation of $G\alpha_s$ and adenylyl cyclase after tricyclic antidepressant and electroconvulsive treatment in rat cerebral cortex (Chen and Rasenick, 1995b). Given the increased association between $G\alpha_s$ and adenylyl cyclase after antidepressant treatment (Chen and Rasenick, 1995b; Ozawa and Rasenick, 1989; Donati and Rasenick, 2005), the antidepressant-induced retardation of $G\alpha_s$ FRAP is likely a result of increased association with the relatively slow moving adenylyl cyclase.

We and others had previously observed that lipid raft disruption increased the physical and functional interaction between $G\alpha_s$ and adenylyl cyclase. This was observed with chronic antidepressant treatment (Chen and Rasenick, 1995a; Zhang and Rasenick, 2010) as well as with cholesterol chelation by methyl-β-cyclodextrin (Allen *et al*, 2009; Head *et al*, 2006; Rybin *et al*, 2000) or with caveolin depletion (Allen *et al*, 2009). It is noteworthy, however, that although raft disruption has similar effects on GFP-$G\alpha_s$ and GFP-$G\alpha_{i1}$, chronic antidepressant treatment affects only $G\alpha_s$ (Figure 4a).

The observed antidepressant effects are quite specific, as only the *S*-enantiomer of citalopram demonstrates this effect (Figure 2a). Again, this matches the enantiomeric specificity of escitalopram on cAMP production and $G\alpha_s$ raft localization (Zhang and Rasenick, 2010). The selectivity of antidepressant effect on GFP-$G\alpha_s$ *vs* GFP-$G\alpha_{i1}$ suggests that this effect is specific for $G\alpha_s$ and/or its membrane and cytoskeletal anchors, rather than an effect on G proteins in general. These findings also lead us to suspect a transporter-independent site (an additional site?) of action for antidepressants (both those shown to inhibit uptake as well as atypical drugs), as C6 glioma lack SERT and other monoamine reuptake transporters (Bhatnagar *et al*, 2004).

We also explored the FRAP assay response to other modulators of $G\alpha_s$ signaling. Lipid raft disruption by methyl-β-cyclodextrin has been previously shown to increase $G\alpha_s$-adenylyl cyclase coupling (Donati and Rasenick, 2005a) and also induces a slower and less mobile recovery of GFP-$G\alpha_s$ after photobleaching as demonstrated here. The same is true for colchicine treatment, which disrupts $G\alpha_s$ anchoring to tubulin, releasing $G\alpha_s$ from rafts (Donati and Rasenick, 2005b; Rasenick, 1986; Rasenick and Wang, 1988; Rasenick *et al*, 2004).

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 that the difference in diffusion speed in raft and non-raft domains may be responsible for changes in GFP-$G\alpha_s$ recovery, but this conclusion runs counter to the concept that rafts are rigid, highly ordered domains where slow diffusion would be expected. Instead, we found that outside of lipid rafts, GFP-$G\alpha_s$ mobility was retarded. We suspect that altered protein scaffolding may play a significant role in this effect. Other groups have shown through methyl-β-cyclodextrin treatment that cholesterol chelation restricts diffusion of a variety of raft and non-raft membrane-associated fluorescent proteins. 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-β-AR and GFP-AC8 were significantly slower than peripheral membrane proteins GFP-$G\alpha_s$ and GFP-$G\alpha_{i1}$ (Figures 4b and c). Note that the translocation of $G\alpha_s$ from rafts alone does not explain the retarded diffusion seen after antidepressant treatment, as the palmitoylation deficient, nonmembrane-associated GFP-$G\alpha_s$ mutant C3S shows much faster mobility than GFP-$G\alpha_s$, either before or after antidepressant treatment.

Curiously, the effect size of FRAP response varies considerably between antidepressants despite similar drug concentration and clinical efficacy among compounds (Anderson, 2000). This difference was not previously noted in assays of cAMP production or $G\alpha_s$ raft localization (Ozawa and Rasenick, 1989; Donati and Rasenick, 2005), and is perhaps revealed now because of the increased sensitivity and greater sample sizes afforded by the higher-throughput FRAP assay. It is noteworthy in this study that the heterogeneity of effect does not depend on drug class (TCA, SSRI, etc), and is variable within classes. As we suggest that the translocation of $G\alpha_s$ from lipid rafts is independent of reuptake transporter, this finding is not surprising. Metabolism of these drugs is not strictly related to class type, and may explain some of these findings, especially given that effect size is dose dependent (Caccia, 1998).

Amphetamine, which inhibits monoamine reuptake but lacks clinically useful antidepressant activity, does not demonstrate this effect on GFP-$G\alpha_s$ FRAP recovery. Or do haloperidol and olanzapine, antipsychotics of different chemical classes, or the benzodiazepine, diazepam.

Note that this study has not attempted to evaluate putative antidepressant compounds acting on the glutamate system. These compounds may have both pre- and post-synaptic effects (Musazzi *et al*, 2013) and depending on the compound, may show extremely rapid effects (Krystal *et al*, 2013). These will be the subjects of a future study.

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 (Figures 5a and b). Consistent with this, diffusion of GFP-$G\alpha_s$ appearing in the cytosol

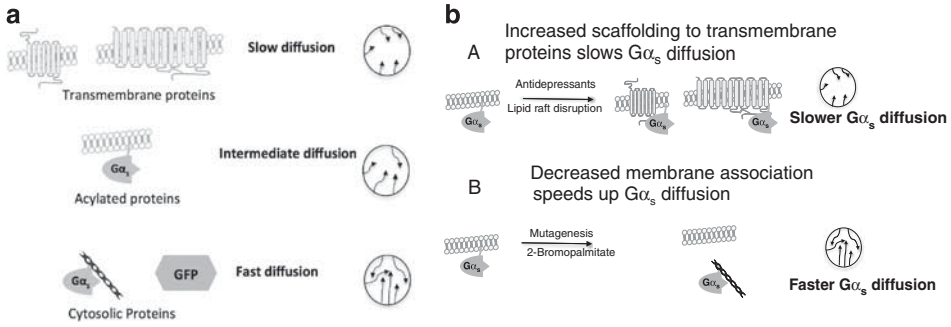

Figure 5 A model for protein diffusion and changes in GFP-Gα_s mobility. (a) Protein diffusion is mediated by its cellular scaffolding. In particular, FRAP reveals that the mechanism of protein anchoring to plasma membranes is predictive of the speed at which it diffuses. Cytosolic proteins diffuse rapidly, especially GFP, which has no natural interacting partners in the cell. Acylated proteins associated with the plasma membrane inner bilayer diffuse slower, and transmembrane proteins show the slowest diffusion. (b) Antidepressant treatment and lipid raft disruption increase GFP-Gα_s coupling to transmembrane scaffolds, which slows GFP-Gα_s diffusion. In contrast, cytosolic GFP-Gα_s diffuses relatively fast.

recovers much faster than that in the membrane. This was also confirmed using the exclusively cytosolic, palmitoylation-deficient, GFP-Gα_s mutant C3S, and with cells treated with 2-bromopalmitate, a palmitoylation inhibitor (Figure 4c). These cytosolic Gα_s have significantly less scaffolds than their membrane-associated counterparts, which is why we suspect they are able to diffuse at greater speeds. Not surprisingly, they diffuse more slowly still than un-fused GFP, as cytosolic Gα_s still has some associations, such as tubulin (Schappi *et al*, 2014; Yu *et al*, 2009).

Others have noted altered membrane distribution of additional proteins involved in signaling, such as SERT and 5HT-2A, both in depression and in response to antidepressant treatment (Rivera-Baltanas *et al*, 2014). As with Gα_s, these changes likely reflect alterations in membrane anchoring, whether protein–protein, protein–cytoskeleton, or both, and the ability of antidepressants to modify these parameters.

A commonly cited function of lipid rafts is to organize and scaffold signaling pathways in close proximity to foster efficient signaling (Allen *et al*, 2007). Gα_s signaling is thought to act the opposite, experiencing more potent transduction out of rafts (Allen *et al*, 2009). In this sense, it is not surprising that raft-associated Gα_s would diffuse faster than non-raft Gα_s and further suggests that generalization concerning the roles of raft and non-raft membrane domains is problematic. Rafts are a descriptive concept that generalize a variety of membrane and protein scaffolds. The precise site at which antidepressants modify this process is still under investigation, but the ability to see these actions in cells devoid of monoamine transporters raises the possibility of a locus of antidepressant action at an additional membrane domain. The raft association of these drugs may be important for their effects, and this may be evidenced either by modifying anchoring sites for Gα_s or by modification of the components to which Gα_s binds, either in raft or non-raft membrane fractions.

Finally, it is noteworthy that the retardation of FRAP subsequent to 3-day treatment of cells was a consistent hallmark of compounds with antidepressant properties. This suggests that lateral diffusion of GFP-Gα_s, as measured by FRAP, is a reliable indicator that can be used to identify novel antidepressant compounds.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)