JBC Papers in Press. Published on July 18, 2016 as Manuscript M116.727263 The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M116.727263 Antidepressants and Lipid Rafts

Antidepressants Accumulate in Lipid Rafts Independent of Monoamine Transporters to Modulate Redistribution of the G protein, $G\alpha_s$

Running title: Antidepressants and Lipid Rafts

Samuel J. Erb^{*}, Jeffrey M. Schappi[‡], and Mark M. Rasenick^{*‡§}

^{*}Department of Biopharmaceutical Sciences, [‡]Department of Physiology and Biophysics, and [§]Department of Psychiatry, University of Illinois at Chicago, Chicago, Illinois 60612.

Corresponding author:

Mark M. Rasenick Phone: (312) 996-6641 Fax: (312) 996-1414 raz@uic.edu

Key words: cyclic AMP (cAMP), depression, drug action, G protein, G protein-coupled receptor (GPCR), gas chromatography-mass spectrometry (GC-MS), glia, heterotrimeric G protein, intracellular trafficking, lipid, lipid raft, mass spectrometry (MS), membrane trafficking, monoamine transporter, plasma membrane, protein-drug interaction, protein translocation, protein-lipid interaction, selective serotonin reuptake inhibitor (SSRI), serotonin, serotonin transporter

ABSTRACT

Depression is a significant public health problem for which currently available medications, if effective, require weeks to months of treatment before patients respond. Previous studies have shown that the G protein responsible for increasing cAMP ($G\alpha_s$) is increasingly localized to lipid rafts in depressed subjects and that chronic antidepressant treatment translocates $G\alpha_s$ from lipid rafts. Translocation of $G\alpha_s$, which shows delayed onset after chronic antidepressant treatment of rats or of C6 glioma cells, tracks with the delayed onset of therapeutic action of antidepressants. Since antidepressants appear to specifically modify $G\alpha_s$ localized to lipid rafts, we sought to determine whether structurally diverse antidepressants, accumulate in lipid rafts. Sustained treatment of C6 glioma cells, which transporters. showed lack 5HT marked concentration of several antidepressants in raft fractions, as revealed by increased absorbance and by mass fingerprint. Closely related molecules antidepressant activity, without did not concentrate in raft fractions. Thus, at least two classes of antidepressants accumulate in lipid rafts and effect translocation of $G\alpha_s$ to the non-raft membrane fraction where it activates the cAMPsignaling cascade. Analysis of the structural determinants of raft localization may both help to explain the hysteresis of antidepressant action and lead to design and development of novel substrates for depression therapeutics.

INTRODUCTION

Depression is the leading cause of long term disability in the industrialized world (1). Although depression is a significant health problem in the United States and antidepressants are heavily prescribed (2), the mechanism of action for these drugs is not understood. Further, nearly a third of those treated with these drugs do not achieve remission of their depression (3). Although most of these drugs do interfere with monoamine uptake or catabolism, they exert this effect within hours even thought most of the compounds require weeks before alleviation of symptoms is observed (4). Thus, other targets for antidepressant drugs may exist (4).

Chronic antidepressant treatment engages signaling pathways apart from increasing monoamine density in the synaptic cleft. One of these is an increased accumulation of cellular Downloaded from http://www.jbc.org/ by guest on October 6, 2020

cAMP and sequelae thereof such as increased CREB phosphorylation and increased transcription of cAMP-regulated genes (e.g. BDNF) (5). Moreover, positron emission tomography (PET) evidence suggests that cAMP is diminished throughout the brain of depressed human subjects (6). Thus, it is possible that some antidepressant effects are mediated through induction of the cAMP generating system, including $G\alpha_s$ and adenylyl cyclase (AC).

Previous studies demonstrated that chronic antidepressant treatment translocates $G\alpha_s$ from lipid rafts, whereupon it engages in a more facile activation of AC (7,8). Lipid rafts are regions of the plasma membrane rich in caveolin, cholesterol, sphingolipids, and cytoskeletal and GPI-anchored proteins (9,10) that allow the clustering or sequestration of signaling molecules (11). The rigid structure afforded by increased cholesterol content tightly coordinates saturated membrane lipids and acylated proteins. As many GPCRs are lipid raft localized and $G\alpha_s$ is palmitoylated, signaling through $G\alpha_s$ is impaired by lipid raft microdomains (12), presumably through inhibiting association(s) between raft and non-raft housed molecules (13,14).

Dampened signaling, through $G\alpha_s$ and/or $G\alpha_s$ coupled receptors, is consistent with the observed increase in $G\alpha_s$ association with rafts as well as lowered levels of cAMP seen in MDD Accordingly, $G\alpha_s$ content in non-raft (15). membrane domains increases after chronic treatment with fluoxetine, desipramine, and escitalopram (16) and cAMP is increased (17). Sustained activation of $G\alpha_s$ is also associated with increased microtubule dynamics and a resulting increase in neurite outgrowth (18, 19).Furthermore, lipid raft disruption displaces many raft proteins, but chronic antidepressant treatment displaces only $G\alpha_s$ (7). However, the precise biochemical mechanisms that account for the antidepressant mediated translocation of $G\alpha_s$ from lipid rafts was not well defined and presented a significant knowledge gap in our understanding of the complex pharmacology of antidepressants.

We hypothesized that antidepressants accumulate gradually in lipid rafts and modulate the distribution of $G\alpha_s$ in the membrane. Results in this study are consistent with that hypothesis and reveal novel binding domains for

antidepressants that may be consistent with delayed therapeutic response.

RESULTS

Gradual accumulation of antidepressant drugs in plasma membrane microdomains is independent of SERT.

While there are many potential targets for monoamine-centric drugs, none offer an explanation for the hysteresis (6-8 weeks) between initiation of therapy and clinical efficacy. C6 glioma cells were used in these experiments because they do not express monoamine transport proteins (Figure 1), yet sustained treatment with antidepressant drugs translocates $G\alpha_s$ from lipid rafts to non-raft regions of the plasma membrane (7,8,20). Furthermore, glia may contribute to both the etiology and treatment of depression (21,22).

One binding site for many of the antidepressant drugs (*e.g.* TCAs and SSRIs) is the serotonin reuptake transport protein (SERT). In order to determine whether SERT expression affects the redistribution of $G\alpha_s$, C6 cells stably expressing $G\alpha_s$ -GFP were engineered to also express SERT.

 $G\alpha_s$ -GFP is identical in its activation by GPCR and activation of adenylyl cyclase with wild type $G\alpha_s$ (23). When kept to a moderate level of expression (2-3X endogenous $G\alpha_s$), $G\alpha_s$ -GFP is transparent to cellular physiology, allowing a window on its movements in response to treatment with antidepressants ($G\alpha_s$ moves out of lipid rafts) (24).

 $G\alpha_s$ -GFP translocation from lipid rafts in response to escitalopram, as measured by FRAP, does not improve upon expression of hSERT (Figure 1B and 1C respectively). Moreover, HEK cells do not respond to chronic stimulation with escitalopram, nor when stably transfected with hSERT (Figure 1D and 1E respectively). These data suggest some additional cellular component is responsible for the redistribution of $G\alpha_s$ and it was hypothesized that the antidepressant drugs accumulate, gradually, in lipid rafts.

Gradual accumulation of antidepressant drugs in plasma membrane microdomains correlates with Ga_s subcellular redistribution. Due to our earlier observations that $G\alpha_s$ translocates from rafts after extended exposure to antidepressants (8), and the observations by Rupprecht and colleagues (25), we hypothesized that antidepressants preferentially associate with lipid rafts, and that all monoamine-centric antidepressants share this property.

We assessed the accumulation of representative drugs from each antidepressant class MAOI (phenelzine), TCA (desipramine, imipramine, and amitriptyline), and SSRI (escitalopram/inactive stereoisomer R-citalopram and fluoxetine), as well as the atypical antipsychotic aripiprazole, which has some independent antidepressant properties, and the atypical antipsychotic, olanzapine, which does not (26). We expected that antidepressants would gradually accumulate in raft fractions of C6 cells over time to mediate the translocation of G α_s out of the lipid raft and that the non-antidepressant compounds tested would not have this property.

Lipid raft fractions were isolated via sucrose density gradient and the UV-Vis spectrum taken for each fraction. Each antidepressant absorbs at a characteristic wavelength for which measurements were normalized to protein content in the sample and the fold change calculated relative to treatment naïve controls. The accumulation of drugs over time in lipid raft fractions of the plasma membrane appears to be a class specific mechanism (e.g. SSRIs and MAO inhibitors) (Figure 2A). Olanzapine, an antipsychotic lacking primary antidepressant properties (27) appears to accumulate as well, which may be due to its highly hydrophobic nature (Figure 2B), However, analysis of raft fractions by GC-MS revealed that accumulation of drugs in rafts is independent of hydrophobicity, as olanzapine and aripiprazole do not accumulate.

Escitalopram, and its therapeutically inactive enantiomer, *R*-citalopram, were selected for further investigation. To parallel the experiments by Eisensamer and colleagues (25), escitalopram was added to sucrose density gradients prepared from membrane fractions. Escitalopram, but not *R*-citalopram, associated with lipid raft fractions of the plasma membrane (Figure 2C). To minimize background measurements as much as possible for this method of detection, we normalized the readings to protein (280 nm) and subtracted the control absorbance. These measurements were then corroborated via mass spectrometry.

GC-MS is sensitive and selective, due in large part to the separation efficiency achieved in the analysis of small molecules. C6 cells were treated for 72 hrs with 10 µM of antidepressant and the lipid raft fraction extracted for determination of drug presence. Analysis of the total ion chromatograms (TIC) of lipid raft extractions, showed only phenelzine, fluoxetine, and escitalopram accumulated in lipid rafts after 72 hrs treatment (Figure 3); R-citalopram, imipramine, amitriptyline, aripiprazole, and olanzapine did not accumulate even though, with the exception of R-citalopram, each of these drugs show an "antidepressant signature" in translocating $G\alpha_s$ from lipid rafts. Base peaks of the molecular ion profile (MIP) from each TIC elution profile were matched to the NIST database to identify the drug presence among membrane fractions.

The accumulation of escitalopram, fluoxetine, and phenelzine, but not *R*-citalopram or olanzapine parallels their capacity to mediate movement of $G\alpha_s$ from lipid rafts (20). Since neither the tricyclic antidepressants (desipramine, imipramine, and amitriptyline) nor aripiprazole accumulate in rafts, this phenomenon may be class-specific for antidepressants. Furthermore, accumulation is independent of the lipophillicity of the compound (Figure 2B).

Gradual accumulation of escitalopram in plasma membrane microdomains is time and concentration dependent.

The finding that antidepressants and not other related drugs, along with the stereospecificity of antidepressant accumulation led us to select escitalopram for closer analysis.

Tracking the accumulation of escitalopram in the lipid raft fraction derived from C6 cells with GC-MS analysis revealed that escitalopram accumulates in a concentration and time dependent manner. Detectable accumulation occurred following 1 μ M treatment for 72 hrs or 100 nM treatments for 120 hrs and at 24, 48, and 72 hrs treatments with 10 μ M escitalopram (Figure 4). Treatment with 10 μ M antidepressant for 72 hrs is a standard assay condition (8) and

These measurements are consistent with drug time and dose required for $G\alpha_s$ translocation from rafts, measured either directly (8) or by FRAP (20). Moreover, stereo specificity and the fact that escitalopram does not accumulate over time in HEK cells indicates a specific target (Figure 4C). Furthermore, antidepressant mediated redistribution of $G\alpha_s$ independent of SERT suggests a molecular drug-binding target that is distinct from the monoamine transport system. Only a protein target(s) could account for both the enantioselectivity as well as the lack of hydrophobic contribution toward the gradual accumulation of SSRIs in lipid rafts.

DISCUSSION

 $G\alpha_s$ is a membrane-associated protein that inhabits cholesterol rich lipid raft micro-domains (15). Data presented above suggest that sustained treatment of cells with several antidepressants results in the accumulation of those compounds in lipid rafts and correlates with the membrane redistribution of $G\alpha_s$. Since the antidepressant drugs used in this study were not strongly hydrophobic, and escitalopram, but not *R*citalopram (equal lipophillicity), accumulates in lipid rafts, it is likely that an unidentified lipid raft protein is the binding site for antidepressants.

Lipid rafts contain many of the anchoring cytoskeletal-associated membrane structures and facilitate molecular association(s) of a vast array of different membrane-embedded and associated proteins to initiate intracellular signaling. While lipid rafts can facilitate this clustering of signaling molecules, the rigid structure afforded by increased cholesterol content appears to have a globally dampening effect on $G\alpha_s$ signaling by inhibiting association(s) between raft and non-raft based molecules (13). Dampened signaling, through $G\alpha_s$ and/or $G\alpha_s$ coupled receptors, is consistent with the observed increase in $G\alpha_s$ association with rafts as well as the increased lipid-raft localization of $G\alpha_s$ seen in postmortem major depressive disorder (MDD) brain (15).

Consistent with these observations, chronic treatment with antidepressant drugs

results in $G\alpha_s$ translocation from lipid rafts to non-raft regions of the plasma membrane, and the extent of translocation is dependent upon both drug dose and treatment duration. This has been observed in both rats (7) and cell culture (8,16,30). Moreover, lipid raft disruption through cholesterol depletion or cytoskeletal disruption displaces many raft proteins, but GPCR activation or antidepressant treatment displaces only $G\alpha_s$ (7). Binding to the reuptake transport machinery and inhibition of neurotransmitter reuptake is rapid, but requires several weeks of treatment in order to achieve clinical efficacy and this is duplicated (albeit in a more rapid timescale) in C6 glioma cells.

Estimates of the ratio of glia to neurons vary considerably, but evidence suggests that glia control the number and stability of neuronal synapses formed (31,32). Furthermore, chronic antidepressant treatment has been shown to increase the expression and release of glial cell derived neurotrophic factor (GDNF) (33-35). Curiously, the molecular entities most commonly associated with antidepressants, serotonin and norepinephrine transporters, are not endogenously expressed in C6 cells (36), which still respond to antidepressants by mediating lipid raft translocation of $G\alpha_s$.

All antidepressants examined thus far move $G\alpha_s$ from lipid rafts. This does not imply a single mechanism of action, but does suggest that antidepressants have a similar molecular footprint to exploit for the purposes of diagnostics and therapeutics. It is possible that the active sites for some antidepressants are downstream from their membrane binding sites. However, we predicted antidepressants would accumulate in the lipid raft regions of the plasma membrane unless the drug translocates across the membrane to bind an A seemingly simplistic intracellular target. explanation for the antidepressant-mediated translocation of $G\alpha_s$ from the lipid raft is the accumulation of drugs in lipid raft regions of the plasma membrane as $G\alpha_s$ moves out.

Previous reports on the concentration of psychoactive drugs in the lipid raft do not correlate exactly with our findings (25). Any discrepancies may well be due to the method of detection. Furthermore, these earlier studies used HEK cells transfected with 5-HT₃ receptors, which were suggested to bind the drugs used in the study. $G\alpha_s$ is not translocated from native HEK cells after antidepressant treatment and membranes prepared from kidney do not show augment of $G\alpha_s$ -activated adenvlyl cyclase in antidepressant-treated rats (37). Finally, in the Eisensamer studies, drug accumulation was determined by absorbance after exogenous addition to membrane fractions, similar to experiments shown in figure 2C of this study. As revealed in this study, results from acute drug addition to membranes are quite different from time-dependent accumulation the of antidepressants in lipid raft fractions (Figure 3). Future studies to refine these observations might employ moclobemide, an antidepressant that did not concentrate in rafts in the Eisensamer studies (21).

Different/multiple mechanisms are likely for the actions of to exist different antidepressants, but all drugs examined, this far, translocate $G\alpha_s$ from lipid rafts. The observed behavior of the antipsychotic, olanzapine was expected, as it does not move $G\alpha_s$ out of rafts Although, further support for distinct (20).molecular drug targets, comes from the enantiomer-selective accumulation of escitalopram, but not the therapeutically inactive enantiomer *R*-citalopram, and that accumulation appears restricted to the SSRIs and MAO inhibitors.

Thus, it appears that at least one action of antidepressants is to accumulate in lipid rafts and mediate the movement of $G\alpha_s$ out of lipid rafts. This may represent a novel biochemical hallmark for antidepressant action. Furthermore, identification of the antidepressant-sensitive molecular anchor for $G\alpha_s$ in lipid rafts may lead to the development of more targeted therapies for depression, including compounds that may have a much more rapid course of action.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, and penicillin/streptomycin were purchased from Sigma-Aldrich, St Louis, MO. Cell culture flasks were from NUNC (VWR International, West Chester, PA). Escitalopram and *R*-citalopram were kindly provided from H. Lundbeck A/S,

Copenhagen, Denmark. Desipramine hydrochloride and olanzapine were purchased from Tocris Bioscience, Ellisville, MO. Phenelzine sulfate, fluoxetine hydrochloride, amitriptyline hydrochloride, imipramine hydrochloride, and aripiprazole were purchased from Sigma-Aldrich, St Louis, MO.

Drug Treatments. C6 cells were cultured in DMEM, 4.5 g of glucose/L, 10% newborn calf serum (Hyclone Laboratories, Logan, UT), $100 \text{ mg} \text{ x} \text{ mL}^{-1}$ bacteriostatic penicillinstreptomycin at 37 °C in humidified 5% CO₂ atmosphere to a confluence of $\sim 40\%$ before drug treatments were begun. Treatment with 10 µM for 72 hrs is a standard assay condition (8), however, effects of these drugs in this cellular system can be observed at concentrations as low as 50 nM after 72 hrs (20). Culture media and drug were changed daily and no apparent change in cell morphology was observed during treatment. Before assay, cells were rinsed twice with pre-warmed 1X phosphate buffered saline (PBS) to remove debris and wash away unbound drugs.

Lipid Raft Isolation. Cells were washed and harvested in ice-cold 1X PBS. Lipid raft fractions were prepared as previously described (38). Briefly, C6 cells were extracted in 1 mL of icecold lysis buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 1mM DTT; 0.25 M Sucrose; 1% Triton X-100: Protease inhibitor cocktail). Following 30 min incubation on ice, the lysates were homogenized and 1 mL gently mixed (v/v)with ice-cold 80% (w/v) sucrose in TME (10mM Tris HCl; 1mM MgCl₂; 1mM EDTA; pH 7.5; 1mM DTT; protease inhibitors), and loaded in the bottom of a centrifuge tube. Samples were overlaid by syringe and fine needle with 1 mL each of 30% sucrose, 15% sucrose, and finally 5% sucrose. Sucrose gradients were spun at 40,000 rpm in an SW55-Ti rotor in a Beckman Ultra centrifuge at 4°C overnight for 16-18 h. Lipid rafts exist between 5% and 15% sucrose layers as opaque white clusters. Raft fractions were collected and sucrose removed via sequential mixing (v/v) in wash buffer (10mM HEPES pH 7.4, 150mM NaCl, 1mM DTT) and centrifugation at 21000 rpm at 4°C for 20 min.

(~4-5X) until a pellet emerges. Lipid raft pellets were reconstituted in 0.5 mL of TME buffer (10mM Tris HCl, 1mM MgCl₂, 1mM EDTA pH 7.5, 1mM DTT, protease inhibitors). Protein content was determined by absorbance at 280 nm on a Nanodrop UV-Vis spectrophotometer.

Accumulation of Antidepressants measured by UV-Vis. UV absorbance of antidepressants was used to determine their association with membrane fractions similar to before (25), but in the absence of HPLC purification. C6 cells treated (72 hrs) with 10 μ M escitalopram, Rcitalopram, fluoxetine, desipramine, phenelzine, or olanzapine were extracted by Tx100/114 and the cytosolic, non-raft membrane, and lipid raft fractions analyzed by UV absorbance and normalized to protein content ($\lambda = 280$ nm).

Furthermore, 500 μ L sucrose density gradient fractions were incubated with a final concentration of 10 μ M escitalopram or Rcitalopram. S- and R-citalopram absorbance (λ = 238 nm) in each fraction was assessed before and after incubation, measurements normalized to protein, and the fold change reported.

Drug hydrophobicity. Partition coefficients of drugs were determined as previously described (39) in a 1:1 v/v octanol to ddH₂O and the UV-Vis spectrum recorded for each phase. Absorbances: phenelzine (256 nm), desipramine (252 nm), imipramine (295 nm), amitriptyline (262 nm), fluoxetine (226 nm), citalopram (238 nm), aripiprazole (298 nm), or olanzapine (270 nm).

Gas Chromatography Mass Spectrometry (GC-MS). Analyses were performed using an Agilent HP-6890 gas chromatograph, equipped with an Agilent 19091S-602 HP-1MS capillary column (25 m, 0.20 mm, 0.33 µm, 7 inch cage), and interfaced with an Agilent HP-5973 mass selective detection (MSD) spectrometer equipped with a Single Flame Ionization Detector, Single 100 psi EPC Split/Splitless Injection Ports, 7673C-6890 Auto sampler: 6890 Control Electronics, 6890 Injector, 100 Position Tray and 6890 Mounting Bracket. Helium was used as the carrier gas at 1.0 mL x min⁻¹ in corrected constant flow mode. Primary oven temperature was

programmed at 70 °C for 2 min. and increased at 20 °C x min^{-1} to 230 °C where it was held for 10 min. The front inlet thermal modulator was set to 20 °C higher relative to the primary oven and 18.91 psi. Constant flow injection of 1 µL was used and inject split mode to Splitless. The injector, transfer line, and ion source temperatures were maintained at 250, 280, and 230 °C, respectively, throughout each analysis. Data acquisition was performed in the full scan mode from m/z 50 to 550 with an acquisition rate of 20 Hz. Molecular ion profiles (MIP) were matched against the standard mass spectral database of the National Institute of Standards and Technology (NIST).

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

1) Cells were collected and membranes fractionated into Tx-100 soluble and Tx-114 soluble fractions.

2) Extraction of accumulated antidepressant drugs in lipid rafts (Tx-114 fraction) may be assessed on large volume samples as previously described (40), but is not appropriate for small volumes Membrane fractions were chloroformhere. methanol precipitated as previously described (41) and the water, chloroform, and methanol phases vacuum centrifuged to recover accumulated drug. Desiccant was dissolved into 1 mL of methanol, and injected directly onto a GC Capillary Column (Agilent J&W HP-1ms). interfaced with an Agilent HP-5973 mass selective detection (MSD) spectrometer.

3) Quantitation of accumulated drug was performed by the internal standard method. Peakarea ratios were determined for the controls. Drug concentrations were calculated from the standard curve values. All data were acquired and analyzed by Agilent software, Enhanced G1701BA Chemstation version B.00.00. Molecular ion profiles (MIP) were matched against the standard mass spectral database of the NIST.

Fluorescence recovery after photobleaching C6 glioma cells stably expressing (FRAP). hSERT were a kind gift from Dr. Kim Neve, Oregon Health & Science University (OHSU). These cells were further transfected with GFP- $G\alpha_s$ and cells expressing the fluorescent construct were selected with G418. Cells were plated on glass microscopy dishes and treated with 10mM escitalopram or desipramine for three days. For imaging, drug was washed out for one hour prior and media was replaced with low serum (2.5%)NCS) phenol red-free DMEM to limit fluorescent background. Temperature was maintained at 37°C using a PeCon temperature controlled stage during imaging. Imaging utilized a Zeiss LSM 710 confocal microscope at 512 x 512 resolution with an open pinhole to maximize signal but minimize photobleaching. One hundred fifty data points, approximately 300 ms apart (including 10 pre-bleach values) were measured for each cell. Zeiss Zen software was used to calculate FRAP recoverv half-time utilizing a one-phase association fit, correcting for total photobleaching of the analyzed regions.

Western blotting. Westerns were conducted according to standard protocols with a rabbit polyclonal anti-SERT (1:1,000; EMD Millipore, Billerica, MA, USA, catalog # AB9726), rabbit polyclonal anti-Caveolin 1 (1:10,000; BD Biosciences, Franklin Lakes, NJ, USA catalog # 610059), and rabbit monoclonal anti- β -tubulin (1:5,000; made in house).

Statistical Analysis. All measurements are presented as the mean (a minimum of n=3) \pm standard error of the mean (SEM). Calculation error was propagated throughout each calculation. We further subjected each data set to statistical analyses using GraphPad Prism (version 5.0), using a one-way analysis of variance (ANOVA) followed by a post-hoc Student's t-test (two groups) or Dunnett's t-test (multiple groups) (95% C.I.).

ACKNOWLEDGEMENTS

The authors thank Drs. Karl Larsen, at the University of Illinois at Chicago, and Thomas Hoye, at the University of Minnesota, who kindly provided access to their Agilent GC-MS instruments. The authors also thank Dr. Kim Neve for C6 SERT cells and Lundbeck Inc. for escitalopram and R-citalopram.

FUNDING AND DISCLOSURE

Support provided by VA Merit award, BX001149; NIH R01AT009169, NIH/NIAAA. P50 AA022538 and T32 MH067631. MMR has received research support from Eli Lilly and Lundbeck, Inc. and is a consultant to Otsuka Pharmaceuticals. He also has ownership in Pax Neuroscience.

AUTHOR CONTRIBUTIONS

SJE designed, coordinated, performed and analyzed the experiments in Figures 2, 3, and 4 and wrote the paper. JMS designed, performed, and analyzed experiments shown in Figure 1. MMR designed experiments, analyzed data, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

- 1. Chen, G., Twyman, R., and Manji, H. K. (2010) p11 and gene therapy for severe psychiatric disorders: a practical goal? *Science translational medicine* **2**, 54ps51
- 2. Tanne, J. H. (2009) Antidepressants surpass antihypertensives as most commonly prescribed drugs in US. *BMJ* **339**
- Rush, A. J., Trivedi, M. H., Wisniewski, S. R., Nierenberg, A. A., Stewart, J. W., Warden, D., Niederehe, G., Thase, M. E., Lavori, P. W., Lebowitz, B. D., McGrath, P. J., Rosenbaum, J. F., Sackeim, H. A., Kupfer, D. J., Luther, J., and Fava, M. (2006) Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: A STAR*D report. *Am J Psychiat* 163, 1905-1917
- Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., Kubera, M., Bob, P., Lerer, B., and Maj, M. (2009) The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. *Metab Brain Dis* 24, 27-53
- 5. Duman, R. S., Heninger, G. R., and Nestler, E. J. (1997) A molecular and cellular theory of depression. *Arch Gen Psychiatry* **54**, 597-606
- 6. Fujita, M., Hines, C. S., Zoghbi, S. S., Mallinger, A. G., Dickstein, L. P., Liow, J. S., Zhang, Y., Pike, V. W., Drevets, W. C., Innis, R. B., and Zarate, C. A., Jr. (2012) Downregulation of brain phosphodiesterase type IV measured with 11C-(R)-rolipram positron emission tomography in major depressive disorder. *Biological psychiatry* **72**, 548-554
- 7. Toki, S., Donati, R. J., and Rasenick, M. M. (1999) Treatment of C6 glioma cells and rats with antidepressant drugs increases the detergent extraction of G(s alpha) from plasma membrane. *Journal of neurochemistry* **73**, 1114-1120
- 8. Zhang, L., and Rasenick, M. M. (2010) Chronic treatment with escitalopram but not R-citalopram translocates Galpha(s) from lipid raft domains and potentiates adenylyl cyclase: a 5-hydroxytryptamine transporter-independent action of this antidepressant compound. *The Journal of pharmacology and experimental therapeutics* **332**, 977-984
- 9. Brown, D. A., and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* 14, 111-136
- 10. Kalinowska, M., Castillo, C., and Francesconi, A. (2015) Quantitative profiling of brain lipid raft proteome in a mouse model of fragile X syndrome. *PLoS One* **10**, e0121464
- 11. Kasahara, K., and Sanai, Y. (2000) Functional roles of glycosphingolipids in signal transduction via lipid rafts. *Glycoconj J* **17**, 153-162
- 12. Insel, P. A., Head, B. P., Patel, H. H., Roth, D. M., Bundey, R. A., and Swaney, J. S. (2005) Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. *Biochem Soc Trans* **33**, 1131-1134

Antidepressants and Lipid Rafts

- 13. Allen, J. A., Halverson-Tamboli, R. A., and Rasenick, M. M. (2007) Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* **8**, 128-140
- 14. Allen, J. A., Yu, J. Z., Dave, R. H., Bhatnagar, A., Roth, B. L., and Rasenick, M. M. (2009) Caveolin-1 and lipid microdomains regulate Gs trafficking and attenuate Gs/adenylyl cyclase signaling. *Molecular pharmacology* **76**, 1082-1093
- 15. Donati, R. J., Dwivedi, Y., Roberts, R. C., Conley, R. R., Pandey, G. N., and Rasenick, M. M. (2008) Postmortem brain tissue of depressed suicides reveals increased Gs alpha localization in lipid raft domains where it is less likely to activate adenylyl cyclase. *The Journal of neuroscience* : the official journal of the Society for Neuroscience **28**, 3042-3050
- 16. Donati, R. J., and Rasenick, M. M. (2005) Chronic antidepressant treatment prevents accumulation of gsalpha in cholesterol-rich, cytoskeletal-associated, plasma membrane domains (lipid rafts). *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **30**, 1238-1245
- 17. Ozawa, H., and Rasenick, M. M. (1991) Chronic Electroconvulsive Treatment Augments Coupling of the GTP-Binding Protein Gs to the Catalytic Moiety of Adenylyl Cyclase in a Manner Similar to That Seen with Chronic Antidepressant Drugs. *Journal of neurochemistry* **56**, 330-338
- 18. Yu, J. Z., Dave, R. H., Allen, J. A., Sarma, T., and Rasenick, M. M. (2009) Cytosolic G{alpha}s acts as an intracellular messenger to increase microtubule dynamics and promote neurite outgrowth. *The Journal of biological chemistry* **284**, 10462-10472
- 19. Sarma, T., Koutsouris, A., Yu, J. Z., Krbanjevic, A., Hope, T. J., and Rasenick, M. M. (2015) Activation of microtubule dynamics increases neuronal growth via the nerve growth factor (NGF)- and Galphas-mediated signaling pathways. *The Journal of biological chemistry* **290**, 10045-10056
- 20. Czysz, A. H., Schappi, J. M., and Rasenick, M. M. (2015) Lateral diffusion of Galphas in the plasma membrane is decreased after chronic but not acute antidepressant treatment: role of lipid raft and non-raft membrane microdomains. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **40**, 766-773
- 21. Sanacora, G., Treccani, G., and Popoli, M. (2012) Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62**, 63-77
- 22. Di Benedetto, B., Rupprecht, R., and Czeh, B. (2013) Talking to the synapse: how antidepressants can target glial cells to reshape brain circuits. *Curr Drug Targets* **14**, 1329-1335
- 23. Yu, J. Z., and Rasenick, M. M. (2002) Real-time visualization of a fluorescent G(alpha)(s): dissociation of the activated G protein from plasma membrane. *Molecular pharmacology* **61**, 352-359
- 24. Allen, J. A., Yu, J. Z., Donati, R. J., and Rasenick, M. M. (2005) Beta-adrenergic receptor stimulation promotes G alpha s internalization through lipid rafts: a study in living cells. *Molecular pharmacology* **67**, 1493-1504

Antidepressants and Lipid Rafts

- 25. Eisensamer, B., Uhr, M., Meyr, S., Gimpl, G., Deiml, T., Rammes, G., Lambert, J. J., Zieglgansberger, W., Holsboer, F., and Rupprecht, R. (2005) Antidepressants and antipsychotic drugs colocalize with 5-HT3 receptors in raft-like domains. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 10198-10206
- 26. Nelson, J. C., and Papakostas, G. I. (2009) Atypical antipsychotic augmentation in major depressive disorder: a meta-analysis of placebo-controlled randomized trials. *The American journal of psychiatry* **166**, 980-991
- 27. Rothschild, A. J., Bates, K. S., Boehringer, K. L., and Syed, A. (1999) Olanzapine response in psychotic depression. *The Journal of clinical psychiatry* **60**, 116-118
- 28. Vetulani, J., and Sulser, F. (1975) Action of various antidepressant treatments reduces reactivity of noradrenergic cyclic AMP-generating system in limbic forebrain. *Nature* **257**, 495-496
- 29. Chen, J., and Rasenick, M. M. (1995) Chronic treatment of C6 glioma cells with antidepressant drugs increases functional coupling between a G protein (Gs) and adenylyl cyclase. *Journal of neurochemistry* **64**, 724-732
- 30. Donati, R. J., Thukral, C., and Rasenick, M. M. (2001) Chronic treatment of C6 glioma cells with antidepressant drugs results in a redistribution of Gsalpha. *Molecular pharmacology* **59**, 1426-1432
- 31. Ullian, E. M., Christopherson, K. S., and Barres, B. A. (2004) Role for glia in synaptogenesis. *Glia* 47, 209-216
- 32. Yan, K., Popova, J. S., Moss, A., Shah, B., and Rasenick, M. M. (2001) Tubulin stimulates adenylyl cyclase activity in C6 glioma cells by bypassing the beta-adrenergic receptor: a potential mechanism of G protein activation. *Journal of neurochemistry* **76**, 182-190
- 33. Hisaoka, K., Maeda, N., Tsuchioka, M., and Takebayashi, M. (2008) Antidepressants induce acute CREB phosphorylation and CRE-mediated gene expression in glial cells: a possible contribution to GDNF production. *Brain Res* **1196**, 53-58
- 34. Hisaoka, K., Tsuchioka, M., Yano, R., Maeda, N., Kajitani, N., Morioka, N., Nakata, Y., and Takebayashi, M. (2011) Tricyclic antidepressant amitriptyline activates fibroblast growth factor receptor signaling in glial cells: involvement in glial cell line-derived neurotrophic factor production. *The Journal of biological chemistry* **286**, 21118-21128
- 35. Golan, M., Schreiber, G., and Avissar, S. (2011) Antidepressants elevate GDNF expression and release from C(6) glioma cells in a beta-arrestin1-dependent, CREB interactive pathway. *Int J Neuropsychopharmacology* **14**, 1289-1300
- 36. Eshleman, A. J., Stewart, E., Evenson, A. K., Mason, J. N., Blakely, R. D., Janowsky, A., and Neve, K. A. (1997) Metabolism of catecholamines by catechol-O-methyltransferase in cells expressing recombinant catecholamine transporters. *Journal of neurochemistry* **69**, 1459-1466
- 37. Menkes, D. B., Rasenick, M. M., Wheeler, M. A., and Bitensky, M. W. (1983) Guanosine triphosphate activation of brain adenylate cyclase: enhancement by long-term antidepressant treatment. *Science (New York, N.Y.)* **219**, 65-67

- 38. Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., and Lisanti, M. P. (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *The Journal of biological chemistry* **270**, 15693-15701
- 39. Chiou, C. T., Schmedding, D. W., and Block, J. H. (1981) Correlation of water solubility with octanol-water partition coefficient. *J Pharm Sci* **70**, 1176-1177
- 40. Winecker, R. E. (2010) Quantification of antidepressants using gas chromatography-mass spectrometry. *Methods in molecular biology* **603**, 45-56
- 41. Wessel, D., and Flugge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* **138**, 141-143
- 42. Wenig, P., and Odermatt, J. (2010) OpenChrom: a cross-platform open source software for the mass spectrometric analysis of chromatographic data. *BMC Bioinformatics* **11**, 405

FIGURE LEGENDS

Figure 1. Ga_s -GFP FRAP is slowed by chronic antidepressant treatment and this is not changed by co-expression of hSERT. Ga_s -GFP C6 cells were treated with 10 µM escitalopram for 72 hrs. A) Whole cell homogenate from C6 cells stably transfected with hSERT (C6hSERT), but not wild type C6 cells, display reactivity to an anti-SERT antibody by western blot (*i.e.* C6 glioma cells do not natively express SERT). B) Half-time to recovery of Ga_s -GFP is increased (mobility slowed) after chronic escitalopram treatment, suggesting increased coupling with adenylyl cyclase. C) C6hSERT were transfected with Ga_s -GFP, selected with G418, and treated with 10 µM escitalopram for 72 hrs. Half-time to recovery of Ga_s -GFP is increased after chronic escitalopram treatment regardless of hSERT expression. Sample size represents the number of cells assayed, with a minimum of 18 and a maximum of 77 cells assayed per experiment. Data were analyzed by student's T-test and data are represented as mean ± SEM. (***, p < 0.001; ****, p<0.0001).

Figure 2. Treatment of C6 glioma cells with antidepressant drugs reveals their capacity to accumulate in plasma membrane microdomains. A) Cells were treated for 3 days with the indicated compound (10 μ M) and lipid raft fractions were prepared from membranes. Drug accumulation was determined by the characteristic absorbance of the drug. The fold change in UV absorbance for phenelzine (256 nm), desipramine (252 nm), imipramine (295 nm), amitriptyline (262 nm), fluoxetine (226 nm), citalopram (238 nm), aripiprazole (298 nm), or olanzapine (270 nm) normalized to protein (280 nm) and the untreated sample suggests that accumulation in lipid rafts is a class specific mechanism. B) UV absorbance was used to determine partition coefficients for psychoactive compounds. Partitioning between octanol and water reveals that each is relatively amphiphillic, with exception of aripiprazole and olanzapine. C) Sucrose density gradient fractions from treatment naïve C6 cells were incubated overnight with escitalopram or R-citalopram. Following methanol-chloroform precipitation of proteins, the fold change in UV absorbance reveals that escitalopram, but not R-citalopram, is retained in the raft regions of the membrane as revealed by anti-Caveolin 1 immunoreactivity (1:5000). (n=3; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

Figure 3. Representative GC-MS chromatograms and quantification of accumulated drug in 3 day treated C6 glioma cells.

Cells were treated for 3 days with the indicated compound (10 μ M) and lipid raft fractions were prepared from membranes. TICs were pre-processed with denoising, Savitsky-Golay, and component detection algorithm (CODA) filters (42). A-I) Representative TIC peaks for all drugs tested, confirms that antidepressant drugs accumulate in lipid rafts of C6 cells; lipid raft (red), lipid raft + accumulated drug (green), and 10 μ M drug standard (blue). J) Drug accumulation was determined and quantified by comparing peak intensities from the total ion chromatograms (TIC) of GC-MS analyses on accumulated drug with standard curves generated from methanol standards. Calculated moles of drug were normalized to protein content and reported as μ mol x mg⁻¹ protein. Phenelzine (41.51 ± 4.52), fluoxetine (26.24 ± 1.41), escitalopram (48.13 ± 5.35), and to a lesser extent desipramine (0.98 ± 0.51) were observed to accumulate in lipid rafts, whereas the inactive stereoisomer R-citalopram and the antipsychotic Olanzapine did not. (n=3; ***, p < 0.001).

Figure 4. GC-MS analysis of escitalopram-treated C6 glioma cells, confirms accumulation in plasma membrane microdomains. A) Lipid rafts isolated from C6 cells treated for 3 days with escitalopram from 10 nM to 10 μ M revealed escitalopram accumulates in a concentration-dependent manner beginning with 1 μ M for 72 hrs or 100 nM for 120 hrs and achieving maximal accumulation with 10 μ M at 72 hrs. B) Lipid rafts isolated from C6 cells treated with 10 μ M escitalopram revealed escitalopram accumulates over time in lipid rafts beginning at 24 hrs and plateauing at 72 hrs. C) TIC chromatogram overlay of drug accumulation in lipid rafts of C6 cells treated with 10 μ M escitalopram vs. R-citalopram compared with lipid rafts isolated from escitalopram-treated HEK cells. (n=3; *, p < 0.05; **, p < 0.01; ***, p < 0.001).









Figure 3



Figure 4

Antidepressants Accumulate in Lipid Rafts Independent of Monoamine Transporters to Modulate Redistribution of the G protein, G α_s Samuel J. Erb, Jeffrey M. Schappi and Mark M. Rasenick

J. Biol. Chem. published online July 18, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.727263

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts