Title: Antidepressants produce persistent Gαs associated signaling changes in lipid rafts following drug withdrawal

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Abstract:

Termination of antidepressant therapy often has negative consequences. While symptoms of antidepressant withdrawal are widely recognized, the molecular processes that underlie them are not well characterized. We show that certain aspects of Gαs signaling remain suppressed following antidepressant withdrawal, even after others have reverted to baseline. Antidepressant treatment causes translocation of Gαs protein from lipid rafts, to non-raft membrane regions. This results in augmented Gαs signaling, including facilitated activation of adenylyl cyclase (AC) and increased cAMP accumulation. Using c6 or SK-N-SH cells, and a lipid raft-localized cAMP sensor, we show that Gαs signaling is reduced in lipid rafts, even while signaling is enhanced elsewhere in the cell. These signaling changes mirror the changes in Gαs localization observed following antidepressant treatment. Furthermore, we show that suppression of Gαs signaling in lipid rafts persists at least 24 hr after cessation of antidepressant treatment. Gαs localization was quantified following membrane isolation and sequential detergent extraction. We show that suppression of lipid raft Gαs signaling persists for an extended time period after antidepressant withdrawal, while increased non-raft membrane Gαs signaling reverts partially or fully upon cessation of antidepressant treatment. Translocation of Gαs out of lipid rafts is also persistent. These events may reflect cellular adaptations to antidepressant treatment which contribute to antidepressant discontinuation syndromes, and may aid in the discovery of new treatments and strategies to mitigate the symptoms of depression and antidepressant withdrawal.

Significance statement: This work explores, for the first time, the effects of antidepressants on Gαs signaling following drug withdrawal. This provides novel insight into the cellular and molecular processes affected by antidepressant drugs, and their persistence after discontinuation of treatment.
Introduction

Major depressive disorder (MDD) is currently the leading cause of disability worldwide, and its impact is expected to continue growing (World Health Organization, 2017). No single treatment is fully effective in all people, with as many as two out of three individuals failing to remit after initial treatment (Rush, et al., 2006a). Follow-up treatments are also often ineffective, with many patients failing to remit even after second- and third-line treatment options have been exhausted (Insel and Wang 2009). These failures are exacerbated by the long delay between initiation of treatment and subsequent antidepressant response, with some drugs taking 8 weeks before effects can be evaluated (Rush, et al., 2006b). This means many individuals will undergo months or even years of failed therapy before finding relief, or drop out of treatment altogether (Sharma, et al., 2019). These challenges necessitate a deeper understanding of the processes leading to a positive antidepressant response, and the specific factors that distinguish individuals who will not respond to antidepressant treatment.

Following cessation of long-term antidepressant treatment, a constellation of symptoms known as antidepressant discontinuation syndrome can occur (Gabriel and Sharma, 2017). These symptoms may include sleep disturbances, anxiety, flu-like symptoms, and sensory abnormalities, including electric shock-like experiences (Baldwin, et al., 2006). Specific symptoms depend on the drug used, and vary between individuals (Fava, et al., 2015). Tricyclic antidepressant such as desipramine tend to produce more severe symptoms, including akathisia and parkinsonian reactions (Charney, et al., 1982; Garner, et al., 1993; Haddad, 2001). Nonetheless, antidepressant drugs with diverse primary mechanisms of action, including the serotonin and norepinephrine reuptake inhibitor venlafaxine, the selective serotonin reuptake inhibitor paroxetine, and the monoamine oxidase inhibitor phenelzine, among others, can all produce an antidepressant withdrawal syndrome (Ogle and Akkerman, 2012). Symptom severity tends to increase with longer treatment duration and higher dosage (Haddad, 1997; Warner, et al., 1993).
Antidepressant discontinuation syndromes can be debilitating and last for over two weeks, and not all patients are fully informed of this risk before treatment initiation (Bull, et al., 2002; Warner, et al., 2006). Although there has been some research into the mechanistic basis of this syndrome, its etiology remains poorly understood (Blier and Tremblay, 2006; Murata, et al., 2010; Zabegalov, et al., 2018). A better understanding of the residual effects that persist following antidepressant withdrawal is sorely needed, and will facilitate improved patient care following antidepressant treatment.

Lipid rafts are a subcellular membrane microdomain high in cholesterol content, and with distinct signaling characteristics compared to other membrane regions (Simons and Toomre, 2000; Allen et al., 2007). A wide variety of antidepressant drugs are known to cause translocation of Gαs out of lipid rafts (Senese, et al., 2018). This action is specific to Gαs, as other Gα proteins are not similarly affected (Donati and Rasenick, 2005). Antidepressants with distinct primary targets cause these effects, and can do so even in model systems lacking that target. For example, the selective serotonin reuptake inhibitor (SSRI) escitalopram causes Gαs translocation in cells lacking the serotonin transporter (SERT), while the rapid acting antidepressant ketamine causes translocation even after NMDA receptor knockdown (Eisensamer et al., 2005; Wray, et al., 2018). In the case of escitalopram this effect is stereospecific, as the inactive stereoisomer r-citalopram fails to affect Gαs localization or signaling (Czysz, et al., 2015).

This translocation produces a distinct change in Gαs signaling, notably increased coupling to adenylyl cyclase and resultant potentiation of downstream signaling (Chen and Rasenick 1995a,b). Potentiation of cAMP generation by antidepressants has been observed in both preclinical and clinical studies (Mooney, et al., 2013; Singh, et al., 2018). These changes likely represent cellular adaptations to extended antidepressant treatment. Multiday treatments are required before these effects manifest, and these changes are still observed at least 30 min after antidepressants have been withdrawn (Zhang and Rasenick, 2010). These signaling changes may provide novel insight into the cellular adaptations
occurring in response to antidepressants with diverse mechanisms of action.

The cAMP signaling cascade has been linked to depression and antidepressant action in various contexts (Dwiwedi and Pandey, 2008). Suicide completers have reduced central adenylyl cyclase activity, cAMP and downstream signaling via PKA (Cowburn et al., 1994; Pandey et al., 2005; Fujita, et al., 2017). Furthermore, in post-mortem cortical tissue from individuals diagnosed with unipolar depression, Gαs is localized in microenvironments with impaired adenylyl cyclase coupling (Donati et al., 2008). Conversely, 8 week treatment with an SSRI restored cAMP generation in the brains of depressed subjects (Fujita, et al., 2017).

We sought to determine, in a cellular model, whether previously observed antidepressant effects on Gαs signaling and translocation persist after drug withdrawal. Furthermore, we differentiated antidepressant-induced signaling changes in lipid rafts and non-raft regions using fluorescent cAMP sensors. The lipid raft-targeted cAMP sensor is expressed specifically in rafts due to the addition of a dually myristoylated/palmitoylatyed peptide sequence. This sequence restricts fluorophore expression to high density TX-100 insoluble regions (i.e. lipid rafts) and engenders significant caveolin colocalization (Zacharias, et al., 2002). This raft localization is reduced by the raft disruptor methyl-β-cyclodextrin (Zacharias, et al., 2002), confirming the lipid raft specificity of this targeting sequence. We determined that cellular hallmarks of antidepressant action, including translocation of Gαs from lipid rafts, persist after drug withdrawal in this model system. Further, we demonstrated that in contrast to antidepressant-induced increases in whole cell cAMP, lipid raft cAMP signaling was suppressed following antidepressant withdrawal. The ability to model antidepressant discontinuation in vitro may facilitate the development of effective antidepressant compounds without the risk of discontinuation syndromes.

**Materials and Methods:**

**Cell Culture:**
HEK-293 and c6 cells were obtained from the American Type Culture Collection (ATCC, VA). SK-N-SH were a generous donation from the lab of Dr. Ankur Saxena at UIC. HEK-293 and c6 cells were cultured in DMEM with 4.5 g/l glucose and L-glutamine without sodium pyruvate (Corning, NY), supplemented with 10% Gibco newborn calf serum (Thermo Fischer Scientific, MA). SK-N-SH cells were cultured in MEM with Earle’s salts and L-glutamine (Corning, NY), supplemented with 10% heat inactivated fetal bovine serum (Corning, NY).

**Drug treatments:**

10 mM stock solutions of all drugs were made as follows. Phenelzine sulphate (Sigma-Aldrich, MO), fluoxetine HCl (Sigma-Aldrich, MO), venlafaxine HCl (Sigma-Aldrich, MO), escitalopram oxalate (Sigma Aldrich, MO) and desipramine HCl (Santa Cruz Biotechnology Inc., TX) were all dissolved in ddH2O. R-citalopram oxalate was a generous gift from Lundbeck (Denmark), and dissolved in ddH2O. Due to low aqueous solubility, paroxetine HCl (Sigma-Aldrich, MO) and MDL 12330A (Tocris Bioscience, UK) were dissolved in DMSO. Ketamine HCl 100 mg/ml solution (Hospira Inc., IL) was diluted with ddH2O. Drugs were diluted from 10 mM to 10x final assay concentration in appropriate culture media for each cell type.

All antidepressants were administered for 3 days, except for ketamine which requires only a 15 min treatment (Wray *et al.*, 2018). Antidepressants were removed from culture flasks or plates either 30 min before start of experiment, or 24 hr before start of experiment, as indicated. An antidepressant is defined as having a ‘full reversal’ if the measured effect was significantly different between 3 day treatment and 24 hr after withdrawal, but not different from vehicle treated cells. A ‘partial reversal’ is designated when 24 hr withdrawal is not different from either 3 day treatment or vehicle treatment. ‘No reversal’ indicates the 24 hr withdrawal effect remained significantly different from vehicle treated cells, but not different from the 3 day treatment.
Lipid raft extraction:

Following drug treatment, two T150 culture flasks (Thermo Fisher Scientific, MA) containing approximately 25 million cells total, were washed twice with TME buffer (10 mM Tris HCl, 1 mM MgCl, 1 mM EDTA, pH 7.5) to remove residual media, scraped in TME buffer containing protease inhibitor cocktail II (MilliporeSigma, MA) and homogenized using a glass dounce and a benchtop drill.

Homogenized cells sat on ice for 30 minutes.

Homogenized cells were centrifuged at 60,000 x g for 60 min at 4C. After aspirating supernatant, each pellet was resuspended in 0.5 ml of TME containing protease inhibitor and 1.0% TX-100 (Thermo Fischer Scientific, MA), and again homogenized. Homogenized samples sat on ice for 30 min.

Samples were again centrifuged at 60,000 x g for 60 min at 4C, generating the TX-100 soluble fraction in the supernatant (non-raft membrane fraction). After aspirating supernatant, each pellet was resuspended in 0.5 ml of TME containing protease inhibitor and 1.4% TX114 (Thermo Fischer Scientific, MA), and again homogenized. Homogenized samples then sat on ice for 30 min. Finally, samples were centrifuged at 60,000 x g for 60 min at 4C, generating the TX-114 soluble fraction in the supernatant (lipid raft membrane fraction).

This protocol was modified for membranes prepared for Alphascreen cAMP experiments (Fig. 2E). The presence of TX-114 interferes with cAMP measurement using this assay (unpublished observation).

Therefore, following extraction of non-raft membranes with TX-100, the TX-100 insoluble pellet containing lipid raft membranes was resuspended in Alphascreen stimulation buffer. Per 20 ml this buffer consists of: 19.81 ml HBSS, 100 μl HEPES pH 7.5, 20 μl 500 mM IBMX, 66.7 μl 30% BSA solution dissolved in ddH2O. This buffer was supplemented with (final concentration): 25 mM MgCl, 375 mM NaCl, 250 μM ATP, 2.5 μM GDP and 2.5 nM GTP.
Protein Quantification:

Fractions were diluted and normalized for total protein content following determination of protein concentration using the Pierce BCA assay kit (Thermo Fischer Scientific, MA). Gαs, Cav-1 and cADDis sensor content were quantified for each sample using the Wes instrument (ProteinSimple, Inc., CA).

Primary antibody for Gαs detection is Anti-Gs protein, alpha subunit N192/12 (Antibodies Incorporated, CA), diluted 1:300 from starting concentration. Primary antibody for Cav-1 is rabbit Anti-Caveolin-1 antibody (Abcam, UK), diluted 1:250 from starting concentration. Primary antibody for fMP cADDis cAMP sensor (i.e. lipid raft cAMP sensor) is mNeon Tag Antibody #53061 (Cell Signaling Technology, MA), diluted 1:50 from starting concentration.

For detection of Gαs and Cav-1, samples were heated to 98°C for 5 min immediately before quantification. For detection of the cADDis cAMP sensor, samples were heated to 37°C for 30 min, with agitation every 10 min, in order to reduce high molecular weight aggregates. Cav-1 and cADDis cAMP sensor were quantified from the same samples, and as such Cav-1 serves as the protein loading control for the cADDis cAMP sensor expression data.

Representative readouts showing protein signal detected with each antibody are provided (Supp. Fig. 2 and 3). Area under the curve analysis is performed for each peak corresponding to target protein, and these values are normalized to the mean AUC value for the vehicle treated control fractions for each run.

cADDis cAMP assay:

cAMP accumulation in c6 cells was determined as described previously, with minor modification (Wray, et al., 2018). Briefly, c6 cells were cultured in T75 tissue culture flasks (Thermo Fischer Scientific, MA) such that they would reach ~80-90% confluence 24 hr before cAMP measurement. Cells were treated
with antidepressant or vehicle in culture flasks, with treatments starting at least 24 hr after plating.

Cells were dissociated from tissue culture flask using Cell-Stripper non-enzymatic cell dissociation reagent (Corning Inc., NY) 24 hr before cAMP measurement, and plated into Costar black-sided clear bottom sterile tissue culture-treated 96-well plates (Corning Inc., NY). For SK-N-SH and c6 cells ~48,000 cells were plated into each well. As HEK-293 cells divide more rapidly only ~30,000 cells were plated per well. Immediately after plating cells, 20 μl of bacculovirus expressing either the cytoplasmic Green Upward cADDis sensor (Montana Molecular, MT), or the lipid raft-restricted FMP Green Downward cADDis sensor (Montana Molecular, MT) was added to each well. Sodium butyrate (Sigma-Aldrich, MO) was added to each well to promote sensor expression, at a final concentration of 7.5 mM for c6 cells, and 2.5 mM for HEK-293 and SK-N-SH cells.

Culture media was replaced with warmed, serum free Fluorobrite DMEM (Thermo Fischer Scientific, MA) 30 min before cAMP measurement, to reduce background fluorescence and remove antidepressant. Plate was incubated for 30 min at room temperature while protected from light to prevent sensor bleaching. GFP signal intensity was quantified using either a Synergy H4 plate reader, a Synergy Neo2 plate reader (Biotek Instruments Inc., VT), or a SpectraMax i3x plate reader (Molecular Devices, CA). After average baseline fluorescence was determined for each well, either isoproterenol or vehicle was added, and fluorescence intensity was measured again.

For time course experiments a fluorescence measurement was taken every 30 s after isoproterenol addition. For isoproterenol dose response experiments fluorescence was quantified 5 min after isoproterenol addition in HEK-293 cells, and 30 min after isoproterenol addition in c6 and SK-N-SH cells, as these timepoints produced the most robust signal (see time courses in Fig. 3, 5, 7, 8, 9 and 10). For colchicine and methyl-β-cyclodextrin (MβCD) experiments, either 10 μM colchicine or 10 mM MβCD was added to the assay plate 15 min before isoproterenol addition. MβCD powder (Sigma-Aldrich, MO)
was dissolved in ddH2O to 4x final assay concentration (40 mM), while colchicine (Sigma-Aldrich, MO) stock solution was diluted in ddH2O to 10x final assay concentration (100 μM). Following 15 min MβCD or colchicine exposure, isoproterenol was added and allowed to incubate for 30 min before final fluorescence measurement. Due to poor aqueous solubility, MDL 12330A was added at 2x final concentration (2 mM), and fluorescence output was measured for 30 min after addition.

**AlphaScreen cAMP assay:**

AlphaScreen cAMP experiments were performed per manufacturer’s instructions (Perkin Elmer, MA), with slight modification. These experiments used the AlphaScreen cAMP detection kit with lipid raft containing membranes prepared as described above. Briefly, cAMP standard curve, stimulation buffer, BSA solution, isoproterenol and forskolin stock solutions were prepared fresh on the day of the assay. 20 μg of lipid raft membrane homogenates were added to each well of a white 384-well Perkin Elmer Optiplate. Agonist and acceptor beads were added to wells containing membrane homogenates and allowed to incubate for 30 min. Final isoproterenol concentration was 1 μM and final forskolin concentration was 10 μM. Signaling was terminated by addition of donor beads prepared in lysis buffer. Plate was then incubated for 1 hr at room temperature while protected from light, before measurement using the SpectraMax i3x plate reader with Alpha module (Molecular Devices, CA). Detected fluorescence signal for each well was fitted to a cAMP standard curve prepared with each experiment in order to determine sample cAMP concentrations.

**Live cell imaging:**

High resolution images of cells expressing fluorescent cAMP sensors were obtained using the LSM 880 confocal microscope (ZEISS, Germany), in Airyscan detection mode with a 40x objective. C6 cells were plated on glass microscopy dishes 48 hr before imaging. Cells were infected with either Green Upward
cADDis sensor (cytoplasmic), or FMP Green Downward cADDis sensor (lipid raft) expressing baculovirus (Montana Molecular, MT). Infection took place 24 hr before live cell imaging. Culture media was replaced with serum free Fluorobrite DMEM (Thermo Fischer Scientific, MA) 30 min before imaging to decrease background fluorescence.

Statistics:

Area under the curve (AUC) was determined for chemiluminescent peaks automatically identified by Protein Simple’s Compass for SW software (version 4.0.0). These AUC values were compared to assess changes in GoS protein expression levels. Concentration-effect results from cAMP assays were fitted to sigmoidal concentration-effect curves (Hill slope = 1) in GraphPAD Prism (version 8.2.1). These curves were used to determine EC50, maximal effect and baseline values. 3 day antidepressant treatments and their corresponding 24 hr withdrawal conditions were always run on the same plate, and as such share the same vehicle control curve, but are presented as separate subfigures for clarity. Venlafaxine and paroxetine (cytoplasmic sensor), escitalopram and desipramine (cytoplasmic sensor) as well as escitalopram and r-citalopram (lipid raft sensor) were also tested together and share vehicle control data. In time course experiments, signal intensity was typically measured once every 30 s for the stated experiment run time. For experiments using the upward cytoplasmic cADDis cAMP sensor raw fluorescence output is corrected using the baseline fluorescence of each well (ΔF/F₀). Due to drug induced changes in baseline fluorescence intensity using the downward fMP cADDis cAMP sensor, raw fluorescence output is corrected using the mean fluorescence of vehicle treated wells (ΔF/Fveh). Data are presented as ± SEM with between 3-6 replicates as indicated, and compared using unpaired t-test except when stated otherwise.

Results:

Desipramine and escitalopram translocate GoS from c6 glioma lipid rafts; desipramine’s effect persists
for over 24 hours after drug withdrawal

We first sought to determine the effects of desipramine and escitalopram on Gαs distribution in c6 cells, immediately after treatment and 24 hr after drug withdrawal. 3 day treatment of c6 cells with 10 μM desipramine significantly reduces Gαs distribution in lipid rafts (72.67 ± 1.54% of control; Fig. 1A). This reduction persists at least 24 hr following desipramine withdrawal (71.25 ± 3.01% of control; Fig. 1A). 3 day 10 μM desipramine treatment has no significant effect on Gαs expression in non-raft membrane regions (90.97 ± 2.72% of control; Fig. 1B), however 24 hr after desipramine withdrawal, non-raft Gαs content is decreased compared to vehicle treated control cells (80.53 ± 3.04% of control; Fig. 1B).

3 day treatment of c6 cells with 10 μM escitalopram also reduces Gαs in lipid rafts (77.32 ± 4.33% of control; Fig. 1C). In contrast to desipramine treated cells, this returns to baseline levels 24 hr after escitalopram withdrawal (95.94 ± 3.24% of control; Fig. 1C). 3 day 10 μM escitalopram treatment does not affect Gαs in non-raft membrane regions (110.74 ± 4.07% of control; Fig. 1D), and there is no change 24 hr after escitalopram withdrawal (111.99 ± 4.86% of control; Fig. 1D).

3 day treatment of c6 cells with 10 μM r-citalopram did not affect Gαs distribution in lipid rafts (Fig. 1E), or in non-raft membranes (Fig. 1F). Both measures remained unchanged 24 hr after r-citalopram withdrawal (Fig. 1E and 1F).

Desipramine and escitalopram treatments reduce baseline and isoproterenol stimulated cAMP in lipid rafts

We next tested whether changes in Gαs localization were reflected by changes in lipid raft cAMP signaling. The FMP cADDis cAMP sensor (Montana Molecular, MT) is highly expressed in lipid raft membranes due to the addition of a dually myristoylated/palmitoylated peptide sequence (Fig. 2A).
Fluorophores tagged with this sequence generally colocalize with the lipid raft marker caveolin, and are highly expressed in TX-100 insoluble (i.e. lipid raft) membrane fractions (Zacharias, et al., 2002). In c6 cells expressing this lipid raft-localized cAMP sensor (Fig. 2A and 2B), baseline fluorescence is significantly increased following 3 day 10 μM desipramine treatment (118.46% ± 2.59% of control; Fig. 2C), and 24 hr after desipramine withdrawal (115.99% ± 2.56% of control; Fig. 2C). As the lipid raft cAMP sensor gains fluorescence intensity in response to decreasing cAMP, this increased baseline fluorescence following desipramine treatment suggests lower baseline cAMP concentrations proximal to the lipid raft sensor. Considering that changes in sensor expression can also affect this baseline reading, we also quantified sensor expression following desipramine treatment. Desipramine did not affect overall sensor expression in c6 cells, or its distribution between raft/non-raft membranes (Fig. 2B). Therefore, the desipramine induced alterations in baseline fluorescence can’t be explained by altered sensor expression or localization, and likely reflect reduced baseline cAMP proximal to the lipid raft sensor.

If desipramine alters sensor expression, or affects fluorescence output non-specifically, then a difference in maximal fluorescence should be apparent when cAMP levels are reduced to a minimum level. cAMP can be reduced below baseline levels using chemical inhibitors of adenylyl cyclase, and this strategy has been used previously to determine the fluorescence range of cytoplasmic and lipid raft-localized fluorescent cAMP sensors (Agarwal et al., 2018).

We tested the effects of a saturating concentration of the adenylyl cyclase inhibitor MDL 12330A on fluorescence output of C6 cells expressing the lipid raft cAMP sensor. In cells with no antidepressant treatment, MDL 12330A increased fluorescence over the 30 min treatment window (Supp. Fig. 5A). This demonstrates that reductions in baseline cAMP result in increased fluorescence with this sensor.

We also compared the maximal fluorescence of c6 cells expressing this sensor, treated initially with either vehicle or desipramine, and then treated with MDL 12330A. The maximal fluorescence was not different between vehicle and desipramine pretreated cells after 30 min (Supp. Fig. 5B). This shows that
desipramine does not affect the maximal fluorescence of this lipid raft cAMP sensor, and that reduced lipid raft cAMP is the most likely explanation for the increased baseline fluorescence following desipramine treatment. Results using this sensor are reported using the final fluorescence reading from vehicle treated wells as a baseline (ΔF/F<sub>veh</sub>), and all drug induced fluorescence changes are reported as percent change compared to the baseline fluorescence of vehicle control wells. 3 day 10 μM desipramine treatment reduced both baseline cAMP in range of the lipid raft sensor (27.61 ± 3.01% increase over vehicle; Fig. 2D), as well as the maximal isoproterenol response (46.20 ± 2.72% decrease from baseline with vehicle vs. 34.87 ± 3.09% decrease from baseline with desipramine; Fig. 2D). The reduction in maximal isoproterenol response is not due to limited sensor range, as forskolin treatment reduces fluorescent intensity to a greater degree than the maximal change observed with isoproterenol (Supp. Fig. 4), in line with higher maximal cAMP concentrations typically observed with forskolin compared to isoproterenol.

Isoproterenol stimulated cAMP peaks rapidly in c6 cells, and remains near peak value for at least 30 min (Fig. 3C). The desipramine-induced suppression of c6 cell lipid raft cAMP is evident immediately after addition of 1 μM isoproterenol, and remains separated over the entire 30 min testing duration (Fig. 3C).

Both effects on lipid raft cAMP produced by 3 day 10 μM desipramine treatment persist 24 hr after drug withdrawal. Baseline cAMP remains suppressed (19.24 ± 2.87% increase over vehicle; Fig. 3A) and maximal isoproterenol stimulation is still decreased (35.49 ± 3.11% decrease from baseline following desipramine withdrawal; Fig. 3A). The suppression of lipid raft cAMP observed 24 hr after desipramine withdrawal is apparent over the entire 1 μM isoproterenol time course (Fig. 3D).

As these effects were not attenuated 24 hr after desipramine withdrawal, we next tested whether lipid raft cAMP effects persist 3 days after desipramine withdrawal. In c6 cells that have been pretreated for 3 days with 10 μM desipramine, neither baseline fluorescence (1.88 ± 2.82% decrease from vehicle) or
maximal isoproterenol stimulation (48.72 ± 2.53% decrease from baseline with vehicle vs. 46.43 ± 2.93% decrease from baseline after desipramine withdrawal) were significantly changed 3 days after withdrawal compared to vehicle treated cells (Fig. 3B).

3 day 10 μM escitalopram treatment produces effects similar to desipramine on lipid raft cAMP. This treatment reduces baseline lipid raft cAMP (25.55 ± 6.73% increase over vehicle; Fig. 4A), as well as maximal isoproterenol stimulated lipid raft cAMP (46.13 ± 6.06% decrease from baseline with vehicle vs. 18.96 ± 4.43% decrease from baseline with escitalopram; Fig. 4B). Escitalopram treatment had a strong trend (p = 0.0588) towards increased isoproterenol potency for cAMP generation (1.77 ± 1.76 nM isoproterenol EC50 with vehicle vs. 107.40 ± 91.58 pM isoproterenol EC50 with escitalopram; Fig. 4A), a trend not observed following desipramine treatment.

24 hr after drug withdrawal, escitalopram treatment produced no significant effects on lipid raft cAMP in c6 cells, with baseline (16.91 ± 7.82% over vehicle), maximal isoproterenol stimulation (39.15 ± 8.64% decrease from baseline) and isoproterenol EC50 (1.83 ± 1.95 nM) not significantly different from vehicle treated cells (Fig. 4B).

Lipid raft cAMP signaling was also probed in c6 cells following 3 day 10 μM r-citalopram treatment. R-citalopram did not affect baseline lipid raft cAMP, maximal isoproterenol stimulation, or isoproterenol EC50 (Fig. 4C). These measures were still unaffected 24 hr after r-citalopram withdrawal (Fig. 4D).

**Whole cell cAMP increases subsequent to escitalopram or desipramine treatment**

We compared antidepressant effects on lipid raft cAMP with effects on whole cell cAMP concentration in c6 cells expressing a cytoplasmic cAMP sensor (Fig. 5A). As drug treatments did not affect baseline fluorescence generated by the cytoplasmic sensor, fluorescence output is normalized to the pretreatment fluorescence output of each well (ΔF/Fo). The maximal whole cell cAMP concentration
produced by isoproterenol in c6 cells is increased following 3 day treatment with 10 μM desipramine (110.01 ± 4.41% increase over baseline with vehicle vs. 133.60 ± 5.63% with desipramine; Fig. 5C). This effect is noticeable almost immediately after addition of 1 μM isoproterenol, and remains elevated over 30 min after isoproterenol addition (Fig. 5E).

This is in contrast to treatment with 3 day 10 μM escitalopram, which increased isoproterenol potency (1.17 ± 0.39 nM isoproterenol EC50 with vehicle treatment vs. 231.8 ± 73.52 pM isoproterenol EC50 with escitalopram treatment; Fig. 6A) with no change in maximal accumulation (110.01 ± 4.41% increase over baseline with vehicle vs. 114.53 ± 3.84% with escitalopram; Fig. 6A).

This effect of escitalopram treatment is dose dependent (Supp. Fig. 1A), with 3 day 1 μM escitalopram shifting isoproterenol EC50 by 2.3-fold compared to vehicle, and 10 μM escitalopram producing a 6.1-fold EC50 shift (Supp. Fig. 1C). 100 nM and 10 nM escitalopram treatments were ineffective.

Unlike Gαs translocation from lipid rafts, desipramine effects on whole cell cAMP accumulation are not sustained after drug withdrawal (110.01 ± 4.41% increase over baseline with vehicle vs. 120.46 ± 4.83% after desipramine withdrawal; Fig. 5D and Fig. 5F). The increase in isoproterenol potency induced by 3 day 10 μM escitalopram treatment is also reversed 24 hr after withdrawal (1.17 ± 0.39 nM isoproterenol EC50 with vehicle vs. 1.03 ± 0.24 nM isoproterenol EC50 after escitalopram withdrawal; Fig. 6B). Lower escitalopram concentrations also fail to affect whole cell cAMP 24 hr after withdrawal (Supp. Fig. 1B).

3 day 10 μM r-citalopram treatment in c6 cells did not affect whole cell cAMP response, with both maximal isoproterenol stimulation and isoproterenol potency remaining unchanged after r-citalopram treatment (Fig. 6C), and 24 hr after r-citalopram withdrawal (Fig. 6D).
**Sustained antidepressant effects on cAMP accumulation vary with drug**

In addition to escitalopram and desipramine, we also tested the effects of fluoxetine, phenelzine, venlafaxine, ketamine and paroxetine on whole cell cAMP. The effects of these drugs are summarized in Table 1. Fluoxetine treatment (3 day 10 μM) increased maximal isoproterenol stimulation, and this was partially reversed 24 hr after fluoxetine withdrawal (118.84 ± 8.04% increase over baseline with vehicle vs. 146.77 ± 4.30% with fluoxetine vs. 130.75 ± 5.72% after fluoxetine withdrawal). Phenelzine treatment (3 day 10 μM) increased maximal isoproterenol stimulation, and this effect was fully persistent 24 hr after phenelzine withdrawal (122.40 ± 10.17% increase over baseline with vehicle vs. 167.78 ± 10.28% with phenelzine vs. 151.21 ± 3.80% after phenelzine withdrawal). Venlafaxine treatment (3 day 10 μM) increased maximal isoproterenol stimulation, and this was partially reversed 24 hr after venlafaxine withdrawal (122.00 ± 3.62% increase over baseline with vehicle vs. 137.77 ± 4.54% with venlafaxine vs. 125.04 ± 11.06% after venlafaxine withdrawal). Ketamine treatment (15 min 10 μM) increased maximal isoproterenol stimulation, and this was fully reversed 24 hr after ketamine withdrawal (134.80 ± 7.83% increase over baseline with vehicle vs. 159.58 ± 6.67% with ketamine vs. 124.11 ± 6.28% after ketamine withdrawal). Paroxetine treatment (3 day 10 μM) did not increase maximal cAMP accumulation, and there was still no effect 24 hr after paroxetine withdrawal (122.00 ± 3.62% increase over baseline with vehicle vs. 113.04 ± 5.48% with paroxetine vs. 115.45 ± 6.06% after paroxetine withdrawal). Aside from escitalopram (Fig. 4B), none of the tested antidepressants alter the potency of isoproterenol for cytoplasmic cAMP generation (Table 1).

**Desipramine potentiates isoproterenol stimulated cytoplasmic cAMP in SK-N-SH cells without affecting lipid raft cAMP**
The effects of antidepressants on lipid raft cAMP in C6 cells were next compared with effects in the neuronal SK-N-SH cell line. SK-N-SH cells expressing a lipid raft-restricted cAMP sensor did not have a measurable response to isoproterenol (Fig. 7A), at any point during the 30 min following isoproterenol addition (Fig. 7D). In contrast, forskolin produced a large lipid raft cAMP response in SK-N-SH cells expressing this sensor, demonstrating that the lipid raft sensor, and adenylyl cyclase, function normally in this cell line (Fig. 7C).

3 day 10 μM desipramine treatment did not affect baseline lipid raft cAMP in these cells, and did not facilitate an isoproterenol response (Fig. 7A). No changes occurred on either measure 24 hr after desipramine withdrawal (Fig. 7B and Fig. 7E).

In contrast, SK-N-SH cells expressing a cytoplasmic cAMP sensor produced a robust isoproterenol response (104.44 ± 4.59% increase over baseline; Fig. 8A). This response peaked within 5 min of 1 μM isoproterenol addition, and then decayed slightly over 30 min (Fig. 8C). The maximal isoproterenol effect was significantly increased by 3 day 10 μM desipramine treatment (151.22 ± 1.25% increase over baseline; Fig. 8A). This effect is evident within 5 min of isoproterenol addition, and persists for the 30 min testing period (Fig. 8C).

24 hr after desipramine withdrawal, these measures reverted to vehicle control levels, with both baseline cytoplasmic cAMP (10.17 ± 2.25% over vehicle) and maximal isoproterenol stimulated cAMP (99.60 ± 3.61% over baseline) not significantly different from vehicle treated cells (Fig. 8B and Fig. 8D).

The lack of a measurable isoproterenol response in SK-N-SH cells expressing the lipid raft-targeted sensor (Fig. 7A) suggests that this sensor measures local lipid raft cAMP concentrations rather specifically. The robust whole cell cAMP response (Fig. 8A), did not bleed over into a detectable response with the lipid raft sensor.
Desipramine does not affect lipid raft or whole cell cAMP signaling in HEK-293 cells

In addition to glial c6 and neuronal SK-N-SH cells, we also sought to determine the effects of antidepressants on lipid raft signaling in human kidney derived HEK-293 cells. HEK-293 cells expressing a lipid raft-restricted cAMP sensor, like c6 cells, produce a measurable increase in cAMP following isoproterenol stimulation (25.87 ± 6.40% decrease from baseline; Fig 9A). This effect peaks within 3-5 min of 1 μM isoproterenol addition, then decays over the 30 min testing period (Fig. 9C).

3 day 10 μM desipramine treatment did not affect baseline lipid raft cAMP in these cells (5.21 ± 2.96% increase over vehicle), nor maximal isoproterenol effect (29.97 ± 3.39% decrease from baseline; Fig. 9A), despite affecting both of these measures in c6 cells expressing this sensor (Fig. 2D). 24 hr after desipramine withdrawal, these measures remained unchanged (Fig. 9B). There is no measurable effect of desipramine at any time point after 1 μM isoproterenol addition (Fig. 9C and Fig. 9D).

Desipramine also failed to affect cAMP signaling in HEK-293 cells expressing a cytoplasmic cAMP sensor, with baseline and maximal isoproterenol stimulated cAMP unchanged compared to vehicle, both after 3 day 10 μM desipramine treatment (Fig. 10A), and 24 hr after desipramine withdrawal (Fig. 10B).

C6 cells reach a maximal lipid raft cAMP response approximately 3 minutes after isoproterenol addition, and maintain this response over the 30 min testing period (Fig. 3C and 3D). SK-N-SH cells have essentially no response to isoproterenol regardless of treatment conditions (Fig. 7D and 7E). The response to isoproterenol in HEK-293 cells peaks 1-2 min after isoproterenol addition, but then decays over the 30 min testing period (Fig. 9C and 9D). At the 30 min time point HEK-293 cells treated with vehicle and 1 μM isoproterenol have similar cAMP levels (Fig. 9C and 9D).

Within 3-5 min of 1 μM isoproterenol addition, HEK-293 cells produced a more rapid cytoplasmic cAMP response (Fig. 10C) compared to c6 cells (Fig. 5E). This peak in HEK-293 cells is relatively transient, such
that 15 min after isoproterenol addition HEK-293 and c6 cells have comparable responses. Nonetheless, desipramine treatment fails to affect HEK-293 cytoplasmic cAMP response at any point during the 30 min testing period, either after 3 day treatment (Fig. 10C), or 24 hr after desipramine withdrawal (Fig. 10D).

The cholesterol chelator MβCD and the microtubule disruptor colchicine produce effects similar to desipramine on cAMP signaling in c6 cells

Microtubule disruption with colchicine affects lipid raft organization and induces translocation of Gαs out of lipid rafts (Donati and Rasenick, 2005). In c6 cells expressing either the lipid raft cAMP sensor (Fig. 11A), or the cytoplasmic cAMP sensor (Fig. 11B), response to colchicine was similar to the effects observed following desipramine treatment. 15 min 10 μM colchicine treatment suppressed baseline lipid raft cAMP (27.73 ± 8.48% increase over vehicle), but did not significantly affect maximal isoproterenol response (56.08 ± 6.28% decrease from baseline with vehicle vs. 31.71 ± 16.87% with colchicine), despite trending towards a reduced effect (Fig. 11A). Colchicine treatment did not affect baseline fluorescence detected by the cytoplasmic sensor (Fig. 11B), but increased the maximal isoproterenol response compared to vehicle treated cells (80.02 ± 13.31% increase from baseline with vehicle vs. 132.60 ± 6.40% with colchicine).

The cholesterol chelator, methyl-beta cyclodextrin (MβCD), also disrupts lipid raft organization (Zidovetzki and Levitan, 2007), and reduces raft-localized Gαs (Allen, et al., 2009). Similar to desipramine, MβCD trends towards reduced baseline cAMP detected by the lipid raft sensor in c6 cells (32.57 ± 14.65% increase over vehicle; Fig. 11C), and also reduces the maximal isoproterenol response (48.47 ± 4.81% decrease from baseline with vehicle vs. 14.61 ± 10.34% with MβCD; Fig. 11C).
Together with the desipramine data presented above, these results suggest that treatments which induce translocation of \( \text{G} \alpha_s \) out of lipid rafts will generally decrease cAMP detected by a lipid raft-localized sensor, while simultaneously increasing total cellular cAMP response.

**Discussion**

Results from this study suggest that, for some antidepressants, effects on \( \text{G} \alpha_s \) signaling and localization persist following drug withdrawal. Desipramine-induced reduction of lipid raft \( \text{G} \alpha_s \) persists for over 24 hr after drug withdrawal in c6 cells (Fig. 1A). In contrast, escitalopram’s effects on lipid raft \( \text{G} \alpha_s \) localization revert to baseline within 24 hr of escitalopram withdrawal (Fig. 1C). This effect is stereospecific, as the inactive stereoisomer r-citalopram has no effect on \( \text{G} \alpha_s \) localization (Fig. 1E and 1F). Although the ratio of non-raft/raft \( \text{G} \alpha_s \) localization returns to baseline 24 hr after withdrawal of either drug, both non-raft and raft \( \text{G} \alpha_s \) are reduced following withdrawal of desipramine, but not escitalopram (Fig. 1). Therefore, this cellular model of antidepressant action reveals a persistent desipramine effect not observed with escitalopram.

The sustained reduction of lipid raft \( \text{G} \alpha_s \) following desipramine withdrawal corresponds with a persistent increase in baseline fluorescence emitted by a lipid raft-localized cAMP biosensor (Fig. 2C). These changes in baseline fluorescence do not result from changes in overall sensor expression or localization, as desipramine treatment did not significantly affect expression or raft localization (Fig. 2B).

In addition to the desipramine-induced increase in baseline fluorescence, desipramine treatment also reduced the maximal change in fluorescence following isoproterenol challenge in c6 cells (Fig. 2D). This suggests that desipramine treatment inhibits the maximal effect of isoproterenol on cAMP generated proximal to the lipid raft cAMP sensor. Consistent with this interpretation, lipid raft membranes purified from desipramine-treated c6 cells produce less cAMP following isoproterenol challenge (Fig. 2E). Together, these data suggest that the increased fluorescence emitted by the lipid...
raft cAMP sensor following desipramine pretreatment corresponds with a reduction in baseline cAMP proximal to lipid rafts, as well as a reduction in maximal isoproterenol-stimulated cAMP in this region.

The cholesterol chelator MβCD, and the microtubule disruptor colchicine, increase baseline fluorescence emitted by the lipid raft cAMP sensor, and attenuate maximal isoproterenol response (Fig. 11). Both of these compounds liberate Gαs from lipid rafts, albeit through divergent mechanisms (Donati and Rasenick, 2005; Zidovetzki and Levitan, 2007; Allen, et al., 2009). As such, reduction of lipid raft-localized Gαs appears to be a sufficient strategy to reduce lipid raft cAMP. This also suggests that the cAMP detected by the lipid raft sensor is primarily produced following activation of raft-localized Gαs.

The reduction of lipid raft cAMP following 3 day desipramine treatment persists for over 24 hr after desipramine withdrawal in c6 cells, but reverts to baseline 72 hr after withdrawal (Fig. 3A and 3B). In contrast, escitalopram effects do not persist after escitalopram withdrawal (Fig. 4A and 4B). Translocation of lipid raft Gαs following desipramine and escitalopram treatments (Fig. 1A and 1C), adheres to a similar time course. This reveals a strong correlation between the reduction in lipid raft Gαs and the reduction of lipid raft cAMP, following treatment with these antidepressants.

Desipramine increases the maximal effect of isoproterenol-stimulated whole-cell cAMP in c6 cells (Fig. 3B), however this effect begins to revert to baseline within 24 hr (Fig. 4A). Desipramine has no effect on the potency of the β-agonist isoproterenol, nor does any other antidepressant tested, save escitalopram (Table 1). This represents an initial demonstration that during the process of removing Gαs from lipid rafts, antidepressants inhibit Gαs evoked signaling in those rafts, while contemporaneously enhancing cAMP signaling in the whole cell.

The reduction in lipid raft cAMP observed here is consistent with predictions from earlier work showing that many antidepressants reduce Gαs distribution in lipid rafts (Toki, et al., 1999; Donati and Rasenick, 2005; Zhang and Rasenick, 2010) without altering cellular content of this protein. This process is specific
to antidepressants, as non-antidepressants including olanzapine, haloperidol, lithium and diazepam lack these effects (Czysz, et al., 2015; Donati, et al., 2015). Antidepressant drugs also accumulate in lipid raft membranes of c6 cells (Erb, et al., 2016). As the c6 cell line lacks monoamine transporters (Eshleman, et al., 1997), the lipid-raft binding target for these antidepressants remains unknown. Nonetheless, the signaling effects of antidepressant accumulation in lipid rafts are becoming more well understood, namely reduced cAMP proximal to lipid rafts, facilitated Gαs signaling elsewhere in the cell, and cAMP dependent increases in signaling factors including pCREB and BDNF (Singh, et al., 2018) as well as the BDNF receptor, TrkB (Casarotto et al., 2021). Note that lipid raft translocation of Gαs, and the resultant sustained increases in cAMP and BDNF are hallmarks of antidepressant action that unfold over the course of extended drug treatment. The identified pharmacologic target of many of these drugs (e.g. monoamine transporters or catabolizing enzymes) are affected immediately by antidepressants, while the clinical effects show a hysteresis of up to 2 months.

Escitalopram's effect on isoproterenol potency was surprising, and differed from all other antidepressants tested (Table 1). This effect was dose dependent (Supp. Fig. 1A), and stereospecific (Fig. 4). Furthermore, escitalopram had a strong trend (p = 0.0588) towards increasing isoproterenol potency in lipid rafts, while simultaneously decreasing isoproterenol's maximal effect in raft domains (Fig. 4A). This suggests a dual action of escitalopram on signaling downstream of Gαs in lipid rafts, both driving Gαs out of this region, while at the same time facilitating β-adrenergic receptor signaling.

Similar to the effect observed in c6 cells, desipramine treatment increased the maximal isoproterenol effect on whole cell cAMP in the neuronal SK-N-SH cell line (Fig. 8A), an effect that did not persist after desipramine withdrawal (Fig. 8B). Unlike c6 cells, SK-N-SH had no measurable response to isoproterenol in lipid rafts, and did not respond to desipramine treatment in rafts (Fig. 7A). This suggests that SK-N-SH lack functional β-adrenergic receptors in lipid rafts, while an intact forskolin response demonstrates
these cells have the capacity to generate cAMP detectable by the lipid raft sensor (Fig. 7C). In contrast, HEK-293 cells respond robustly to isoproterenol stimulation, both when measuring whole cell cAMP (Fig. 10A) and lipid raft cAMP (Fig. 9A). Nonetheless, neither of these measures are affected by desipramine treatment, consistent with the lack of antidepressant effect on Gαs signaling in rat kidney (Menkes, et al., 1983).

The inverse effect of desipramine pretreatment on whole cell and lipid raft cAMP in c6 cells was surprising, due to the assumption that cAMP would generally diffuse freely and rapidly throughout the cell. If this were true, lipid raft sensors in SK-N-SH would detect overall cellular increases in isoproterenol stimulated cAMP. They do not (Fig. 7). In fact, several reports provide evidence that cAMP concentration is regulated independently across subcellular domains, and that cAMP diffuses at a rate significantly lower than that predicted for free diffusion (Agarwal, et al., 2016; Saucerman, et al., 2006). In fact, cAMP concentration gradients have been observed between nanometer-sized subcellular domains in HEK-293 cells (Bock, et al., 2020), confirming that cAMP does not diffuse freely throughout the cytoplasm.

In HEK-293 cells PGE1 increases cAMP, gradually, to a steady state in cytoplasmic regions distant from the plasma membrane (Rich, et al., 2001a; Rich, et al., 2001b). The rapid reduction in membrane cAMP was prevented by pretreatment with a phosphodiesterase inhibitor, suggesting that differences in the rate of cAMP breakdown contribute to differential concentrations between various cellular subdomains (Rich, et al., 2001a; Rich, et al., 2001b; Oliveira, et al., 2010). The rate of cAMP production by adenylyl cyclase is also affected by minute changes in local pH, particularly in caveolae (Willoughby, et al., 2005). Distinct expression of proteins which form complexes with adenylyl cyclase between membrane subdomains (e.g. calmodulin, PKA, AKAP) may also contribute to subregion specific cAMP regulation (Simpson, et al., 2006; Di Benedetto, et al., 2008; Zaccolo and Pozzan, 2002). Together these localized
differences in cAMP breakdown and production allow for distinct, compartmentalized regulation of cellular cAMP.

Cell-specific variation in whole cell vs. lipid raft cAMP among c6, SK-N-SH and HEK-293 cells demonstrates cell type specificity for desipramine’s effects. Both the neuronal SK-N-SH and glial c6 cells generated higher maximal whole cell cAMP levels following desipramine treatment, while HEK-293 cells were unaffected. Only c6 cells displayed a reduction in lipid raft cAMP following desipramine treatment, suggesting these cells (and perhaps, glia generally) have somewhat unique antidepressant-responsive elements in this membrane region. This is consistent with the observation that both c6 cells and primary astrocytes, but not HEK-293 cells, display magnified cAMP responses following treatment with the antidepressant ketamine (Wray, et al., 2019).

Although antidepressant efficacy has traditionally been assumed to involve neuronal targets, glial cells have also been heavily implicated in antidepressant action and discontinuation syndromes. SSRIs such as citalopram evoke a calcium response in astrocytes, an effect that persists for longer time periods than other neurotransmitter induced calcium signaling (Schipke, et al., 2011). Antidepressants also induce gliogenesis in mouse embryonic stem cells (Kusakawa, et al., 2010), an effect consistent with increased expression of neurotrophic factors following antidepressant treatment in c6 cells (Singh, et al., 2018). Furthermore, astrocytes derived from neural stem cells of depressed subjects responded to n-3 PUFA with cellular responses similar to those described in this study (Yu et al., 2020). Finally, altered choline-to-creatine ratio observed in the anterior cingulate of individuals experiencing antidepressant discontinuation syndrome has been attributed to disrupted astrocytic function (Kaufman, et al., 2003), affirming the involvement of glial cells not only in antidepressant action, but also the residual effects after antidepressant discontinuation.

The use of baseline fluorescence output to determine changes in baseline cAMP has the potential to be
confounded by changes in sensor expression. We control for this possibility by quantifying sensor expression directly following vehicle and desipramine pretreatments (Fig. 2B), and show that sensor expression and localization are unaltered. Using a chemical inhibitor of adenylyl cyclase to reduce baseline cAMP, we also show that desipramine does not affect the maximal fluorescent output of this sensor (Supp. Fig. 5).

Furthermore, fluorescence alterations detected with lipid raft cAMP biosensors are consistent with reduced lipid raft cAMP generation detected with the AlphaScreen cAMP assay (PerkinElmer, MA). Although we did not detect a baseline cAMP difference between vehicle and desipramine treated lipid raft membranes using the AlphaScreen assay, this is likely due to the lipid raft purification process (Fig. 2E). The cytoplasmic contents are removed during membrane purification, so baseline measurements reflect cAMP that is generated during the Alphascreen assay, rather than resting cAMP levels in intact and alive cells with the fluorescent sensor. Desipramine reduced isoproterenol stimulated cAMP to a greater degree using AlphaScreen detection compared to the fluorescent lipid raft sensor. This result was surprising, but may indicate that some cAMP generated during isoproterenol stimulation in a live cell6 cell diffuses from non-raft membrane regions, such that it is detected by the lipid raft sensor.

This work underscores the importance of measuring G-protein signaling in relevant subcellular locales. The lipid raft specific signaling events described here complement recent studies exploring the importance of G-protein signaling from endosomes (Lyga, et al., 2016; Thomsen, et al., 2016), in recycling tubules (Bowman, et al., 2016), and the trans-golgi network (Godbole, et al., 2017). Persistent endosomal cAMP signaling is a consistent finding after receptor internalization (Ferrandon, et al., 2009; Tsvetanova and von Zastrow, 2014; Jean-Alphonse et al., 2017). Persistent antidepressant induced changes in cAMP signaling are likely independent from this endosomal signaling, as the antidepressant induced changes are evident within minutes of isoproterenol addition.
Dysregulated cAMP signaling is a hallmark feature of depression. Depressed individuals have widespread reductions in cAMP levels throughout the brain (Fujita, et al., 2017), and depressed suicide subjects have reduced adenylyl cyclase IV expression and activity in post-mortem temporal cortex (Reiach, et al., 1999). The reduced cAMP level observed in unmedicated depressed patients is corrected to healthy control levels following effective SSRI treatment (Fujita, et al., 2017), while the rapid acting antidepressant ketamine facilitates cAMP signaling in glial cells (Wray, et al., 2018). In animal models, chronic but not acute antidepressant treatments increase brain cAMP levels (Menkes et al., 1983; Ozawa and Rasenick, 1989), and downstream signaling factors including CREB (Nibuya, et al., 1996). Furthermore, phosphodiesterase 4B inhibitors specifically increase cAMP concentrations, and produce antidepressant-like effects in preclinical models (Zhang, et al., 2006; Zhang, et al., 2017).

Together these studies indicate that antidepressant induced increases in cAMP are an important determinant of treatment effectiveness, while reductions are generally indicative of unmedicated depression. Our results complement these findings, showing for the first time that antidepressant-induced increases in cAMP reverse relatively quickly following antidepressant withdrawal in both glial and neuronal cells. They also validate Gαs translocation from lipid rafts and the sequelae of cAMP signaling events accompanying this, as consistent biomarkers of antidepressant action. The residual depression of lipid raft cAMP signaling may represent an as of yet unrecognized cellular mechanism underlying antidepressant discontinuation syndromes. In future studies we hope to determine how a wider spectrum of antidepressants affect lipid raft signaling, and whether this action correlates with severity of discontinuation symptoms.

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Author Contributions:

Participated in research design: NBS and MMR

Conducted experiments: NBS

Performed data analysis: NBS and MMR

Wrote or contributed to the writing of the manuscript: NBS and MMR

Footnotes:

The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

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Footnotes:

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Figure legends

**Figure 1: Desipramine and escitalopram translocate Gα<sub>s</sub> from lipid rafts**

Treatment of c6 cells with 10 μM desipramine for 3 days reduces Gα<sub>s</sub> presence in lipid rafts, and this effect persists for over 24 hr after drug withdrawal (1A). Desipramine has no immediate effect on Gα<sub>s</sub> in non-raft membrane regions, however there is a significant decrease 24 hr after drug withdrawal (1B). Treatment with 10 μM escitalopram for 3 days also reduces lipid raft Gα<sub>s</sub>, however this effect is reversed 24 hr after drug withdrawal (1C). There is no change in non-raft membrane regions immediately after escitalopram treatment, or 24 hr after withdrawal (1D). R-citalopram has no effect on lipid raft Gα<sub>s</sub> (1E) or in non-raft membranes (1F). * and ** indicate p < 0.05 and p < 0.01 respectively, compared to vehicle control. # indicated p < 0.05 compared to antidepressant treated cells. n = 3-4

**Figure 2: Desipramine and escitalopram reduce baseline and isoproterenol stimulated cAMP in lipid rafts**

A c6 cell expressing the lipid raft-localized FMP Green Downward cADDis cAMP sensor (2A). 3 day treatment of c6 cells with 10 μM desipramine does not affect sensor localization or expression (2B). This treatment increases baseline fluorescence in c6 cells (2C). Desipramine pretreatment increases baseline and isoproterenol stimulated fluorescence in lipid rafts (2D). Isoproterenol (1 μM) stimulated cAMP generation following desipramine pretreatment in lipid raft membranes prepared from c6 cells is reduced, as measured by the AlphaScreen assay (2E). **** indicates p < 0.0001, compared to top end of
vehicle control curve. *** indicates $p < 0.001$ compared to vehicle baseline fluorescence. # indicates $p < 0.05$, compared to bottom end of vehicle control curve. ++ indicates $p < 0.01$ compared to vehicle treated membranes. n = 6-12

**Figure 3: Reduction of lipid raft cAMP by desipramine persists for over 24 hr, but reverses 72 hr after withdrawal**

The increased fluorescence following 3 day 10 μM desipramine persists 24 hr after desipramine withdrawal in c6 cells (3A and 3D), but returns to untreated levels 3 days after desipramine withdrawal (3B). 1 μM isoproterenol maximally reduces fluorescence intensity after 3 min in c6 cells, and this response is maintained for at least 30 min (3C and 3D). The increase in fluorescence following desipramine pretreatment in c6 cells is apparent over the entire 30 min isoproterenol time course (3C), as well as 24 hr after desipramine withdrawal (3D). ** indicates $p < 0.01$, compared to top end of vehicle control curve. # indicates $p < 0.05$, compared to maximal isoproterenol effect in vehicle pretreated wells. n = 6

**Figure 4: Reduction of lipid raft cAMP by escitalopram reverses 24 hr after withdrawal**

3 day treatment with 10 μM escitalopram significantly reduces baseline and isoproterenol stimulated cAMP in c6 lipid rafts (4A), however this is no longer observed 24 hr after escitalopram withdrawal (4B). 3 day treatment with 10 μM r-citalopram does not affect baseline or isoproterenol stimulated cAMP in c6 lipid rafts, either immediately after drug removal (4C), nor 24 hr after drug withdrawal (4D). ** indicates $p < 0.01$, compared to top end of vehicle control curve. ## indicates $p < 0.01$, compared to maximal isoproterenol effect in vehicle pretreated wells. n = 6

**Figure 5: Desipramine increases maximal isoproterenol stimulated whole cell cAMP**

A c6 cell expressing the cytoplasmic Green Upward cADDis cAMP sensor (5A). Baseline fluorescence
intensity is not affected by desipramine treatment in c6 cells expressing this sensor (5B). 3 day treatment of c6 cells with 10 μM desipramine significantly increases maximal isoproterenol stimulated cAMP production (5C and 5E). This effect is no longer significantly different from vehicle treated cells 24 hr after desipramine withdrawal (5D and 5F). 1 μM isoproterenol maximally increases fluorescence intensity in c6 cells after 3 min, and this response is maintained over the entire 30 min isoproterenol time course (5E and 5F). # indicates p < 0.05 compared to top end of vehicle control curve. n = 4

Figure 6: Escitalopram increases potency of isoproterenol stimulated whole cell cAMP

3 day treatment with 10 μM escitalopram increases the potency of isoproterenol stimulated cAMP production, but has no effect on maximal accumulation in c6 cells expressing the cytoplasmic cAMP sensor (6A). This is reversed 24 hr after escitalopram withdrawal (6B). R-citalopram has no effect on isoproterenol stimulated cytoplasmic cAMP in c6 cells (6C and 6D). # indicates p < 0.05 compared to top end of vehicle control curve. n = 4

Figure 7: Desipramine does not affect lipid raft cAMP in SK-N-SH cells

3 day 10 μM desipramine treatment has no effect on baseline lipid raft cAMP levels immediately following drug withdrawal (7A), nor 24 hr later (7B) in the neuronal SK-N-SH cell line. This cell line has no quantifiable change in lipid raft-localized cAMP levels following isoproterenol treatment, and this is not modified by desipramine treatment (7A and 7B). Forskolin produces a concentration dependent response in SK-N-SH cells expressing this sensor (7C). SK-N-SH have virtually no response to 1 μM isoproterenol at any point along the 30 min time course (7D and 7E).

Figure 8: Desipramine potentiates isoproterenol stimulated whole cell cAMP in SK-N-SH cells

Desipramine treatment potentiates isoproterenol stimulated cAMP in SK-N-SH cells expressing the cytoplasmic cAMP sensor (8A and 8C), but this effect reverses to vehicle treated levels 24 hr after
desipramine withdrawal (8B and 8D). Response to 1 μM isoproterenol peaks after 3 min then declines over the 30 min time course (8C and 8D). # indicates p < 0.05 compared to top end of vehicle control curve. n = 4

Figure 9: Desipramine does not affect lipid raft cAMP signaling in HEK-293 cells

HEK-293 cells expressing the lipid raft cAMP sensor respond to isoproterenol (9A and 9B), but this response is not affected by desipramine treatment (9A and 9C), and signaling remains unchanged 24 hr after desipramine withdrawal (9B and 9D). Response to 1 μM isoproterenol peaks after 2 min then declines over the 30 min time course (9C and 9D). n = 4

Figure 10: Desipramine does not affect whole cell cAMP signaling in HEK-293 cells

HEK-293 cells expressing the cytoplasmic cAMP sensor respond to isoproterenol (10A and 10B), but this response is not affected by desipramine treatment (10A and 10C), and signaling remains unchanged 24 hr after desipramine withdrawal (10B and 10D). Response to 1 μM isoproterenol peaks after 1 min then declines over the 30 min time course (10C and 10D). n = 4

Figure 11: Lipid raft disruptors MβCD and colchicine reduce baseline cAMP in lipid rafts while colchicine potentiates isoproterenol stimulated whole cell cAMP in c6 cells.

Colchicine reduces baseline lipid raft cAMP in c6 cells (11A) while also potentiating maximal isoproterenol induced whole cell cAMP (11B). 15 min 10 mM MβCD treatment reduces baseline and isoproterenol stimulated lipid raft cAMP (11C). * indicates p < 0.05 compared to top end of vehicle control curve. # indicates p < 0.05 compared to top end of vehicle control curve. n = 4

Table 1: Effects of antidepressant treatment and withdrawal on c6 whole cell cAMP
### Isoproterenol stimulated cAMP accumulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Efficacy</th>
<th>Potency</th>
<th>24 hr reversal</th>
</tr>
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<tbody>
<tr>
<td>Desipramine</td>
<td>*</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>Escitalopram</td>
<td>*</td>
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<tr>
<td>Fluoxetine</td>
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<td>Venlafaxine</td>
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<tr>
<td>R-citalopram</td>
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Antidepressants were applied for 3 day at 10 μM (desipramine, escitalopram, fluoxetine, phenelzine, venlafaxine, paroxetine or r-citalopram), or for 30 min at 10 μM (ketamine). Desipramine, fluoxetine, venlafaxine and ketamine all increased maximal whole cell isoproterenol response. Phenelzine’s effect was fully persistent 24 hr after withdrawal, while desipramine, fluoxetine and venlafaxine reversed partially, and ketamine reversed fully. In contrast, escitalopram increased isoproterenol potency but not maximal response, an effect that reversed fully 24 hr after withdrawal. Paroxetine and r-citalopram had no effect. * indicates p < 0.05 compared to vehicle treatment. n = 3 - 4
Figure 1

A

Gαs expression (% of control)

Vehicle Desipramine Desipramine + 24 hr Withdrawal

**

#

B

Gαs expression (% of control)

Vehicle Desipramine Desipramine + 24 hr Withdrawal

*

C

Lipid Raft Fraction

Gαs expression (% of control)

Vehicle Escitalopram Escitalopram + 24 hr Withdrawal

**

#

D

Non-Raft Membrane Fraction

Gαs expression (% of control)

Vehicle Escitalopram Escitalopram + 24 hr Withdrawal

E

Lipid Raft Fraction

Gαs expression (% of control)

Vehicle R-citalopram R-citalopram + 24 hr Withdrawal

F

Non-Raft Membrane Fraction

Gαs expression (% of control)

Vehicle R-citalopram R-citalopram + 24 hr Withdrawal
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7

A and B: Graphs showing the effect of isoproterenol on 
ΔF/F_veh vs. log[isoproterenol].

C: Graph showing the effect of forskolin on 
ΔF/F_veh vs. log[forskolin].

D and E: Graphs showing the effect of various treatments on 
ΔF/F_veh vs. time.
Figure 8
Figure 9
Figure 10

A

B

C

D

Vehicle
10 μM Desipramine

Vehicle
10 μM Desipramine + 24 hr Withdrawal

Vehicle + Isoproterenol

Desipramine Withdrawal + Vehicle

Desipramine Withdrawal + Isoproterenol

Time After Isoproterenol Addition (s)
Figure 11
Antidepressants produce persistent Gαs-associated signaling changes in lipid rafts following drug withdrawal

Authors: Dr. Nicolas B. Senese and Dr. Mark M. Rasenick

Journal Title: Molecular Pharmacology

Supplementary Figure Legends

Supplemental Figure 1: Escitalopram dose dependently increases isoproterenol potency in c6 cells

3 day 10 μM escitalopram treatment increases the potency of isoproterenol stimulated cAMP accumulation in the cytosol of c6 cells (Supp. 1A). Log(EC50) for this response in vehicle pretreated cells is -8.919 ± 0.08765, but reduced to -9.702 ± 0.1899 following 3-day treatment with 10 μM escitalopram. This effect is reversed 24 hr after escitalopram withdrawal (Supp. 1B). Both 1 and 10 μM treatments increase isoproterenol's potency on this measure (Supp. 1C), while lower concentrations are ineffective. EC50 fold shift calculated by comparing EC50 after escitalopram treatment with vehicle EC50. n = 4

Supplemental Figure 2: Gαs raft and non-raft protein detection

Chemiluminescent signal detected from lipid raft and non-raft membrane fractions probed with anti-Gαs primary antibody. Area under the curve for each band is quantified and analyzed in Figure 1. “V” corresponds to fractions from vehicle pretreated c6 cells, “D” to cells that have undergone 3 day 10 μM desipramine treatment (A and B), “E” to cells that have undergone 3 day 10 μM escitalopram treatment (C and D), “R” to cells that have undergone 3 day 10 μM r-citalopram treatment (E and F), and “W” to cells 24 hr after respective antidepressant withdrawal.
**Supplemental Figure 3: Caddis sensor and Cav-1 protein raft and non-raft detection**

Chemiluminescent signal detected from whole cell lysate, cytoplasm, lipid raft, and non-raft membrane fractions probed with anti-mNeon primary antibody (A and B), or anti-Caveolin 1 primary antibody (C and D). The lipid raft localized Caddis cAMP sensor (Montana Molecular) is detected by anti-mNeon antibodies (A and B). Area under the curve for each band is quantified and analyzed in Figure 2.

**Supplemental Figure 4: Forskolin reduces fluorescence intensity more than isoproterenol using a lipid raft localized cAMP sensor**

Forskolin produces a larger maximal reduction in fluorescence intensity than isoproterenol in c6 cells expressing a lipid raft restricted cAMP sensor. n = 11

**Supplemental Figure 5: Maximal fluorescence output following adenylyl cyclase inhibition is not different between vehicle and desipramine pretreated C6 cells expressing a lipid raft localized cAMP sensor**

A 1 mM saturating concentration of the adenylyl cyclase inhibitor MDL 12330A was applied to C6 cells following either vehicle treatment, or 3 day 10 μM desipramine treatment. MDL increases fluorescence intensity over the 30 min treatment duration in cells with no antidepressant pretreatment (A). Maximal fluorescent intensity is not different between vehicle and desipramine treated cells following MDL application (B). **** indicates p < 0.0001 for main effect of MDL over time in the vehicle pretreatment condition (one-way ANOVA; F (60, 183) = 3.565). n = 4