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Chronic Antidepressant Treatment Prevents Accumulation of Gsα in Cholesterol-Rich, Cytoskeletal-Associated, Plasma Membrane Domains (Lipid Rafts)

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Previous studies demonstrated that Gsα migrates from a Triton X-100 (TTX-100) insoluble membrane domain to a TTX-100 soluble membrane domain in response to chronic treatment with the antidepressants desipramine and fluoxetine. Antidepressant treatment also causes a Gsα redistribution in cells as seen by confocal microscopy. The current studies have focused on examining the possibility that the association between Gsα and the plasma membrane and/or cytoskeleton is altered in response to antidepressant treatment, and that this is relevant to both Gsα redistribution and the increased coupling between Gsα and adenylyl cyclase seen after chronic antidepressant treatment. Chronic treatment of C6 cells with two fuctionally and structurally distinct antidepressants, desipramine and fluoxetine, decreased the Gsα content of TTX-100 insoluble membrane domains by as much as 60%, while the inactive fluoxetine analog LY368514 had no effect. Disruption of these membrane domains with the cholesterol chelator methyl-β-cyclodextrin altered the localization of many proteins involved in the cAMP signaling cascade, but only Gsα localization was altered by antidepressant treatment. In addition, microtubule disruption with colchicine elicited the movement of Gsα out of detergent-resistant membrane domains in a manner identical to that seen with antidepressant treatment. The data presented here further substantiate the role of Gsα as a major player in antidepressant-induced modification of neuronal signaling and also raise the possibility that an interaction between Gsα and the cytoskeleton is involved in this process.
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INTRODUCTION

For over half a century, electroconvulsive shock (ECS) and various classes of antidepressant drugs have been used for the treatment of depression and other psychiatric disorders. The possibility that these diverse agents converge on a single postsynaptic target has been an area of great research interest. In 1983, we first reported that long-term administration of various antidepressants enhanced guanylyl-5′-imidodiphoshate (Gpp(NH)p)- and fluoride-stimulated adenylyl cyclase activity in rat cortex and hypothalamus membranes (Menkes *et al*, 1983). This suggested that the stimulatory α-subunit of the G protein, Gs, was at least an indirect target of antidepressant action, and that antidepressant treatment facilitated the activation of adenylyl cyclase by Gs. These initial findings have been substantiated by later *in vivo* and *in vitro* studies (Chen and Rasenick, 1995a; De Montis *et al*, 1990; Kamada *et al*, 1999; Ozawa and Rasenick, 1989, 1991; Perez *et al*, 1991, 1989). Consistent with these findings, it has been reported that chronic antidepressant treatment increases the expression and activity of cAMP response element binding protein (CREB) in the rat brain (Duman *et al*, 1997; Nibuya *et al*, 1996; Takahashi *et al*, 1999; Thome *et al*, 2000).

G protein signaling complexes at the plasma membrane have been identified as associated with specific components of the membrane and cytoskeleton (Huang *et al*, 1997). These domains, which contain receptors, G proteins and effector enzymes, and other proteins, are likely to be constrained from lateral mobility within the plasma membrane (Neubig, 1994) in part by cytoskeletal structures that form 'corrals' on the inner membrane face (Kuo and Sheetz, 1993). Certain G proteins have also been shown to bind to tubulin (Wang *et al*, 1990) and microtubules (Sarma

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et al, 2003). This appears to both modulate cytoskeletal dynamics and cell signaling.

The localization of G proteins to specific detergent insoluble membrane domains such as caveolae (Li *et al*, 1995) and rafts has generated interest as to how these cholesterol and sphingolipid-rich detergent-resistant membrane domains modulate G protein targeting and function (Bayewitch *et al*, 2000; Brown and London, 2000; Moffett *et al*, 2000; Ostrom and Insel, 2004). Interestingly, the different G protein α subunits are localized in distinct plasma membrane domains (Oh and Schnitzer, 2001). While both Gsα and Giα can move in and out of caveolae, they are predominantly found in lipid rafts complexed with Gβγ. On the other hand, Gqα couples to caveolin and is found predominantly in caveolae without Gβγ. Current studies in this laboratory have found that Gsα raft localization is increased after isoproteronol stimulation (Allen *et al*, 2004, submitted).

Recent studies have demonstrated that Gsα from C6 rat glioma cells migrates from a caveolae and raft-enriched Triton X-100 (TTX-100) insoluble membrane domain (from here on referred to as a Triton Insoluble Floating Fraction (TIFF) due to the ability to float on sucrose gradients) to a TTX-100 soluble membrane domain in response to chronic antidepressant treatment (Toki *et al*, 1999). The TTX-100 detergent extractability of Gsα from C6 cell or rat brain membranes was increased upon treatment with amitriptyline, desipramine, and fluoxetine. Gsα, which is normally enriched in TIFFs (that include caveolae), was removed from these domains by 50% in antidepressant-treated C6 cells. There was no shift in the membrane localization of Giα to a more TTX-100 soluble membrane domain after antidepressant treatment, suggesting that the antidepressant effect on G protein membrane localization is Gsα specific (Toki *et al*, 1999). These data are reinforced by the findings of Bayewitch *et al* (2000) who showed that chronic exposure to agonists of Giα-coupled receptors leads to a decrease in the cholate solubility of these G protein subunits and a 'superactivation' of adenylyl cyclase. Further studies involving the visualization of Gsα demonstrated that Gsα is localized to the plasma membrane as well as the cytosol in both desipramine-treated and nontreated control cells (Donati *et al*, 2001). In nontreated cells, Gsα is distributed throughout the entire length of the cell processes with an enrichment at the distal end. Antidepressant-treated cells show reduced localization of Gsα in cellular processes (especially the distal ends), while Gsα is increased in the perinuclear region of the cells (Donati *et al*, 2001). Taken together, these studies suggest that the lipid environment and cytoskeletal association of the G protein may play an important role in its localization and function, and that chronic antidepressant treatment alters the membrane localization of Gsα, resulting in augmented coupling to adenylyl cyclase.

The aim of this study is to further investigate the role of antidepressants on the membrane and cytoskeletal localization of Gsα. We examined whether antidepressants effect Gsα membrane localization via disruption of the raft domains or disruption of the microtubule cytoskeleton. In addition to the chronic antidepressant treatment regimen, these cells were treated with a fluoxetine analog that differs only by the position of the CF3 group to determine if structurally similar compounds could exert a similar effect on Gsα localization. Furthermore, the cells were acutely treated with the cholesterol chelator methyl-β-cyclodextrin or the microtubule-disrupting agent colchicine. The results of this study directly reveal that the localization of Gsα is a prime target for antidepressant action. This study also lends support to the hypothesis that the microtubule cytoskeleton and its regulation of G protein signaling is involved in the sequellae of events initiated by antidepressant treatment that results in altered Gsα signaling.

METHODS

Cell Culture and Treatment Paradigms

C6 cells (between passages 30 and 50) were grown in 150 cm^2 flasks and allowed to attach overnight in Dulbecco's modified Eagle's medium, 4. 5 g of glucose/l, 10% newborn calf serum (Hyclone), 100 μg/ml penicillin and streptomycin at 37°C in humidified 10% CO_2 atmosphere. As reported previously, desipramine treatment regimens of 3 μM for 5 days and 10 μM for 3 days yielded similar biochemical results (Chen and Rasenick, 1995b). Therefore, the latter treatment paradigm was used in these experiments because it was easier to maintain the cell cultures for 3 days. The cells were treated with 10 μM of either desipramine hydrochloride (Sigma), fluoxetine, or LY368514 (both gifts from Eli Lilly and Co., structure in Figure 1a) for 3 days or 10 mM methyl-β cyclodextrin (Sigma) for 30 min. The culture media and drug were changed daily. None of the treatments altered cell growth (as determined by the confluency of the cell monolayer and total protein estimation). In a separate experiment, C6 cells were treated with desipramine as above while another group of cells was treated with colchicine (2 and 5 μM) and β-lumicolchicine (5 μM) (both from Sigma) for 2 h prior to harvesting.

Cell Fractionation by Sucrose Density Gradient Sedimentation

Following treatment, cells were washed, scraped and sedimented and the pellets were used to prepare TIFFs by the procedure of Li *et al* (1995) with minor modifications. Briefly, C6 cells were harvested into 0.75 ml of HEPES buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, protease inhibitors) containing 1% TTX-100. Homogenization was carried out with 10 strokes of a Potter–Elvehjem homogenizer. The homogenate was adjusted to 40% sucrose by the addition of an equal volume of 80% sucrose prepared in HEPES buffer and placed at the bottom of an ultracentrifuge tube. A step gradient containing 30, 15, and 5% sucrose was formed above the homogenate and centrifuged at 50 000 rpm for 20 h in an SW65 rotor (≈240 000 *g*). The opaque bands confined between the 15 and 30% sucrose layers were harvested, diluted three-fold with HEPES buffer and pelleted in a microcentrifuge at 16 000 *g*. The pellet was resuspended in HEPES buffer and subjected to further biochemical analysis.

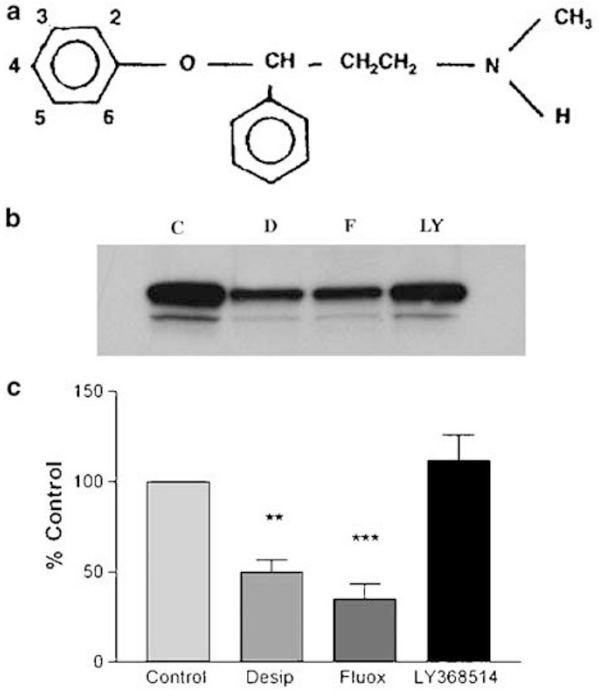

Figure 1 Antidepressant treatment of C6 cells causes a shift in the localization of Gsα from a TTX-100 insoluble lipid-raft-rich domain to a more TTX-100 soluble domain. (a) Chemical backbone of fluoxetine with the CF3 group in the para position (4) and LY368514 with the CF3 group in the ortho position (2). (b) Cells were treated chronically with desipramine (DMI), fluoxetine (FLU), or the inactive fluoxetine analog, LY368514 (LY) (3 days, 10 μM). The detergent insoluble membranes were floated on sucrose density gradients and analyzed by SDS-PAGE and immunoblot for Gsα content. Representative immunoblot from three experiments. (c) Percent change in Gsα in the TTX-100 insoluble lipid-raft-rich fraction. Note that acute treatment has no effect on the redistribution of Gsα. Autoradiographs were compared by densitometry from three separate experiments and the results were plotted using Prism Graphpad 4.0. Data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison post-test. Mean ± SEM (**$p<0.01$, ***$p<0.001$).

SDS-PAGE and Immunoblotting

TIFFs were analyzed by SDS-PAGE followed by Western blotting onto PVDF membranes (Millipore). Proteins were detected with antibodies to protein kinase A (PKA) catalytic and regulatory subunits, adenylyl cyclase type II and V/VI (Santa Cruz), tubulin (Sigma), actin C4 clone (gift from Dr J Lessard), and Gsα (Dupont/NEN) followed by secondary antibody (HRP-linked anti-rabbit or mouse IgG F(ab′)2) (Jackson). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) Western blot detection system (Amersham and Pierce) in accord with the manufacturer's instructions. Autoradiographs were analyzed by densitometry using ImageQuant software (Molecular Dynamics).

Cholesterol Analysis

C6 membranes were prepared as described (Rasenick and Kaplan, 1986) and stored under liquid N_2 until use. C6 whole cell lysates (hypotonic lysis on ice) and 100 000 *g* crude membrane pellets of the lysates (both in 15 mM HEPES, pH 7.4) were submitted to the Clinical Pathology Department at UIC for cholesterol analysis. Samples were analyzed on the SYNCHRON CX System using the Cholesterol Reagent kit in conjunction with the SYNCHRON Systems CX MULTI Calibrator (Beckman Coulter). The reaction results in the hydrolysis of cholesterol esters into free cholesterol and the oxidation of free cholesterol producing hydrogen peroxide. The result is quantified by a peroxidase-generated color reaction.

Table 1 Displacement of Gs-Signaling Proteins from Lipid Rafts by Cyclodextrin or Chronic Desipramine Treatment

Sample	*N*	Cyclodextrin (% Control ± SEM)	Desipramine (% Control ± SEM)
Gsα	5	44.2 ± 16.9[a]	62.4 ± 4.5[b]
ACII	6	55.7 ± 13.4[a]	99.8 ± 15.2
ACV/VI	6	100 ± 14.9	107 ± 8.7
PKAcat	6	53.8 ± 12.6[b]	98 ± 5.1
PKAreg	6	59.8 ± 14.1[a]	99.8 ± 12.8
Actin	2	54 ± 0.0	96 ± 9.0
Tubulin	4	76.8 ± 10.9	94.5 ± 12.9

Quantification of the alteration in the TIFF localization of various transmembrane and membrane-associated proteins involved in Gsα signaling after antidepressant and cyclodextrin treatment. Data were obtained from densitometry of Western blots performed on these samples using antibodies against the given protein. One sample *t*-tests were performed based on the theoretical mean of 100% (Control). Significance was determined as $a = p < 0.05$ and $b = p < 0.02$.

Statistical Methods

Data were analyzed for statistical significance using a one-way ANOVA followed by Student–Newman–Keuls multiple comparison test using Prism 4.0, software package for statistical data analysis (Graph Pad Software Inc.). Additionally, a one sample *T*-test using a hypothetical mean of 100% for the control samples was performed (Table 1). Means are ± SEM and differences for all experiments were considered significant at $p < 0.05$.

RESULTS

Antidepressants Effect Gsα Membrane Domain Localization

Previous results demonstrated that the cellular localization of Gsα is altered in response to chronic, but not acute antidepressant treatment (Donati *et al*, 2001; Toki *et al*, 1999). These studies showed that Gsα localization to TIFFs was decreased after chronic antidepressant, but not chronic antipsychotic drug (chlorpromazine) nor nonantidepressant uptake inhibitor (amphetamine) treatment (Toki *et al*, 1999), and under these same conditions, it moved out of the process tips of C6 glioma cells (Donati *et al*, 2001). The membrane localization of caveolin-1, which is a membrane protein highly enriched in TIFFs, was not altered by similar antidepressant treatments (Toki *et al*, 1999). To confirm these results, we used the inactive fluoxetine analog LY368514 under chronic treatment conditions (Figure 1). LY368514 differs from fluoxetine by having a CF3 group in the para *vs* the ortho position (Figure 1a) and is ineffective

at inhibiting 5HT transport (Wong *et al*, 1995). Figure 1b is a representative immunoblot showing the reduced amount of Gsα in the TIFFs of antidepressant-treated cells compared to drug-free control cells. Notice that LY368514 did not decrease the amount of Gsα in the TIFFs. The results of three such experiments are quantified in Figure 1c where Gsα was reduced to 50 and 35% of control levels by desipramine and fluoxetine, respectively, while LY368514 had no effect on the amount of Gsα in the TIFFs.

Lipid Raft Disruption and Cellular Compartmentalization of Gsα

Cholesterol-depleting compounds, such as cyclodextrin, cause a disruption of the raft/caveolae structure by chelating the cholesterol that gives these domains their rigid structure (Miura *et al*, 2001; Nichols, 2003). Tricyclic antidepressants have been also shown to alter fluorescence anisotropy, suggesting a possible reordering of membrane lipids (Menkes *et al*, 1983). Thus, we sought to compare cyclodextrin and desipramine treatment with respect to effects on a number of membrane proteins within lipid raft domains represented by TIFFs. We chose to use only desipramine in the following studies because the effects of desipramine and fluoxetine on Gsα TIFF localization have been shown to be identical (Figure 1 and Toki *et al*, 1999). C6 glioma cells were treated with cyclodextrin (acute) or desipramine (chronic) as described in the Methods section. For cholesterol analysis, total cell lysates and crude membrane preparations were examined (Figure 2). TTX-100 detergent extracts from the treated C6 cells were subjected to sucrose density ultracentrifugation for TIFF isolation and protein analysis (Figure 3). Methyl-β-cyclodextrin treatment resulted in a significant (40%) decrease in the amount of membrane cholesterol compared to the control sample, while desipramine had no significant effect (Figure 2). Lipid rafts (TIFFs) were examined for proteins involved in the cAMP signaling cascade (adenylyl cyclase types II and V/VI, PKA regulatory and catalytic subunits, Gsα) and cytoskeletal proteins (tubulin and actin). While many of the proteins analyzed (Gsα, ACII, PKAcat, and PKAreg) were significantly removed from TIFFs due to cholesterol depletion of these membrane domains, only Gsα content was significantly changed by chronic desipramine treatment (Figure 3 & Table 1). Thus, even though chronic antidepressant treatment may have an effect on membrane lipids, Gsα appears to be a unique target of that effect.

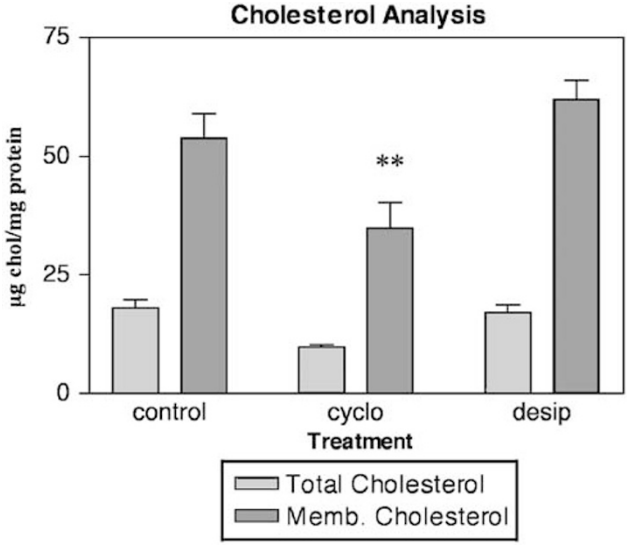

Figure 2 Desipramine treatment does not effect the total or membrane cholesterol content of C6 glioma cells. Total cholesterol levels were measured from C6 cell lysates and membrane cholesterol levels were measured from C6 membrane preparations and corrected for the amount of protein in the preparations. Treatment of cells with cyclodextrin as described in Methods section depleted approximately 50% of the total and membrane cholesterol, while chronic desipramine treatment had no effect on membrane cholesterol. Results from four separate experiments were plotted using Prism Graphpad 4.0. Data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison post-test. Mean ± SEM (**$p < 0.01$).

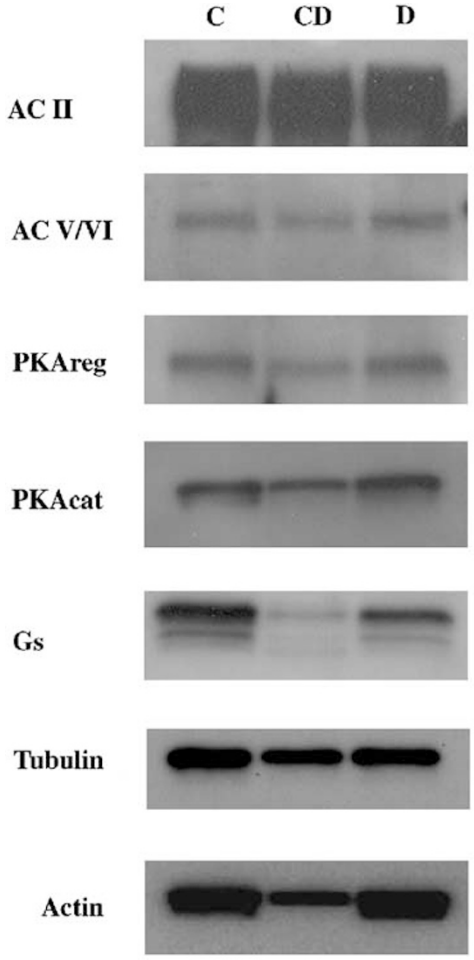

Figure 3 Gsα membrane localization is disrupted by antidepressant treatment, while other members of the cAMP signaling cascade are not effected. C6 glioma cells were treated with desipramine and cyclodextrin as described in Methods. In all, 1% Triton X-100 cell lysates were prepared and centrifuged and TIFFs were isolated, washed, and prepared for SDS-PAGE. Western blots were probed for proteins involved in the cAMP signaling cascade (adenylyl cyclase types II and V/VI, PKA regulatory and catalytic subunits, Gsα) and cytoskeletal proteins (tubulin and actin). Blots are a representative of 4–6 separate experiments with the exception of actin, which is 2. C = control, CD = cyclodextrin, DMI = desipramine.

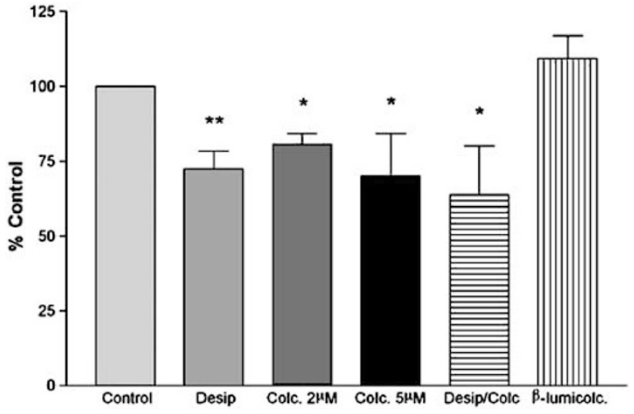

Figure 4 Microtubule disruption and chronic antidepressant treatment have comparable effects on Gsα membrane compartmentalization. C6 cells were treated with desipramine (DMI) as in Figure 1, colchicine (Colc.) (2 or 5 μM for 2 h.), β-lumicolchicine (β-Lum.) (5 μM for 2 h), desipramine followed by 2 μM colchicine (DMI/Colc), or nontreated controls (C). Results from 3–5 separate experiments were plotted on a graph using Prism Graphpad 4.0. Data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison post-test. Mean ± SEM (*$p < 0.05$, **$p < 0.01$).

Effects of Colchicine on Gsα Raft Localization

Earlier experiments showed that rat cerebral cortex synaptic membranes treated with microtubule disrupting drugs (colchicine or vinblastine) increased Gsα-stimulated adenylyl cyclase activity due to an apparent release of Gsα from a cytoskeletal 'tether', which prevented Gsα from a facile interaction with adenylyl cyclase (Rasenick *et al*, 1981). We wanted to determine if microtubule disruption in intact C6 cells would have antidepressant-like effects on Gsα raft localization. Colchicine (up to 10 μM) treatment of C6 cells for up to 4 h did not induce toxic effects (Hough *et al*, 1994). The data in Figure 4 demonstrate that acute treatment (2 h) with colchicine (2 or 5 μM) does in fact have an effect on Gsα raft localization similar to that seen with chronic desipramine treatment, but the two drug treatments are not additive. These treatment regimens resulted in a significant movement of 20–40% of Gsα out of the raft domains. Treatment with the inactive colchicine isomer, β-lumicolchicine, is without effect. The amount of raft localized Gsα compared to control is as follows: desipramine—72.4 ± 6.1%; 2 μM colchicine—80.0 ± 3.6%; 5 μM colchicine—70.0 ± 14%; desipramine/colchicine—63.7 ± 16.4%; and 5 μM β-lumicolchicine—109.0 ± 8.0. Thus, it appears that microtubule disruption had a similar effect on the cellular localization of Gsα as did desipramine treatment. The nonadditive result of these drugs suggests that they may be affecting the membrane distribution of Gsα in a similar manner.

DISCUSSION

Over 20 years ago, we first reported that long-term administration of various antidepressants enhanced guanylyl-5′-imidodiphoshate (Gpp(NH)p)- and fluoride-stimulated adenylyl cyclase activity in rat cortex and hypothalamus membranes (Menkes *et al*, 1983). This suggested that some aspect of Gsα was a target of antidepressant action, and that antidepressant treatment facilitated the activation of adenylyl cyclase by making Gsα more available without altering expression of that protein (Ozawa and Rasenick, 1989; Chen and Rasenick, 1995a). Similar antidepressant-induced increases in Gpp(NH)p-stimulated adenylyl cyclase activity have been observed *in vitro* using C6 glioma cells (Chen and Rasenick, 1995a). Later studies demonstrated that the membrane localization of Gsα is altered after chronic antidepressant treatment, and that the presence of Gsα in lipid raft/caveolar membrane domains may be important to its function as a mediator of antidepressant action (Donati *et al*, 2001; Toki *et al*, 1999).

Postreceptor neuronal cell signaling processes, particularly the downstream effects involving cAMP, have been the focus of many *in vivo* studies seeking to determine mechanisms of antidepressant action (Duman *et al*, 1997; Nibuya *et al*, 1996; Ozawa and Rasenick, 1989; Perez *et al*, 1991, 1989; Takahashi *et al*, 1999; Thome *et al*, 2000). While each of these studies focuses on a different aspect of intracellular signaling, they are all compatible with a sustained increase in cAMP synthesis. Similar effects using *in vitro* models of antidepressant action have also been observed (Chen and Rasenick, 1995a; Donati *et al*, 2001; Toki *et al*, 1999). Toki *et al* (1999) demonstrated that antidepressant treatment results in an alteration in the detergent extractability of Gsα from the plasma membrane of C6 glioma cells and rat cerebral cortex. Altered detergent solubility of Giα and Gβγ has also been demonstrated after chronic activation of Gi/o-coupled opiate receptors (Bayewitch *et al*, 2000). This change in detergent solubility corresponds to adenylyl cyclase 'superactivation'. Most recently, we have demonstrated that Gsα is internalized in the lipid rafts during the process of desensitization. This, at least for Gsα, suggests that rafts are domains where signaling is attenuated (Allen *et al*, 2004, submitted). (Note that the opposite may hold for other G proteins such as Gqα (Bhatnagar *et al*, 2004)). The current study confirms that the TTX-100 extractability of Gsα is altered by chronic antidepressant treatment. Additionally, the data presented here further substantiate the role of Gsα as a cornerstone for antidepressant-induced changes in cell signaling and also shed light on the possibility that an interaction between Gsα-tubulin is involved in this signaling process.

The functionally inactive fluoxetine analog LY368514, which differs from fluoxetine by having a CF3 group in the para *vs* the ortho position and is ineffective at inhibiting 5HT transport (Wong *et al*, 1995), was unable to decrease the amount of Gsα in C6 TIFFs under chronic treatment conditions (Figure 1). Chronic treatment of these cells with either a tricyclic (desipramine) or a selective serotonin reuptake inhibitor (fluoxetine), two fuctionally and structurally distinct antidepressants, was able to decrease the Gsα content of TIFFs by as much as 60% (Figure 1). We have observed that compounds with structural similarity to antidepressants, but without antidepressant effect, have no effect on Gsα localization, both in this study and in a previous study (Toki *et al*, 1999). Thus, it appears that movement of Gsα out of TIFFs is induced by chronic antidepressant treatment. This relocalization after chronic antidepressant treatment has not been observed with other G protein α subunits (Donati *et al*, 2001; Toki *et al*, 1999) and the total cellular content of G protein is not altered by

antidepressant treatment (Chen and Rasenick, 1995a; Emamghoreishi *et al*, 1996; Toki *et al*, 1999).

Recently, the lipid environment in which G proteins and their effectors are localized has been under investigation (Ostrom and Insel, 2004). G proteins appear to be present in both caveolae and cholesterol/sphingolipid-enriched plasma membrane raft domains (Oh and Schnitzer, 2001), and caveolin may play a role in mediating G protein signaling (Bhatnagar *et al*, 2004; Li *et al*, 1995). While both Gsα and Giα can move in and out of caveolae, they are predominantly found in lipid rafts complexed with $G\beta\gamma$ (Oh and Schnitzer, 2001). The direct association of G proteins with caveolin has been disputed (Huang *et al*, 1997); however, these authors conclude that the proteins involved in the hormone-sensitive adenylyl cyclase system are indeed localized to a specialized subdomain of the plasma membrane. Figure 3 clearly shows that many of the proteins involved in the Gsα signaling cascade are localized in the caveolae/lipid raft-enriched TIFFs. The cholesterol chelating drug, methyl-β-cyclodextrin, can be used to disrupt these cholesterol-rich membrane domains and study the proteins localized within them (Miura *et al*, 2001; Nichols, 2003). With the exception of adenylyl cyclase V/VI, all of the other proteins shown in Figure 3 and Table 1 have a decreased TIFF localization after cholesterol depletion, indicating the importance of these domains as scaffolds to concentrate signaling molecules. While Gsα is extremely sensitive to the loss of cholesterol, chronic desipramine treatment does not affect the cholesterol content of the membrane (Figure 2) nor the raft localization of these proteins, with the exception of Gsα (Figure 3). This suggests that the antidepressant-induced disruption of Gsα from TIFFs is not due to any direct effect of these drugs on cholesterol or cholesterol-dependent membrane structure. Rather, it appears that chronic antidepressant treatment directly disrupts the association between Gsα and some other molecule(s) that target Gsα to TIFFs.

Studies by Miyamoto *et al* (1997) revealed that chronic, but not acute treatment of rats with desipramine resulted in decreased microtubule assembly and nucleation in the cerebral cortex. Chronic desipramine treatment has also been reported to phosphorylate certain microtubule-associated proteins (Perez *et al*, 1989). A fraction of PKA II associates with microtubules (Perez *et al*, 1993; Theurkauf and Vallee, 1982) via MAP2, which serves as an anchor and substrate for PKA II (Obar *et al*, 1989; Rubino *et al*, 1989). Thus, chronic PKA II activation as seen during antidepressant treatment leads to MAP2 phosphorylation and a subsequent decrease in microtubule assembly (Perez *et al*, 2000).

The decrease in microtubule assembly and nucleation due to increased PKA II activity seen after chronic antidepressant treatment (Perez *et al*, 2000) should lead to increased tubulin dimers within the cell. Tubulin has been shown to interact directly with Gsα (Wang and Rasenick, 1991; Wang *et al*, 1990; Yan *et al*, 1996, 2001) and transfer GTP to G protein α subunits. Experiments with a GTP photoaffinity analog suggest that tubulin dimers form complexes with the α subunits of Gs, Gi1, or Gq and activate them through a direct nucleotide transfer (transactivation) mechanism (Popova and Rasenick, 2000; Rasenick and Wang, 1988; Roychowdhury *et al*, 1993; Yan *et al*, 2001). Antidepressants may increase the pool of available tubulin dimers leading to the sustained transactivation of Gsα and subsequent association of that Gsα with adenylyl cyclase in a receptor-independent manner. This would allow increased cAMP formation even in the presence of a downregulation of receptors normally coupled to Gsα. Thus, microtubule-disrupting agents should have antidepressant-like effects on Gsα TIFF localization by increasing the pool of tubulin dimers capable of transactivation. Figure 4 demonstrates that colchicine does in fact decrease the amount of Gsα in the TIFF fraction comparable to the extent seen with desipramine treatment.

Alternatively, or in conjunction with the above hypothesis, chronic desipramine treatment may lead to a release of the microtubule tether hindering the coupling of Gsα with adenylyl cyclase. It is well established that treatment of cells with microtubule-disrupting agents like colchicine and vinblastine leads to an increase in G protein-mediated activation of adenylyl cyclase (Kennedy and Insel, 1979; Rasenick *et al*, 1981, 1984; Leiber *et al*, 1993; Jasper *et al*, 1995; Nishigaki *et al*, 1998; Rudolph *et al*, 1979). These potential actions would allow the cAMP signal to be propagated after receptor desensitization mediated by antidepressant treatment (reviewed by Sulser, 1984). Recent evidence has shown that it is the duration of cAMP expression rather than the quantity of the cAMP increase that is important for the downstream activation of CREB-mediated gene transcription (Baker *et al*, 2004), and there is evidence that chronic antidepressant treatment leads to an increase in the amount of CREB and phospho-CREB in the cell (Duman *et al*, 1997; Nibuya *et al*, 1996; Takahashi *et al*, 1999; Thome *et al*, 2000).

Previous studies have suggested that depletion of serum cholesterol in both humans and non-human primates leads to depressive symptoms (Kaplan *et al*, 1994; Steegmans *et al*, 2000; Golomb *et al*, 2004), although this is not a universal finding (Yang *et al*, 2003). These data, at first glance, appear to contradict the findings that agents such as cyclodextrin that chelate membrane cholesterol have similar effects on Gsα localization as chronic antidepressant treatment. It must be noted, however, that the cyclodextrin effect in this study is on cellular (total and membrane) and not serum cholesterol. Furthermore, the antidepressant drugs tested in this study had no effect on either membrane or total cellular cholesterol. Thus, it is possible that effects of lowered serum cholesterol on mood may have been due to altered levels of steroid cholesterol metabolites.

The structure and function of caveolae and lipid rafts in the nervous system is beginning to be elucidated (Maekawa *et al*, 2003; Masserini *et al*, 1999; Tsui-Pierchala *et al*, 2002) and these structures may become important in defining neurological and psychiatric disease. Data in this report clearly demonstrate that Gsα raft localization is altered by chronic antidepressant treatment, and that this may be due to an alteration of the membrane-associated microtubule cytoskeleton. These data are consistent with the suggestion that chronic antidepressant treatment ultimately leads to an increase in CREB-mediated gene transcription and BDNF signaling. If we couple the recent findings implicating lipid rafts in the maintenance of synaptic density and morphology in rat primary hippocampal neurons (Hering *et al*, 2003) with those demonstrating that chronic rolipram

(a phosphodiesterase inhibitor) treatment increases hippocampal neuritogenesis in mice (Nakagawa *et al*, 2002), it appears that lipid rafts may be important in the formation and maintenance of the synapses of these newly formed neurites. Data from this report clearly show that chronic antidepressant treatment does not alter the cholesterol content of the plasma membrane, only the content of Gsα in lipid rafts, and therefore would not hinder, and may enhance synapse formation in these new neurons. Further study on the G protein/raft/cytoskeleton relationship may help to establish relationships between cell structure and G protein signaling and may also lead to an increased understanding of the cellular basis of mood disorders.

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