

Chronic Treatment with Escitalopram but Not *R*-Citalopram Translocates $G\alpha_s$ from Lipid Raft Domains and Potentiates Adenylyl Cyclase: A 5-Hydroxytryptamine Transporter-Independent Action of This Antidepressant Compound

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ABSTRACT

Chronic antidepressant treatment has been shown to increase adenylyl cyclase activity, in part, due to translocation of $G\alpha_s$ from lipid rafts to a nonraft fraction of the plasma membrane where they engage in a more facile stimulation of adenylyl cyclase. This effect holds for multiple classes of antidepressants, and for serotonin uptake inhibitors, it occurs in the absence of the serotonin transporter. In the present study, we examined the change in the amount of $G\alpha_s$ in lipid raft and whole cell lysate after exposing C6 cells to escitalopram. The results showed that chronic (but not acute) escitalopram decreased the content of $G\alpha_s$ in lipid rafts, whereas there was no change in overall $G\alpha_s$ content. These effects were drug dose- and exposure time-dependent. Although *R*-citalopram has been reported to antagonize some effects of escitalopram, this

compound was without effect on $G\alpha_s$ localization in lipid rafts, and *R*-citalopram did not inhibit these actions of escitalopram. Escitalopram treatment increased cAMP accumulation, and this seemed due to increased coupling between $G\alpha_s$ and adenylyl cyclase. Thus, escitalopram is potent, rapid and efficacious in translocating $G\alpha_s$ from lipid rafts, and this effect seems to occur independently of 5-hydroxytryptamine transporters. Our results suggest that, although antidepressants display distinct affinities for well identified targets (e.g., monoamine transporters), several presynaptic and postsynaptic molecules are probably modified during chronic antidepressant treatment, and these additional targets may be required for clinical efficacy of these drugs.

The selective serotonin reuptake inhibitors (SSRIs) are the most frequently used drugs for treatment of depressive and anxiety disorders. However, the mechanism of action at the molecular and cellular level remains unclear. Certainly, one effect of SSRIs is binding to the 5-HT transporter and preventing reuptake of 5-HT into serotonergic neurons, increasing serotonergic transmission. However, 5-HT reuptake inhibition occurs rapidly, whereas the clinical effects require several weeks of drug administration. So, gradual changes should happen in the brain that can match the delayed re-

sponse of antidepressants. There are several actions of SSRIs that are of delayed onset. SSRIs seem to elicit hippocampal neurogenesis (Santarelli et al., 2003); however, the linkage of this phenomenon to behavior responses to antidepressants has not been established (Wang et al., 2008). Synaptic rearrangement has also been associated with antidepressant action (Guest et al., 2004), and this is often linked to increased brain-derived neurotrophic factor, which, in turn, results from up-regulated cAMP signaling (Malberg and Blendy, 2005; Gass and Riva, 2007).

On the molecular level, it has long been established that chronic antidepressant treatment increases the coupling between $G\alpha_s$ and adenylyl cyclase. Menkes et al. (1983) found that long-term antidepressant treatment enhanced guanylyl-5'-imidodiphosphate [Gpp(NH)p]- and fluoride-stimulated adenylyl cyclase activity in rat cortex and hypothalamus membranes (Menkes et al., 1983). This suggested that $G\alpha_s$ or

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ABBREVIATIONS: SSRI, selective serotonin reuptake inhibitor; 5-HT, 5-hydroxytryptamine (serotonin); Gpp(NH)p, guanyl-5'-imidodiphosphate; SERT, serotonin transporter; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; TTX, Triton X; DTT, dithiothreitol; ACVI, type VI adenylyl cyclase; ANOVA, analysis of variance; LSD, least significant difference.

some G_{α_s} -associated protein, was one of the targets of chronic antidepressant treatment (Ozawa and Rasenick, 1989; De Montis et al., 1990). Further studies from this laboratory demonstrated that chronic antidepressant treatment of rats or C6 glioma cells redistributed the G_{α_s} in the plasma membrane, out of lipid raft fractions (Toki et al., 1999; Donati and Rasenick, 2005) and into nonraft plasma membrane domains. Consistent with this is the observation that G_{α_s} signaling is attenuated in the raft fraction and coupling to adenylyl cyclase is facilitated in nonraft membrane fractions (Allen et al., 2005; Head et al., 2006; Allen et al., 2009). It is noteworthy that these actions occur independently of the actions of SSRIs on serotonin reuptake transporter (SERT), as C6 glioma cells lack SERT. Thus, it is hypothesized that the actions of chronic antidepressants that potentiate the activation of adenylyl cyclase by G_{α_s} are "postsynaptic" in nature and occur independently of SERT.

Escitalopram is a selective SSRI; it is the therapeutically active *S*-enantiomer citalopram, a racemic 1:1 mixture of *S*-(+)-citalopram and *R*-(-)-citalopram (escitalopram and *R*-citalopram; Hyttel et al., 1992). *R*-Citalopram does not display the antidepressant action of citalopram and *R*-citalopram inhibited the action of escitalopram both in vitro and in behavioral studies (Sánchez et al., 2004). This was hypothesized to be due to binding of *R*-citalopram to a separate site on the SERT. It has also been suggested that escitalopram is more potent than citalopram clinically and displays an earlier therapeutic onset (Montgomery et al., 2001; Gorman et al., 2002).

To test whether escitalopram continued to demonstrate antidepressant effects in a SERT-independent manner, we treated C6 glioma cells (which lack SERT) with this compound as well as *R*-citalopram. Escitalopram displayed rapid and robust "antidepressant activity" as measured by translocation of G_{α_s} and increase in G_{α_s} -activated adenylyl cyclase, whereas *R*-citalopram was without effect. These effects of escitalopram were not damped by the presence of *R*-citalopram, suggesting that the SERT-independent effects of escitalopram on G_{α_s} raft localization and enhanced coupling between G_{α_s} and adenylyl cyclase are molecularly disparate from those actions at SERT. This study also indicated that the effects of escitalopram on G_{α_s} are similar, albeit more potent and efficacious than other antidepressant compounds.

Thus, although several selective targets of antidepressants have been identified, it is likely that there are several pre- and postsynaptic molecules that are modified during the chronic treatment with these compounds and these additional components are required for clinical effectiveness.

Materials and Methods

Cell Culture and Drug Treatment. C6 cells in 150-cm² flasks were cultured in Dulbecco's modified Eagle's medium, 4.5 g/l glucose, 10% newborn calf serum (HyClone Laboratories, Logan, UT), and 100 mg/ml penicillin and streptomycin at 37°C in humidified 10% CO₂ atmosphere. The cells were treated with escitalopram, *R*-citalopram (gifts from Lundbeck, Copenhagen, Denmark) or fluoxetine (Sigma-Aldrich, St. Louis, MO) for 1, 3, or 5 days. Both escitalopram and *R*-citalopram were dissolved in dimethyl sulfoxide; the DMSO group acted as another control group. The culture media and drug were changed daily. There was no change in morphology of cells during the period of exposure to antidepressants.

Cell Membrane and Lipid Rafts Preparation. After treatment, cells were washed, and Triton X (TTX)-100 insoluble membrane fractions were prepared as described by Li et al. (1995), with slight modification (Donati and Rasenick, 2005). In brief, two flasks of C6 cells were scraped into 0.75 ml of HEPES buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, and protease inhibitors) containing 1% TTX-100. Cells were homogenized with 10 strokes of a Potter-Elvehjem homogenizer. The homogenate was then mixed with an equal volume of 80% sucrose prepared in HEPES buffer to form 40% sucrose and loaded at the bottom of an ultracentrifuge tube. A step gradient was generated by layering, sequentially, 30, 15, and 5% sucrose over the homogenate. Gradients were centrifuged at 200,000g for 20 h in an SW55 rotor (Beckman, Palo Alto, CA). Two or three opaque bands were confined between the 15 and 30% sucrose layers. These bands were removed from the tube, diluted 3-fold with HEPES buffer, and pelleted in a microcentrifuge at 16,000g. The pellet was resuspended in HEPES buffer and subsequently analyzed by immunoblotting.

To prepare membrane fractions, the cellular homogenate was centrifuged at 1000g for 10 min to remove nuclei, and total cellular membranes were obtained from the supernatant by 100,000g centrifugation for 30 min. The total membrane pellet was resuspended into HEPES buffer containing 1% Triton X-100. Samples of membrane pellet were analyzed for specific proteins by immunoblotting.

Measurement of cAMP Accumulation in Antidepressant-Treated C6 Cells. cAMP accumulation was monitored by measuring [³H]cAMP as described by Obara et al. (2005), with minor modification. In brief C6 cells, treated with drugs as indicated and cultured in 12-well plates, were incubated with 4 μCi/ml [³H]adenine for 24 h at 37°C. Labeled cells were washed once with assay medium (40 mM HEPES-buffered Dulbecco's modified Eagle's medium) and then incubated with the same medium with and without 10 μM isoproterenol or 100 μM forskolin at 37°C for 30 min. Reactions were terminated by addition of ice-cold trichloroacetic acid (5%, final concentration), and cell lysates were incubated at 4°C for 3 h. [¹⁴C]cAMP then was added to correct for the recovery of [³H]cAMP. Cells were scraped into tube on ice. The supernatant was obtained by centrifugation at 15,000g for 30 min and cAMP was isolated by the method of Salomon (1979). cAMP production was expressed as [³H]cAMP per [³H]adenine incorporated into cells (percentage). All assays were performed in triplicate.

Adenylyl Cyclase Assay. Adenylyl cyclase was assayed as described previously (Rasenick et al., 1989). C6 cells were treated as indicated, harvested, and resuspended in 1 ml of HEPES-sucrose buffer (15 mM HEPES, 0.25 M sucrose, protease inhibitors, and 1 mM DTT, pH 7.5). The cells were homogenized by 10 strokes with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1000g for 10 min and the nuclear pellet discarded. Membrane fractions were obtained by 100,000g centrifugation for 30 min. The membranes (pellet) were washed and resuspended in 700 μl of HEPES buffer (15 mM HEPES, protease inhibitors, and 1 mM DTT, pH 7.5) and stored at -80°C until use. Then, 25 μg of membranes was added into a reaction mixture with 10 μM GTPγS, 10 mM NaF (+ 20 μM AlCl₃), or 10 μM isoproterenol for 20 min at 30°C in 100 μl of medium containing 15 mM HEPES, pH 7.5, 0.05 mM ATP, 2.5 μCi/ml [α-³²P]ATP, 5 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.05 mM cAMP, 0.01 mM GTP, 0.25 mg/ml bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 mg/ml creatine phosphate, and 0.14 mg/ml creatine phosphokinase. The reaction was terminated by adding 0.1 ml of a solution containing 2% SDS, 1.4 mM cAMP, and 40 mM ATP. [³²P]cAMP was isolated by the method of Salomon (1979) using [³H]cAMP to monitor recovery. All assays were performed in triplicate.

Immunoblotting. Five micrograms of lipid raft fraction, 20 μg of membrane pellet, or 10 μg of whole cell lysate was subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat

dry milk diluted in Tris-buffered saline/Tween 20 solution (10 mM Tris-HCl, 159 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h. After three washes with Tris-buffered saline/Tween 20, membranes were incubated with polyclonal rabbit $G\alpha_s$ antibody (1:5000 dilution; Millipore, Billerica, MA) or polyclonal ACV/VI (1:300; from Richard Green, University of Illinois, Chicago, IL) or ACII antibody (1:350; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. After secondary antibody incubation, immunoreactivity was detected with an enhanced chemiluminescent detection (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoreactive bands were quantified by ImageQuant software (GE Healthcare) after scanning densitometry. In all experiments, the original membranes were stripped with stripping buffer (100 mM β -mercaptoethanol, 62.5 mM Tris-HCl, and 2% SDS, pH 6.7) and reprobed using a monoclonal mouse anti- β -actin antibody (1:10,000 dilution), followed by immunodetection. To adjust for protein loading variation, $G\alpha_s$ or ACVI was normalized for the level of β -actin. Note that although ACII antibodies showed no changes in control versus treated groups, these antibodies are rather nonspecific in cells where ACII expression is low. Thus, we hesitate to make any conclusion based on data obtained with them.

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

Results

Effect of Escitalopram and *R*-Citalopram on the Cellular Distribution of $G\alpha_s$. $G\alpha_s$ localization in lipid rafts is decreased after chronic antidepressant treatment both in frontal cortex membranes from rats and in C6 glioma cells, and coupling between $G\alpha_s$ and adenylyl cyclase is increased (Toki et al., 1999; Donati and Rasenick, 2005). This occurs without changes in the content of $G\alpha_s$. To test whether escitalopram had similar effects, C6 cells were exposed to escitalopram, *R*-citalopram, or fluoxetine, respectively. The results show that both escitalopram and fluoxetine reduced amount of $G\alpha_s$ in the lipid raft compared with drug-free control cells, whereas *R*-citalopram was without effect (Fig. 1a). As shown in Fig. 1b, total $G\alpha_s$ content was not changed.

Escitalopram Translocates $G\alpha_s$ from Lipid Rafts in a Dose- and Time-Dependent Manner. To further characterize the effect of escitalopram on the amount of $G\alpha_s$ in lipid rafts, C6 cells were treated with 0, 0.2, 1, 5, and 10 μ M escitalopram, respectively, for 3 days. There was an escitalopram dose-dependent displacement of $G\alpha_s$ from lipid rafts (Fig. 2). Escitalopram is more potent than fluoxetine or tricyclic compounds (Toki et al., 1999; Donati et al., 2001). To determine the time dependence of escitalopram treatment,

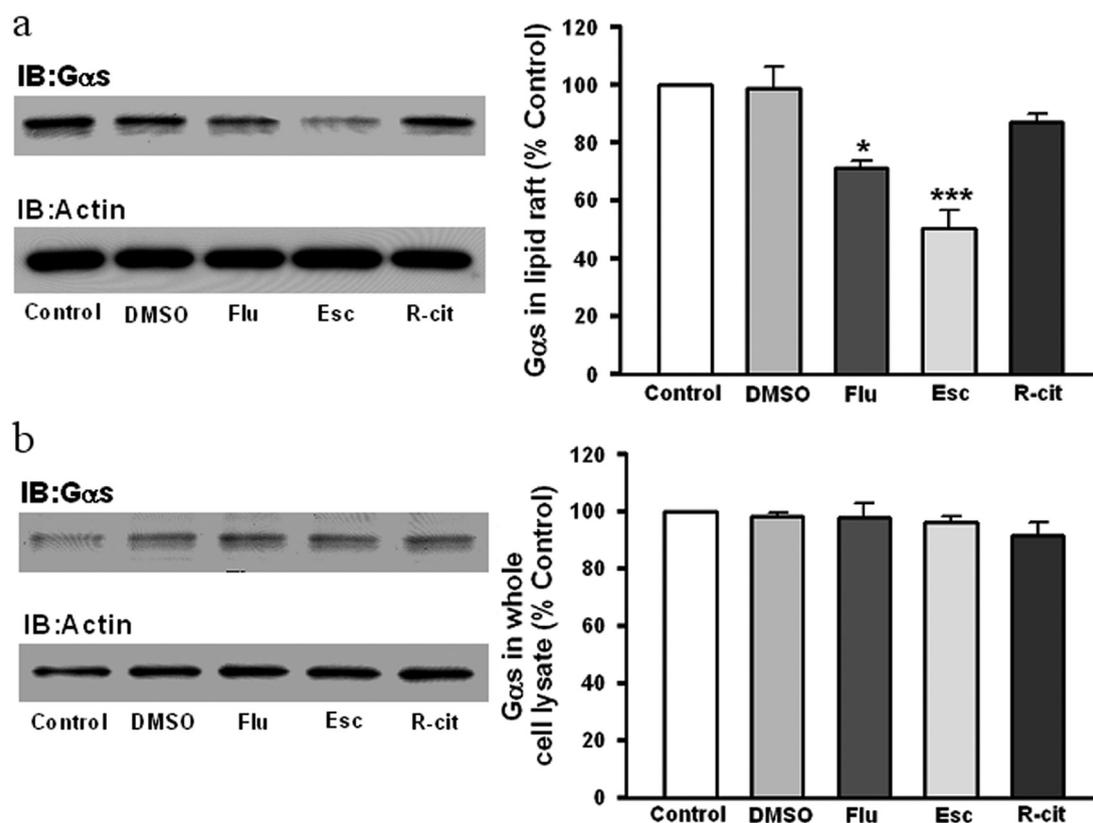


Fig. 1. Effect of escitalopram and *R*-citalopram on the cellular and lipid rafts content of $G\alpha_s$. a, C6 cells were treated chronically with fluoxetine (Flu), escitalopram (Esc), and *R*-citalopram (*R*-cit) (at 10 μ M for 3 days). The detergent-insoluble lipid rafts were obtained by sucrose density gradients fractionation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot for $G\alpha_s$ content. A representative blot of $G\alpha_s$ protein is shown (top); the same blot was reprobed for actin (bottom). The figure shows the percentage of change in $G\alpha_s$ protein above control in the lipid raft membrane fractions from three independent experiments. b, $G\alpha_s$ content in whole cell lysates detected with immunoblots (top) in comparison with actin (bottom). The figure is a quantification of $G\alpha_s$ protein in whole cell lysates and is presented as a percentage of control ($n = 3$). Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means. Data are represented as mean \pm S.E.M. (*, $p < 0.05$; ***, $p < 0.001$ versus control).

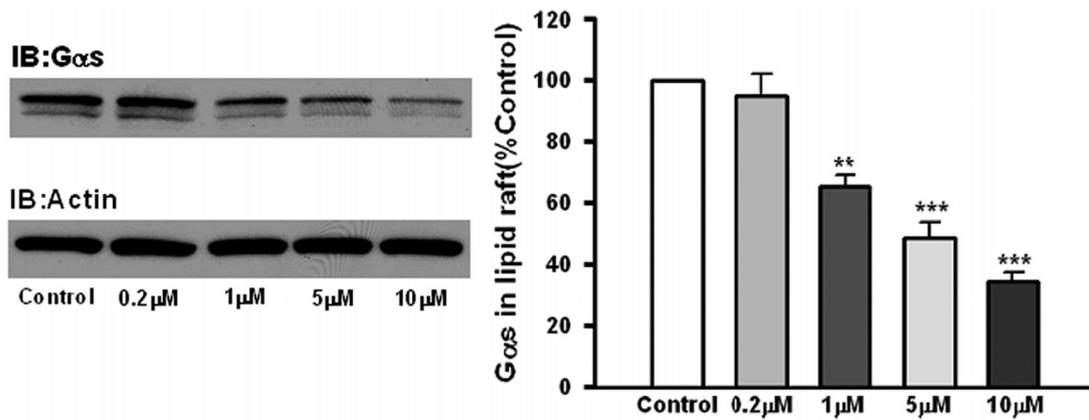


Fig. 2. Escitalopram-induced translocation of $G\alpha_s$ from lipid rafts is dose-dependent. C6 cells were treated with 0.2, 1, 5, and 10 μM escitalopram, respectively, for 3 days, and detergent-insoluble lipid rafts were obtained as described in Fig. 1. The quantity of $G\alpha_s$ in lipid rafts was determined by Western blotting. A representative blot of $G\alpha_s$ protein is shown (top) as well as actin protein (bottom). The figure shows the percentage of change in $G\alpha_s$ protein above control in the lipid raft membrane fractions ($n = 4$). Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means. Data are represented as mean \pm S.E.M. (**, $p < 0.01$; ***, $p < 0.001$ versus control).

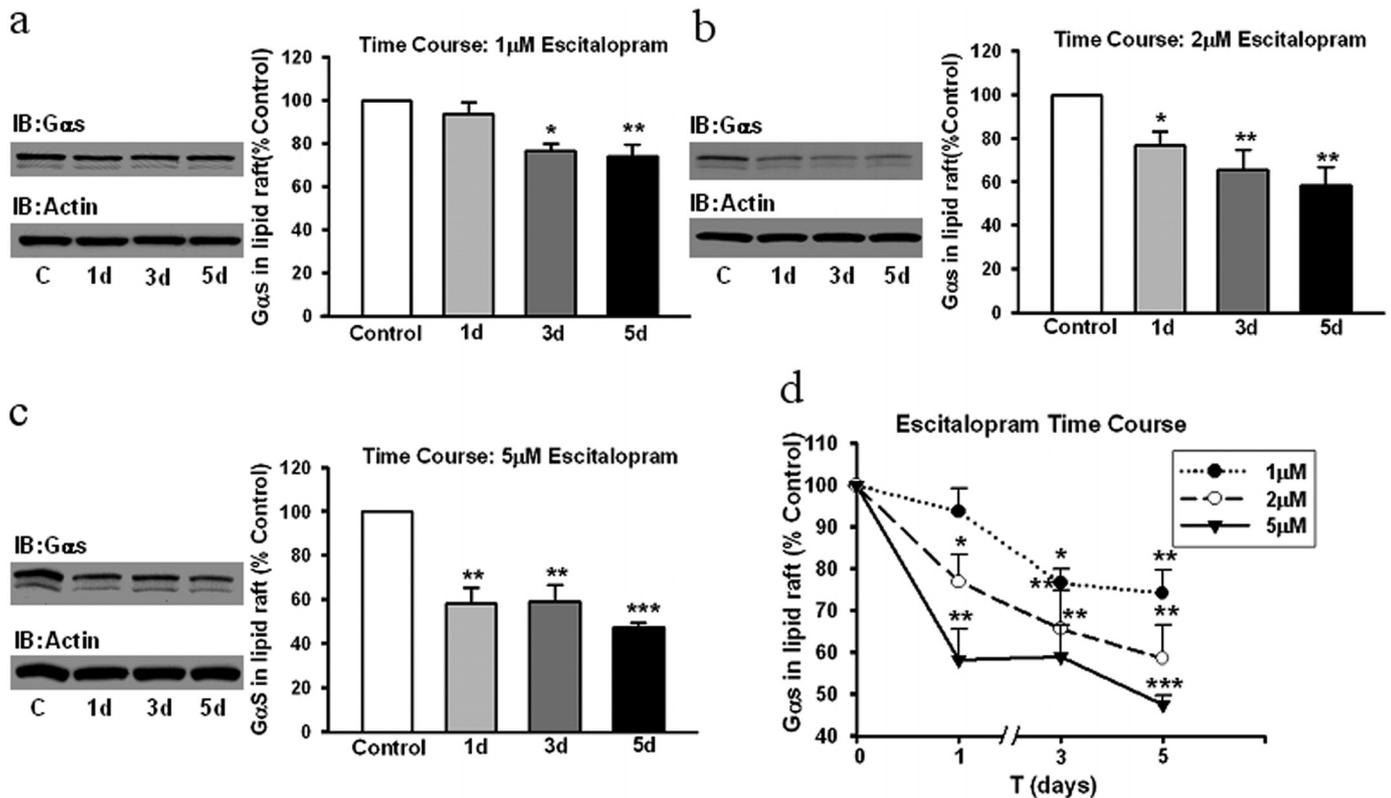


Fig. 3. Time course for escitalopram-induced translocation of $G\alpha_s$ from lipid rafts. C6 cells were cultured for 5 days and drug was initiated in the final 1, 3, or 5 days of this period with escitalopram at the indicated dose. Cells treated for 1 day were grown for 4 days and treated for the final 24 h, and those treated for 3 days were grown for 2 days before escitalopram treatment. Subsequent to treatment, lipid raft fractions were isolated as described above. Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means. Data are represented as mean \pm S.E.M. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control). a to c, $G\alpha_s$ protein contents in lipid rafts were detected by immunoblot (top) and compared with actin as a loading control (bottom) after C6 cells were treated with 1 μM escitalopram (Esc) for 1, 3, and 5 days, respectively (1 and 2 μM Esc; $n = 4$; 5 μM Esc, $n = 3$). C, control. d, compiled time course data for multiple experiments.

C6 cells were exposed to medium containing 1, 2, or 5 μM escitalopram for a total 5 days and drug was initiated in the final 1, 3, or 5 days of this period. Figure 3, a to c, shows representative immunoblots, revealing that the content of $G\alpha_s$ in lipid rafts decreased after cells were treated with 1, 2, or 5 μM escitalopram. Figure 3d shows that a significant reduction of $G\alpha_s$ from lipid rafts occurred in every treatment

regimen compared with 0 day except for 1 day treatment with 1 μM escitalopram.

R-Citalopram Does Not Antagonize the Escitalopram-Induced Redistribution of $G\alpha_s$ from Lipid Rafts. The distribution of $G\alpha_s$ in lipid rafts was analyzed after treatment of cells with 1 μM escitalopram with or without *R*-citalopram (1 or 5 μM). We chose 5 μM *R*-citalopram because

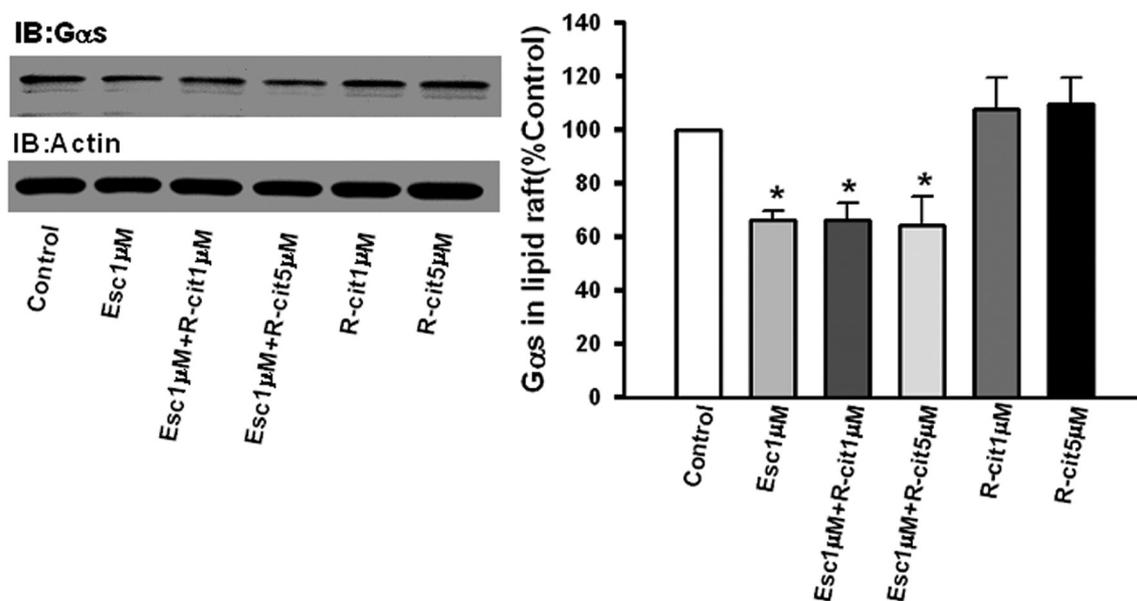


Fig. 4. *R*-Citalopram does not inhibit escitalopram-induced translocation of $G\alpha_s$ from lipid rafts. C6 cells were treated with escitalopram (1 μ M) alone or together with *R*-citalopram (1 or 5 μ M) for 3 days. After treatment, lipid raft fractions were isolated as described above. A representative immunoblot shows $G\alpha_s$ protein (top) and actin protein (bottom) in lipid rafts. The figure shows the percentage of change in $G\alpha_s$ protein above control in the lipid raft membrane fractions ($n = 5$). Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means. Data are represented as mean \pm S.E.M. (*, $p < 0.05$ versus control).

clinical studies have shown that levels of *R*-citalopram are higher than escitalopram in citalopram-treated subjects at steady state. Regardless, *R*-citalopram did not inhibit escitalopram-induced translocation of $G\alpha_s$ from lipid rafts (Fig. 4). Prior studies suggested inhibition of escitalopram by the *R*-enantiomer, but it was suggested that the compound bound to two different sites on SERT (Plenge et al., 2007). The effects seen here are independent of SERT, which is not present in C6 cells, and are consistent with a lack of *R*-citalopram effect.

Escitalopram Treatment Enhances Isoproterenol, but Not Forskolin-Induced cAMP Accumulation in C6 Cells. Previous studies reported that the β -receptor binding and isoproterenol-stimulated adenylyl cyclase were decreased in limbic forebrain of rats by chronic antidepressant treatment (Vetulani and Sulser, 1975). The "postsynaptic" C6 cells show that these effects are temporally unrelated and that down-regulation of β -receptor/ G_s coupling precedes antidepressant-induced augmentation of G_s -activated adenylyl cyclase (Chen and Rasenick, 1995b). Furthermore, citalopram was not seen to alter β -receptor-elicited cyclic AMP accumulation or β -receptor density (Sapena et al., 1994). We examined cAMP accumulation after escitalopram treatment. There is no difference in basal activity between control and escitalopram groups. Nevertheless, escitalopram-treated cells showed marked increase in cAMP accumulation in response to isoproterenol (Fig. 5). Neither escitalopram nor *R*-citalopram has any effect on intrinsic cAMP accumulation, as both basal and forskolin-stimulated cAMP-accumulation are comparable in untreated, escitalopram-treated, and *R*-citalopram-treated cells. Previous work suggests that antidepressant treatment does not change agonist sensitivity for cAMP accumulation (Sapena et al., 1994; Chen and Rasenick, 1995b), rendering it likely that escitalopram acts by increasing coupling between $G\alpha_s$ and adenylyl cyclase in nonraft membrane regions.

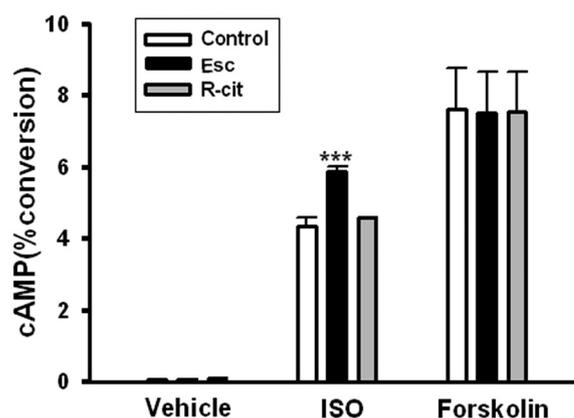


Fig. 5. Chronic escitalopram (Esc) treatment potentiates G protein-coupled receptor-activated cAMP production. C6 cells grown in 12-well plates were exposed to 10 μ M escitalopram or *R*-citalopram after plating for a period of 3 days ($n = 3$). On day 2, cells were also incubated for 24 h with 4 μ Ci/ml [2,8- 3 H]adenine to label the total pool of cellular ATP. Cells were washed with serum-free Dulbecco's modified Eagle's medium (without Esc or [3 H]adenine) and incubated with or without 10 μ M isoproterenol or 100 μ M forskolin at 37°C for 30 min. cAMP production was expressed as [3 H]cAMP relative to [3 H]adenine incorporated into cells (percentage of conversion of [3 H]adenine to [3 H]cAMP). Data presented are the mean values \pm S.E.M. from three independent experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means (***, $p = 0.001$ versus control).

Escitalopram Treatment Enhances $G\alpha_s$ -Stimulated Adenylyl Cyclase Activity in C6 Membranes. To further confirm that $G\alpha_s$ is the target of escitalopram action, fluoride or GTP γ S-induced adenylyl cyclase activity was examined in membranes from cells treated with escitalopram. Escitalopram (at 10 μ M for 3 days) did not alter basal activity of adenylyl cyclase in membranes but resulted in a significant increase in fluoride or GTP γ S-induced adenylyl cyclase activity (Fig. 6). Chronic escitalopram treatment also resulted in a significant increase in isoproterenol-induced adenylyl

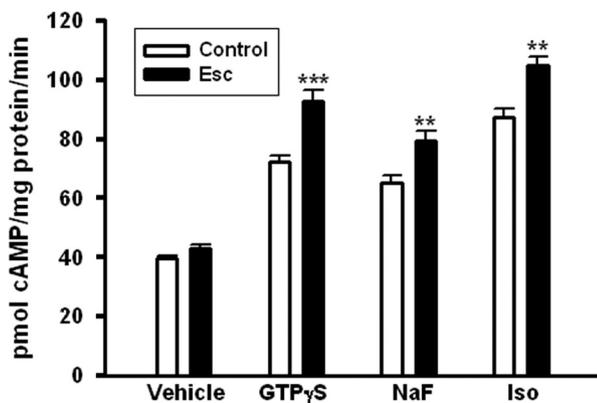


Fig. 6. Escitalopram treatment increases on GTP γ S, fluoride and isoproterenol-induced adenylyl cyclase activity in C6 membranes. C6 cell membranes were made from cells exposed to medium containing 10 μ M escitalopram for 3 days ($n = 4$). The membranes were assayed for adenylyl cyclase activity as described under *Materials and Methods*. Data represented as mean \pm S.E.M. of triplicate determinations from one of four similar experiments are shown. Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means (**, $p < 0.01$; ***, $P < 0.001$ versus control).

cyclase activity (Fig. 6), and these results were consistent with the results from intact cells. Furthermore, the escitalopram-induced increase in $G\alpha_s$ -activated adenylyl cyclase did not result from an intrinsic increase in adenylyl cyclase, as immunoblots for ACVI, the dominant adenylyl cyclase isoform in C6 cells, showed no change subsequent to drug treatment (Fig. 7).

Discussion

5-HT transporters show selective and specific binding for SSRIs, including escitalopram; thus, it is difficult to explain the requirement for chronic treatment with these compounds to observe clinical effects. Previous results have suggested that $G\alpha_s$ /adenylyl cyclase coupling was one of the targets of antidepressant action and that chronic treatment (3 weeks in rats and 3 days in cells) was required to observe this effect (Menkes et al., 1983; Ozawa and Rasenick, 1989; Chen and Rasenick, 1995b). Later studies demonstrated antidepressant treatment-induced increases in $G\alpha_s$ /adenylyl cyclase from a TTX-100-

insoluble membrane domain to a TTX-100-soluble membrane (Toki et al., 1999). These results are consistent with a study revealing that a number of antidepressant drugs concentrate in lipid rafts subsequent to chronic treatment (Eisensamer et al., 2005). Allen et al. (2005) demonstrated that $G\alpha_s$ is internalized from lipid rafts after receptor activation and that disruption of rafts, either by cholesterol depletion or caveolin knockdown, inhibits internalization.

It is suggested that the efficiency of $G\alpha_s$ signaling is attenuated in lipid rafts and this has been demonstrated using genetic manipulation of raft proteins in both cells and animals (Allen et al., 2009). These most recent data demonstrate more efficient coupling of $G\alpha_s$ to adenylyl cyclase in the nonraft plasma membrane domains. A previous study also provided evidence that $G\alpha_s$ signaling was inhibited in TTX-100-insoluble membrane (Li et al., 1995). These are also consistent with the notion that chronic treatment with antidepressants increases the coupling between those signaling molecules in the nonraft membrane regions.

In this vein, we set out to test whether escitalopram altered $G\alpha_s$ localization in lipid rafts and facilitated G_s protein coupling with adenylyl cyclase and how this might be altered by *R*-citalopram. The results showed that escitalopram does prevent $G\alpha_s$ localization in lipid rafts, whereas *R*-citalopram did not change the amount of $G\alpha_s$ in lipid rafts. These results are consistent with clinical effects of these compounds.

Although *R*-citalopram has no reuptake inhibition properties, several *in vitro* and *in vivo* experiments have found that it could counteract the effect of escitalopram (Sánchez et al., 2003; Sánchez et al., 2004). The mechanism through which *R*-citalopram exerts its inhibition on escitalopram is not yet established. However, it is proposed that there exist at least two binding sites on SERT for inhibitors and 5-HT, a primary, high-affinity binding site and a low-affinity allosteric site (Chen et al., 2005). The high-affinity site mediates the action of uptake inhibitors, whereas the low-affinity site modulates the binding of uptake inhibitor and 5-HT. It is suggested that the action of escitalopram on inhibiting uptake of 5-HT is attenuated because the binding of *R*-citalopram with the allosteric site results in a conformational change in SERT (Plenge et al., 2007). These data are consis-

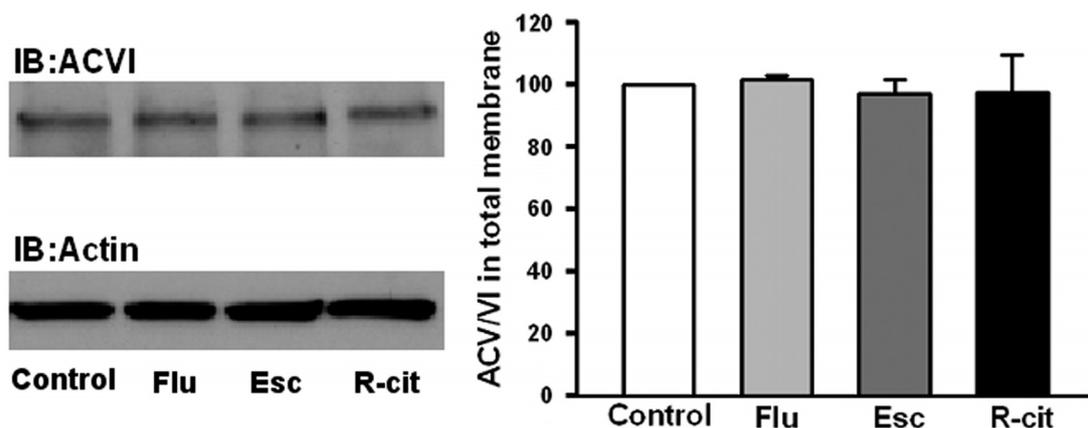


Fig. 7. C6 cells were treated chronically with fluoxetine (Flu), escitalopram (Esc), and *R*-citalopram (*R*-cit) (at 10 μ M for 3 days). Total cellular membranes were obtained as described under *Materials and Methods*. The quantity of ACVI in the membrane was determined by Western blotting. A representative immunoblot shows ACVI (top) and actin protein (bottom) in membrane. The figure is a quantification of ACVI protein, represented as a percentage of control ($n = 3$). Data were analyzed by one-way ANOVA followed by Fisher's LSD test for post hoc comparisons of means. Data are represented as the mean \pm S.E.M.

tent with the notion that escitalopram and *R*-citalopram exert their effects at different binding sites. Concomitant incubation of C6 cells with escitalopram and *R*-citalopram suggested little interaction between these drugs, with no inhibition of the effects of escitalopram by *R*-citalopram (Fig. 4). Note, however, the cells used in this study comprise a model “postsynaptic” system and lack SERT. Thus, the lack of inhibitory effects of *R*-citalopram is not surprising. Perhaps more importantly, these data suggest multiple sites of action for SSRIs (and perhaps other antidepressants).

Several previous studies demonstrated that chronic antidepressant treatment enhanced Gpp(NH)p or forskolin (postreceptor)-stimulated adenylyl cyclase activity on rat brain membrane (Menkes et al., 1983; Ozawa and Rasenick, 1989; De Montis et al., 1990). It is suggested that G_{α_s} -adenylyl cyclase coupling may be an important modulator of antidepressant action. The observed decrease in Gpp(NH)p or forskolin-stimulated adenylyl cyclase in brain membranes of depressed patients was consistent with this notion (Cowburn et al., 1994). Treatment of animals with chronic citalopram did not decrease β -receptor density (Holoubek et al., 2004), and isoproterenol-stimulated cAMP accumulation is increased after escitalopram treatment. Given the increase in fluoride-activated adenylyl cyclase subsequent to escitalopram treatment, the most likely explanation for escitalopram effects is an increase in coupling between G_{α_s} and adenylyl cyclase in nonraft fractions of the plasma membrane. Previous studies show no chronic antidepressant-induced change in amount, activity or distribution of G_{α_i} or G_{α_o} (Chen and Rasenick, 1995b; Toki et al., 1999; Donati et al., 2001). It seems that antidepressants-induced translocation is unique to G_{α_s} . Note, however, these data do not indicate that G_{α_s} is a direct target for antidepressant drugs. Because many of these drugs concentrate in lipid raft fractions during the course of treatment (Eisensamer et al., 2005), it is entirely possible that an “antidepressant binding site” involves some lipid moiety.

It is noteworthy that experiments with human cerebral cortex tissue consistently show dysfunctional cAMP signaling in depressed suicide subjects (Dwivedi et al., 2002; Pandey et al., 2005). Consistent with this, G_{α_s} is enriched in lipid raft fractions from these same suicide samples relative to controls (Donati et al., 2008). In this study, escitalopram increased both fluoride and GTP γ S-induced activation of adenylyl cyclase activity. Immunoprecipitation experiments have suggested an increased physical interaction between G_{α_s} and adenylyl cyclase after chronic antidepressant treatment (Chen and Rasenick, 1995a), and these studies are consistent. Thus, escitalopram changes the cellular localization of G_{α_s} from lipid raft, resulting in enhancement in coupling between G_{α_s} and adenylyl cyclase in nonraft fractions.

We hypothesize that chronic escitalopram treatment alters the association between G_{α_s} and some specific membrane component that target G_{α_s} to lipid rafts. Such altered interactions would render G_{α_s} more available to adenylyl cyclase in nonlipid raft membranes. Lipid rafts are defined not only by their enriched cholesterol but also by their enriched cytoskeletal association. It has been reported that the G protein-coupled receptor- G_s -AC pathway is inhibited by intact cytoskeleton when cytoskeleton interacts with some signaling components localized in lipid rafts and caveolae (Head et al., 2006). Donati and Rasenick (2005) demonstrated that the

microtubule disrupting agent, colchicine, also decrease the proportion of G_{α_s} in lipid rafts, auguring a complex relationship between G_{α_s} in those structures and the cytoskeleton. These data suggested that membrane-associated microtubules may sequester inactive G_{α_s} in lipid rafts. Recently, it was reported that chronic fluoxetine treatment increased microtubule dynamics in rat hippocampus (Bianchi et al., 2009). A greater proportion of the tubulin pool in the depolymerized state leads to more G_{α_s} activated. It has been shown that microtubule-disrupting agents such as colchicine and vinblastine increase G_s protein-mediated activation of adenylyl cyclase (Kennedy and Insel, 1979; Rasenick et al., 1981; Head et al., 2006). Toward this end, effects of antidepressants on microtubules or tubulin in lipid rafts and their association with G_{α_s} have yet to be examined. Comparisons of *R*-citalopram and escitalopram in this regard will prove useful, as the former compound represents an ideal control.

This report contributes to the notion that although several antidepressants have a “specific” binding site (e.g., transporter inhibition, inhibition of monoamine catabolism), these sites may not fully account for their antidepressant activity. This is especially true in light of the chronic treatments required for clinical effects of these drugs and the existence of antidepressants that inhibit neither uptake nor monoamine breakdown. It is likely that several presynaptic and postsynaptic molecular cascades contribute to the antidepressant effects (Millan, 2006) and that the translocation of G_{α_s} to nonraft membrane domains results from one of these. It seems that, similar to many antidepressant compounds, escitalopram also has a postsynaptic action. Chronic treatment effectively moves G_{α_s} out of lipid rafts into a greater coupling with adenylyl cyclase. Although G_{α_s} is unlikely to be a direct target of escitalopram or any other antidepressant, the liberation of that protein from lipid raft anchors represents one consistent molecular aspect of antidepressant treatment. Hopefully, experiments with simple systems will assist in further dissection of the molecular pathways involved in the therapeutic actions of antidepressants as well as those involved in the pathogenesis of depression and other mood disorders.

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References

- Allen JA, Yu JZ, Dave RH, Bhatnagar A, Roth BL, and Rasenick MM (2009) Caveolin-1 and lipid microdomains regulate Gs trafficking and attenuate Gs/adenylyl cyclase signaling. *Mol Pharmacol* **76**:1082–1093.
- Allen JA, Yu JZ, Donati RJ, and Rasenick MM (2005) Beta-adrenergic receptor stimulation promotes G alpha s internalization through lipid rafts: a study in living cells. *Mol Pharmacol* **67**:1493–1504.
- Bianchi M, Shah AJ, Fone KC, Atkins AR, Dawson LA, Heidbreder CA, Hows ME, Hagan JJ, and Marsden CA (2009) Fluoxetine administration modulates the cytoskeletal microtubular system in the rat hippocampus. *Synapse* **63**:359–364.
- Chen F, Larsen MB, Neubauer HA, Sánchez C, Plenge P, and Wiborg O (2005) Characterization of an allosteric citalopram-binding site at the serotonin transporter. *J Neurochem* **92**:21–28.
- Chen J and Rasenick MM (1995a) Chronic antidepressant treatment facilitates G protein activation of adenylyl cyclase without altering G protein content. *J Pharmacol Exp Ther* **275**:509–517.
- Chen J and Rasenick MM (1995b) Chronic treatment of C6 glioma cells with antidepressant drugs increases functional coupling between a G protein (Gs) and adenylyl cyclase. *J Neurochem* **64**:724–732.
- Cowburn RF, Marcusson JO, Eriksson A, Wiehager B, and O’Neill C (1994) Adenylyl cyclase and G-protein subunit levels in postmortem frontal cortex of suicide victims. *Brain Res* **633**:297–304.
- De Montis GM, Devoto P, Gessa GL, Porcella A, Serra G, and Tagliamonte A (1990) Selective adenylyl cyclase increase in the limbic area of long-term imipramine-treated rats. *Eur J Pharmacol* **180**:169–174.

- Donati RJ and Rasenick MM (2005) Chronic antidepressant treatment prevents accumulation of g α in cholesterol-rich, cytoskeletal-associated, plasma membrane domains (lipid rafts). *Neuropsychopharmacology* **30**:1238–1245.
- Donati RJ, Dwivedi Y, Roberts RC, Conley RR, Pandey GN, and Rasenick MM (2008) Postmortem brain tissue of depressed suicides reveals increased G α localization in lipid raft domains where it is less likely to activate adenylyl cyclase. *J Neurosci* **28**:3042–3050.
- Donati RJ, Thukral C, and Rasenick MM (2001) Chronic treatment of C6 glioma cells with antidepressant drugs results in a redistribution of G α . *Mol Pharmacol* **59**:1426–1432.
- Dwivedi Y, Rizavi HS, Conley RR, Roberts RC, Tamminga CA, and Pandey GN (2002) mRNA and protein expression of selective alpha subunits of G proteins are abnormal in prefrontal cortex of suicide victims. *Neuropsychopharmacology* **27**:499–517.
- Eisensamer B, Uhr M, Meyr S, Gimpl G, Deiml T, Rammes G, Lambert JJ, Ziegler-gänsberger W, Holsboer F, and Rupprecht R (2005) Antidepressants and antipsychotic drugs colocalize with 5-HT $_3$ receptors in raft-like domains. *J Neurosci* **25**:10198–10206.
- Gass P and Riva MA (2007) CREB, neurogenesis and depression. *Bioessays* **29**:957–961.
- Gorman JM, Korotzer A, and Su G (2002) Efficacy comparison of escitalopram and citalopram in the treatment of major depressive disorder: pooled analysis of placebo-controlled trials. *CNS Spectrums* **7**:40–44.
- Guest PC, Knowles MR, Molon-Noblot S, Salim K, Smith D, Murray F, Laroque P, Hunt SP, De Felipe C, Rupniak NM, et al. (2004) Mechanisms of action of the antidepressants fluoxetine and the substance P antagonist L-000760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res* **1002**:1–10.
- Head BP, Patel HH, Roth DM, Murray F, Swaney JS, Niesman IR, Farquhar MG, and Insel PA (2006) Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. *J Biol Chem* **281**:26391–26399.
- Holoubek G, Nöldner M, Treiber K, and Müller WE (2004) Effect of chronic antidepressant treatment on beta-receptor coupled signal transduction cascade. Which effect matters most? *Pharmacopsychiatry* **37** (Suppl 2):S113–S119.
- Hyttel J, Bøgesø KP, Perregaard J, and Sánchez C (1992) The pharmacological effect of citalopram resides in the (S)-(+)-enantiomer. *J Neural Transm Gen Sect* **88**:157–160.
- Kennedy MS and Insel PA (1979) Inhibitor of microtubule assembly enhance beta-adrenergic and prostaglandin E $_1$ -stimulated cyclic AMP accumulation in S49 lymphoma cells. *Mol Pharmacol* **16**:215–223.
- Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I, and Lisanti MP (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J Biol Chem* **270**:15693–15701.
- Malberg JE and Blendy JA (2005) Antidepressant action: to the nucleus and beyond. *Trends Pharmacol Sci* **26**:631–638.
- Menkes DB, Rasenick MM, Wheeler MA, and Bitensky MW (1983) Guanosine triphosphate activation of brain adenylyl cyclase: enhancement by long-term antidepressant treatment. *Science* **219**:65–67.
- Millan MJ (2006) Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacol Ther* **110**:135–370.
- Montgomery SA, Loft H, Sánchez C, Reines EH, and Papp M (2001) Escitalopram (S-enantiomer of citalopram): clinical efficacy and onset of action predicted from a rat model. *Pharmacol Toxicol* **88**:282–286.
- Obara Y, Kurose H, and Nakahata N (2005) Thromboxane A $_2$ promotes interleukin-6 biosynthesis mediated by an activation of cyclic AMP-response element-binding protein in 1321N1 human astrocytoma cells. *Mol Pharmacol* **68**:670–679.
- Ozawa H and Rasenick MM (1989) Coupling of the stimulatory GTP-binding protein G α to rat synaptic membrane adenylyl cyclase is enhanced subsequent to chronic antidepressant treatment. *Mol Pharmacol* **36**:803–808.
- Pandey GN, Dwivedi Y, Ren X, Rizavi HS, Mondal AC, Shukla PK, and Conley RR (2005) Brain region specific alterations in the protein and mRNA levels of protein kinase A subunits in the post-mortem brain of teenage suicide victims. *Neuropsychopharmacology* **30**:1548–1556.
- Plenge P, Gether U, and Rasmussen SG (2007) Allosteric effects of R- and S-citalopram on the human 5-HT transporter: evidence for distinct high- and low-affinity binding sites. *Eur J Pharmacol* **567**:1–9.
- Rasenick MM, Hughes JM, and Wang N (1989) Guanosine-5'-O-thiodiphosphate functions as a partial agonist for the receptor-independent stimulation of neural adenylyl cyclase. *Brain Res* **488**:105–113.
- Rasenick MM, Stein PJ, and Bitensky MW (1981) The regulatory subunit of adenylyl cyclase interacts with cytoskeletal components. *Nature* **294**:560–562.
- Salomon Y (1979) Adenylyl cyclase assay. *Adv Cyclic Nucleotide Res* **10**:35–55.
- Sánchez C, Bøgesø KP, Ebert B, Reines EH, and Braestrup C (2004) Escitalopram versus citalopram: the surprising role of the R-enantiomer. *Psychopharmacology* **174**:163–176.
- Sánchez C, Gruca P, and Papp M (2003) R-citalopram counteracts the antidepressant-like effect of escitalopram in a rat chronic mild stress model of depression. *Behav Pharmacol* **14**:465–470.
- Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, et al. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**:805–809.
- Sapena R, Morin D, Zini R, and Tillement JP (1994) Cyclic AMP and inositol phosphate accumulations in rat brain cortical slices following chronic citalopram or desipramine administration. *Drugs Exp Clin Res* **20**:93–102.
- Toki S, Donati RJ, and Rasenick MM (1999) Treatment of C6 glioma cells and rats with antidepressant drugs increases the detergent extraction of G α from plasma membrane. *J Neurochem* **73**:1114–1120.
- Vetulani J and Sulser F (1975) Action of various antidepressant treatments reduces reactivity of noradrenergic cyclic AMP-generating system in limbic forebrain. *Nature* **257**:495–496.
- Wang JW, David DJ, Monckton JE, Battaglia F, and Hen R (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. *J Neurosci* **28**:1374–1384.

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