Estradiol accelerates the effects of fluoxetine on serotonin 1A receptor signaling

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Abstract

A major problem with current anti-depressant therapy is that it takes on average 6–7 weeks for remission. Since desensitization of serotonin $(5-HT)_{1A}$ receptor signaling contributes to the anti-depressive response, acceleration of the desensitization may reduce this delay in response to antidepressants. The purpose of the present study was to test the hypothesis that estradiol accelerates fluoxetine-induced desensitization of $5-HT_{1A}$ receptor signaling in the paraventricular nucleus of the hypothalamus (PVN) of rats, via alterations in components of the $5-HT_{1A}$ receptor signaling pathway. Ovariectomized rats were injected with estradiol and/or fluoxetine, then adrenocorticotropic hormone (ACTH) and oxytocin responses to a $5-HT_{1A}$ receptor agonist (+)8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) were examined to assess the function of $5-HT_{1A}$ receptors in the PVN. Treatment with estradiol for either 2 or 7 days or fluoxetine for 2 days produced at most a partial desensitization of $5-HT_{1A}$ receptor signaling, whereas 7 days of fluoxetine produced full desensitization. Combined treatment with estradiol and fluoxetine for 2 days produced nearly a full desensitization, demonstrating an accelerated response compared to either treatment alone. With two days of combined treatments, estradiol prevented the fluoxetine-induced increase in $5-HT_{1A}$ receptor protein, which could contribute to the more rapid to the desensitization. Furthermore, EB treatment for 2 days decreased the abundance of the 35 kD $G\alpha z$ protein which could contribute to the desensitization response. We found two isoforms of $G\alpha z$ proteins with molecular mass of 35 and 33 kD, which differentially distributed in the detergent resistant microdomain (DRM) and in Triton X-100 soluble membrane region, respectively. The 35 kD $G\alpha z$ proteins in the DRM can be sumoylated by SUMO1. Stimulation of $5-HT_{1A}$ receptors with 8-OH-DPAT increases the sumoylation of $G\alpha z$ proteins and reduces the 33 kD $G\alpha z$ proteins,

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The authors declare no actual or potential financial and other conflicts of interest related to the submitted manuscript.

suggesting that these responses may be related to the desensitization of 5-HT$_{1A}$ receptors. Treatment with estradiol for 2 days also reduced the levels of the G-protein coupled estrogen receptor GPR30, possibly limiting to the ability of estradiol to produce only a partial desensitization response. These data provide evidence that estradiol may be effective as a short-term adjuvant to SSRIs to accelerate the onset of therapeutic effects.

Keywords

SSRIs; GPR30; Gαz; oxytocin; ACTH; sumoylation; detergent resistant microdomain; lipid raft; 5-HT$_{1A}$ receptors

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most widely used antidepressants. SSRIs bind serotonin (5-HT) transporters and block 5-HT reuptake from the synaptic cleft to nerve terminals, resulting in prolonged stimulation of 5-HT receptors. 5-HT$_{1A}$ autoreceptor-mediated feedback regulation of 5-HT release is transiently increased and then becomes desensitized likely contributing to the initial increase in anxiety experienced by some individuals as well as the delay of the therapeutic effects of SSRIs (Artigas et al., 2006). One of the major problems with the use of SSRIs is that the therapeutic effects take an average 6–7 weeks of treatment (Gaynes et al., 2009). Clearly, reducing this delay with adjuvant therapy such as estrogens could improve the clinical benefits of SSRIs.

5-HT$_{1A}$ receptors, including autoreceptors and postsynaptic receptors, are involved in emotional regulation (Albert and Lemonde, 2004; Hoyer et al., 1994). Postsynaptic 5-HT$_{1A}$ receptors may be directly involved in therapeutic effects of SSRIs (Millan, 2003). Both human and rodent studies demonstrate that repeated administration of SSRIs attenuate 5-HT$_{1A}$ receptor agonist-induced increase in oxytocin and adrenocorticotropic hormone (ACTH) secretion, suggesting a desensitization of 5-HT$_{1A}$ receptors in the paraventricular nucleus of the hypothalamus (PVN) (Gomez-Gil et al., 2010; Lerer et al., 1999; Lesch et al., 1991; Li et al., 1996). Since these hormones are related to the pathogenesis of depression, desensitization of 5-HT$_{1A}$ receptors in the PVN may contribute to the therapeutic effects of SSRIs (Coryell et al., 2008; Wasserman et al., 2010). Seven to 14 days of treatment with either the SSRI fluoxetine or paroxetine resulted in desensitization of 5-HT$_{1A}$ receptor signaling in the PVN (Li et al., 1997b; Li et al., 1996; Raap et al., 1999). Although treatment with fluoxetine and paroxetine for up to 14 days did not significantly reduce the density of 5-HT$_{1A}$ receptors in the PVN (Li et al., 1997a; Li et al., 1997b), the levels of Gαz protein in the hypothalamus were reduced with 14 days of treatment with fluoxetine (Raap et al., 1999). Adjuvant treatment that hastens alterations in the expression of components of the 5-HT$_{1A}$ receptor signaling pathway could reduce the time needed to cause desensitization of 5-HT$_{1A}$ receptor signaling in the PVN.

Alterations in components of the 5-HT$_{1A}$ receptor system could underlie the changes in hormone response with anti-depressant therapy. Studies have demonstrated that the oxytocin and ACTH responses to 8-OH-DPAT are mediated by 5-HT$_{1A}$ receptors in the PVN (Bagdy, 1996; Osei-Owusu et al., 2005). 5-HT$_{1A}$ receptors are G-protein-coupled receptors that couple to the Gαi/o protein family of Gα proteins. Our previous studies demonstrated that the 5-HT$_{1A}$ receptors mediating the hormone responses to 8-OH-DPAT are coupled to Gαz protein, a member of Gαi/o protein family (Serres et al., 2000). Reductions in the levels of either functional 5-HT$_{1A}$ receptors or Gαz protein in the PVN could underlie the desensitization of 5-HT$_{1A}$ receptor signaling as indicated by decreased oxytocin and ACTH responses to 8-OH-DPAT. Currently, little data is available regarding the cellular location

and post-translational modifications to Gαz proteins. Knowing the effects of SSRIs on components of the 5-HT$_{1A}$ receptor signaling pathway, could have a significant impact on the understanding of the mechanisms underlying the SSRI-induced desensitization of 5-HT$_{1A}$ receptors in the PVN.

Estrogens are involved in the regulation of emotional states (Alexander et al., 2007). Administration of estradiol, a major endogenous form of estrogen, produces desensitization of 5-HT$_{1A}$ receptor signaling, suggesting that the effects of estradiol on mood regulation may be mediated by alterations in 5-HT$_{1A}$ receptor signaling (Bethea et al., 2000; Mize et al., 2003; Raap et al., 2000). Our previous studies demonstrated a partial desensitization of 5-HT$_{1A}$ receptor signaling in the PVN with 2 days of estradiol treatment and further noted changes in expression of components of the 5-HT$_{1A}$ receptor signaling pathway in the PVN (Garzon et al., 2011; Raap et al., 2000). In addition to the two classes of nuclear estrogen receptors (ER), ERα and ERβ, estradiol also activates the G-protein coupled receptor, GPR30, also known as GPER (Filardo and Thomas, 2005). Our previous studies demonstrated that GPR30, but not ERβ, mediates the estradiol-induced desensitization of 5-HT$_{1A}$ receptors in the PVN (McAllister et al., 2012; Rossi et al., 2010; Xu et al., 2009). In addition, the detergent resistant microdomain (DRM) of plasma membrane, including lipid rafts and calveolae, contains G-protein-coupled receptors, G-proteins and other proteins associated with GPCR signaling, physically bringing together the components for GPCR signaling. Thus, DRM plays a role in assembling GPCR signaling pathways and possibly integrating the interactions among signaling pathways such as 5-HT$_{1A}$ receptor and GPR30 signaling pathways. Understanding the signal pathways will help us to determine the mechanisms underlying the GPR30-mediated desensitization of 5-HT$_{1A}$ receptors.

A complete blockade of hormonal responses to a 5-HT$_{1A}$ receptor agonist requires at least 7 days of SSRI treatment (Li et al., 1996), and estradiol produces a partial desensitization after 2 days of treatment (Garzon et al., 2011). Thus, we hypothesize that combined administration of an SSRI and estradiol accelerates the desensitization of 5-HT$_{1A}$ receptors in the PVN, which is mediated by synergistic effects on alterations in components of the 5-HT$_{1A}$ receptor signaling pathway. To test this hypothesis, we examined the effects of treatment with estradiol and the SSRI fluoxetine on hormonal responses to a 5-HT$_{1A}$ receptor agonist, as an index of the function of 5-HT$_{1A}$ receptors in the PVN. To examine possible molecular mechanisms mediating the SSRI-and estradiol-induced alterations in the 5-HT$_{1A}$ receptor and GPR30 signaling pathways, we further studied the cellular location and post-translational modifications of Gαz and GPR30, as well as the effects of EB or/and fluoxetine on the expression of 5-HT$_{1A}$ receptors, Gαz protein and GPR30 in the PVN.

Methods

Drugs and reagents

Fluoxetine and 17β-estradiol -3-benzoate (EB) were purchased from Sigma (St Louis, Mo). Fluoxetine was dissolved in saline (5 mg/ml) by heating the solution until completely dissolved. 8-OH-DPAT was purchased from Tocris Biosciences (Ellisville, Mo) and was dissolved in saline. N-ethymaleimide (NEM) a cysteine peptidase inhibitor, protease inhibitor cocktail, and phosphatase inhibitor cocktail I and II were purchased from Sigma (St Louis, MO). HRP-conjugated goat-anti-rabbit IgG and goat-anti-mouse IgG antibodies were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). The rabbit-anti-5-HT$_{1A}$ receptor antibody that was kindly provided by Dr. Karie Scrogin at Loyola University Chicago was generated to against a 24 amino acid peptide (NH2-GTSSAPPPKKSLNGQPGSGDWRRC-OH) located in the amino acid 243–267 of rat 5-HT$_{1A}$ receptors (Accession # NM_012585).

Animals

Adult female Sprague-Dawley rats (225–250g) purchased from Harlan Laboratories (Indianapolis, IN) were housed two per cage in a temperature-, humidity-, and light-controlled room (12 h light/dark cycles). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as approved by Loyola University Chicago or the University of Kansas Institutional Animal Care and Use Committees.

Experimental procedure

***Experiment 1*. To determine the effects of estradiol and fluoxetine on the desensitization of 5-HT$_{1A}$ receptors in the PVN—**Female rats were ovariectomized as previously described (Rossi et al., 2010). Five days after ovariectomy, rats were injected with sesame oil (Oil) or 17β-estradiol-3-benzoate (EB) (10μg/0.4ml/kg, sc) and saline or fluoxetine (10 mg/2 ml/kg, sc) for 7 days. Animals in the 2-day treatment groups received oil and/or saline for 5 days and then were injected with EB and/or fluoxetine for 2 days. The two injections (Oil/EB and saline/fluoxetine) were performed on the left and right side of the upper back of rats, respectively. Eighteen hours after the last injection, rats were injected with saline or the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT (40μg/kg, sc) and decapitated 15 min later. The trunk blood was collected into a tube containing 0.5 ml of 0.3M EDTA. Plasma aliquots were stored at −80°C until used for plasma oxytocin and ACTH assays.

***Experiment 2*. To determine the mechanisms mediating the accelerating effects of EB on fluoxetine-induced desensitization of 5-HT$_{1A}$ receptors in the PVN—**Since we observed that combined treatment of EB and fluoxetine for 2 days accelerated the desensitization of 5-HT$_{1A}$ receptors in Experiment 1, we next examined alterations in components of the 5-HT$_{1A}$ receptor signaling pathway in the PVN of rats treated with EB and/or fluoxetine for 2 days. Ovariectomized female rats were injected with EB (10 μg/kg, 0.4ml/kg, sc) and/or fluoxetine (10 mg/kg, sc) for 2 days as described above. Eighteen hours after the last injection, rats were injected with saline or 8-OH-DPAT (200μg/kg, sc) and were then decapitated 15 min after the injection. Using 200μg/kg of 8-OH-DPAT was based on a new dose response study for 8-OH-DPAT (Creech et al., 2012). The trunk blood was collected for plasma estradiol, oxytocin and ACTH assays. The brains were removed and flash-frozen in dry-ice-cooled isopentane followed by freezing in dry ice for an additional 10 min. The brains were wrapped with parafilm and aluminum foil and were stored at −80°C until used for western blot assays. The PVN were used for immunoblot assay to determine protein levels of 5-HT$_{1A}$ receptors, Gαz protein and GPR30 in response to EB and/or fluoxetine treatment. Due to the need for larger amounts of tissue, we used rat cortex, hippocampus and the hypothalamus without PVN for isolation of the detergent resistant microdomain (DRM), subcellular distribution and immunoprecipitation studies. These brain regions have a high density of 5-HT$_{1A}$ receptors and express estrogen receptors including GPR30. Furthermore, their membrane location, G-protein coupling and the regulation by agonist stimulation are similar among the brain regions. Thus, the data observed from these brain regions should be relevant for understanding the regulation of 5-HT$_{1A}$ receptor signaling in general.

Biochemical assays

Hormone assays—Plasma oxytocin and ACTH were examined using a radioimmunoassay as previously described (Li et al., 1996; Rossi et al., 2010). The intra- and inter-assay variances for ACTH assay were 4.2 and 14.6%, respectively. The intra- and inter-assay variances for oxytocin assay were 5% and 9%, respectively. Plasma estradiol concentrations were measured using an estradiol radioimmunoassay kit from Siemens

Medical Solutions Diagnostics (Los Angeles, CA). 0.2 ml of plasma was used for the assay. Estradiol concentrations were measured in duplicate and are presented as the mean of the duplicates.

Isolation of detergent resistant microdomain (DRM) from cortex tissue—To determine whether Gαz protein and GPR30 are located in the DRM, we isolated DRM from the cortex using a protocol described by Kumari and Francesconi (Kumari and Francesconi, 2011) with minor modification. Briefly, rat cortex was homogenized with a homogenator driven by an overheadmotor at 500 rpm for 25 strokes in 10 volume of homogenate buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 320 mM sucrose, 20mM NEM, 1:100 dilution of protease inhibitor cocktail (Sigma) and phosphatase inhibitor II & III cocktails (Sigma)). The homogenate was centrifuged at 800×g for 15 min at 4°C to remove the nucleus and cell debris. The supernatant was then centrifuged at 30,000×g for 30 min at 4°C. The pellet was resuspended in 2.2 ml of extracting buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 (v/v), 20mM NEM, 1:100 dilution of protease and phosphatase inhibitor cocktails) and was then incubated for 10 min in ice (total extraction, Triton X-100 treatment). 2 ml of the Triton X-100-treated homogenate was adjusted to a final concentration of 40% sucrose with an 80% sucrose solution in extracting buffer. The 40% sucrose Triton X-100-treated homogenate was placed on the bottom of a 13 ml centrifugation tube and was overlaid with 6 ml of 30% sucrose on the top. Finally, 2 ml of 5% sucrose buffer was placed on the top of 30% sucrose. The sucrose gradient was then centrifuged at 230,000× g, 4°C for 16 hours. After the centrifugation, 1 ml fractions were collected from top to bottom. To solubilize the membrane protein, 0.1 ml of 10% sodium cholate was added to the fractions, which were then sonicated and shaken for one hour at 4°C. The fractions were aliquoted and stored in −80°C until use. The protein concentration of the fractions was measured using Pierce BCA protein assay kit (Thermo Scientific Inc, Rockford IL). Ten and 300–1,000 μg of protein were used for immunoblot and immunoprecipitation assays, respectively.

Subcellular fractionation of rat hippocampus—To separate endoplasmic reticulum, endosomes, Golgi, plasma membrane, ERGIC (Endoplasmic Reticulum-Golgi Intermediate Compartment) and TGN from the hippocampal tissue, we conducted an OptiPrep discontinuous gradient centrifugation based on the manufacture's protocol (http://www.axis-shield-density-gradient-media.com/organelleindexes.htm, Application 05 and 22), with modification. The hippocampus from four rats was homogenized in 4 volumes of homogenate buffer (0.32M sucrose, 1mM EDTA 10mM Tris-HCl pH 7.4, 20 mM NEM, 1/100 dilution of protease and phosphatase inhibitors) using a Powergen 1000 homogenizer at speed 5, 4°C for ~10 sec. After a centrifugation at 1500× g, 4°C for 15 min, the supernatant was used for OptiPrep discontinuous gradient centrifugation. Iodixanol (OptiPrep, Sigma) was diluted into 7.5, 10, 12.5, 15, 20, 25 and 30% with Diluent (0.25mM sucrose, 6mM EDTA 60mM Tris-HCl pH 7.4, 20 mM NEM, 1/100 dilution of protease and phosphatase inhibitors). 1 ml of each solution from 7.5% to 30% was underlayed sequentially in a 13 ml SW41 centrifuge tube. 1 ml brain homogenate supernatant was layered on the 7.5% iodixanol solution and centrifuged at 200,000× g, 4°C for 3 hours in a SW41 rotor. After the centrifugation, 0.5 ml fractions were collected from the top to the bottom of the tube. The membrane proteins were then solubilized with 50 μl 10% sodium cholate as described above.

Immunoprecipitation (IP)—IP was conducted using 300–1000 μg protein of the brain tissue preparation or fractions in a total volume of 500 μl IP buffer (50 mM Tris, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, containing 20 mM NEM and 1:100 dilution of protease and phosphatase inhibitor cocktails) as previously described (Garzon et al.,

2011). After pre-absorption of endogenous IgG, the protein was incubated in primary antibodies (Table 1) at 4°C overnight with nuitating. To control for non-specific interactions with the IgG antibody and agarose beads, the same amount of IgG (from the same animal source as the antibody used for immunoprecipitation) was added into another tube containing the same amount tissue. For an antibody control, 4 μg antibodies were added into 500 μl IP buffer. The solution was then incubated with 50μl pre-washed agarose-protein G beads (Invitrogen) for 2 hours at 4°C with nuitating. After washing three times with 0.5 ml IP buffer, 25 μl PAGE sample buffer (1.3×) was added and incubated at 95°C for 5 min to elute bound proteins. The elution (~35–40μl) from each IP reaction was resolved with PAGE to identify the proteins, as described below. Samples from SUMO1 IP were blotted with rabbit-anti-Gαz antibody followed by goat-anti-rabbit IgG antibody. The samples from Gαz IP were blotted with sheep-anti-SUMO1 followed by donkeyanti-sheep IgG. The same blots were then blotted with rabbit-anti-Gαz antibody follow by goatanti-rabbit IgG light chain (Jackson ImmunoResearch Lab Inc., West Grove, PA).

Immunoblot assays—To determine the protein levels of 5-HT$_{1A}$ receptors, Gαz proteins and GPR30 in fluoxetine and/or EB treated rats, the PVN was punched from 300μm coronal sections (2–3 sections, AP ~(−1.08) – (−2.04) mm from bregma) of the brain (Paxinos and Watson, 2007). The 300 μm sections were placed on a slide and were kept in the cryostat chamber. A triangle with a dorsal line between two fornices at the top of third ventricle and two lines from ends of the dorsal line to the middle of 3rd ventricle was dissected as shown in our previous paper (McAllister et al., 2012). The membrane fractions of the PVN were prepared and used for immunoblot assay as described previously (Garzon et al., 2011). 5 μg of the membrane samples were used for the immunoblot analysis. The dilutions of the primary antibodies are listed in Table 1. To detect the loading variation, the membranes were re-incubated with mouse-anti-β-actin.

To quantify the proteins, the IOD from each band was first normalized with its β-actin band (target protein band/actin band). Then the percent of each sample relative to the mean of the control samples (saline/oil) in the same blot was calculated. Each sample was measured in three gels and the mean of the triplicates was used to represent the level of the protein (% of saline/oil).

Transfection and recombinant adenoviral treatment—_Tranfection_ of pcDNA4.1-5-HT$_{1A}$ construct into HEK 293 and A1A1V cells was conducted using lipofactamine reagent and lipofactamine/plus reagents, respectively. A full length rat 5-HT$_{1A}$ receptor sequence (encoding 1–1269 bp (Accession No. NM_012585) was constructed into Bam HI and Eco RI sites of pcDNA 4.1 vector. The pcDNA4.1-5HT$_{1A}$ or pcDNA4.1 vector was transfected into HEK 293 and A1A1V cells according to the manufacturer's protocols (Invitrogen, Carlsbad, CA).

Unilateral injection of recombinant adenovirus was conducted as previously described (McAllister et al., 2012; Rossi et al., 2010). Recombinant adenovirus containing antisense for full length Gαz coding sequence was generated as described in our previous study (Li et al., 2004).

Data analysis and statistics—All data were analyzed by analysis of variance (ANOVA) using StatView (Abacus Concepts Inc., Berkeley, CA). If a significant difference was detected, a Student-Newman-Keuls post-hoc test was used to evaluate differences between individual groups. The data are presented as group means ± SEM of 8–14 rats in hormone studies and 6–8 rats in immunoblot assays, unless noted otherwise.

Results

Treatment with EB accelerates fluoxetine-induced desensitization of 5-HT$_{1A}$ receptors in the PVN

To determine whether EB can accelerate fluoxetine-induced desensitization of 5-HT$_{1A}$ receptors in the PVN, we treated ovariectomized rats with EB and/or fluoxetine for 2 or 7 days. The oxytocin and ACTH responses to the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT were used to assess the function of the 5-HT$_{1A}$ receptor signaling pathway in the PVN. Our results (Figure 1A) show that pre-treatment with EB and/or fluoxetine did not alter the basal level of plasma oxytocin. Administration of 8-OH-DPAT significantly increased oxytocin secretion in the vehicle (saline/oil pre-treated) group. Treatment with EB for 2 or 7 days or fluoxetine for 2 days partially reduced the oxytocin response to 8-OH-DPAT to a similar level. The reduction in the oxytocin response to 8-OH-DPAT was more extensive in the rats injected with combined EB and fluoxetine for 2 days and further reduced in rats treated with fluoxetine or EB and fluoxetine for 7 days (two-way ANOVA: main effect for pre-treatment $F_{(6,121)}=14.9$, $P<0.0001$; main effect for 8-OH-DPAT challenge $F_{(1,121)}=181.3$, $P<0.0001$, effect for pre-treatment $\times$ 8-OH-DPAT challenge $F_{(6,121)}=15.09$, $P<0.0001$).

Similar to the oxytocin response, pre-treatment with EB and/or fluoxetine did not alter the basal levels of plasma ACTH. Injection with 8-OH-DPAT significantly increased ACTH secretion in vehicle-treated rats (Figure 1B). Treatment with EB for both 2 and 7 days produced a partial reduction in the ACTH response to 8-OH-DPAT. Treatment with fluoxetine for 2 days did not significantly reduce 8-OH-DPAT-stimulated ACTH secretion, whereas 7-days of fluoxetine treatment blocked the ACTH response. Furthermore, treatment with EB and fluoxetine for both 2 days and 7 days completely blocked the ACTH response to 8-OH-DPAT, i.e., not significantly different from the saline-challenged group (Figure 1B, two-way ANOVA: main effect for pre-treatment $F_{(6,102)}=47.9$, $P<0.0001$; main effect for 8-OH-DPAT challenge $F_{(1,102)}=468.7$, $P<0.0001$, effect for pre-treatment $\times$ 8-OH-DPAT challenge $F_{(6,102)}=15.09$, $P<0.0001$).

We next studied the mechanisms mediating the synergistic effects of combined treatment with EB and fluoxetine for two days. To ensure that the results observed in the experiment 1 are repeatable and obtain more tissue from the PVN for immunoblot assays, we repeated the 2-day treatments with EB and/or fluoxetine and determined the hormone responses to 8-OH-DPAT again. Based on a new dose response study for 8-OH-DPAT (Garzon et al., 2011), we used 200μg/kg in the second experiment. Five days after ovariectomy, plasma estradiol concentrations (7.49 ± 0.74 pg/ml) were reduced compared to physiological estradiol levels (Flugge et al., 1999). Injection of EB for 2 days significantly increased estradiol levels (125.72 ± 11.58 pg/ml) compared to oil-treated rats and were 2–3 fold higher than physiological levels (Flugge et al., 1999). Although treatment with EB or fluoxetine for 2 days did not alter the oxytocin response to 8-OH-DPAT, EB and fluoxetine for 2 days significantly reduced the oxytocin response to 8-OH-DPAT (saline vs 8-OH-DPAT were 2.02±0.26 vs. 51.46±4.53 in saline/oil pretreated rats; 1.67±0.22 vs. 55.44±5.49 in saline/EB pre-treated rats; 1.61±0.21 vs. 54.6±5.71 in fluoxetine/oil pre-treated rats and 2.08±0.53 vs. 39.96±0.71 pg/ml in EB and fluoxetine pre-treated rats; threeway ANOVA showed no significant effects for fluoxetine and EB alone, but a significant effect for 8-OH-DPAT: $F_{(1,42)}=697.44$, $P<0.0001$ and for fluoxetine $\times$ EB: $F_{(1,42)}=5.87$, $P=0.02$ and for fluoxetine $\times$ EB $\times$ 8-OH-DPAT: $F_{(1,42)}=7.0$, $P=0.011$.

Treatment of fluoxetine and EB alters protein levels of 5-HT$_{1A}$ receptors

To examine alterations in the expression of 5-HT$_{1A}$ receptors, we first characterized the rabbit-anti-5-HT$_{1A}$ receptor antibody that was kindly provided by Dr. Karie Scrogin at

Loyola University Chicago. We transfected plasmid containing pcDNA 4.1–5-HT$_{1A}$ receptors or pcDNA 4.1 vector into HEK 293 cells or A1A1V cells (a rat cortical cell line). 48 hours later, the cells were collected and lysed. The 5-HT$_{1A}$ receptors were detected using the 5-HT$_{1A}$ receptor antibody. As shown in Figure 2A, the 5-HT$_{1A}$ antibody detected a ~ 42 kD band in both HEK 293 and A1A1V cells that were transfected with pcDNA 4.1–5HT$_{1A}$ receptor, but not those with pcDNA 4.1 vector (control). Since 5-HT$_{1A}$ receptors contain 422 amino acids, the 42 kD band is the appropriate size band for the 5-HT$_{1A}$ receptors without any post-translational modifications. Besides the 42 kD band, a 55 kD band was also detected in transfected HEK 293, suggesting a post-translationally modified 5-HT$_{1A}$ receptor from transfected protein. The antibody also detected several bands in both 5-HT$_{1A}$ receptor-transfected and control non-transfected cells, which could be endogenous 5-HT$_{1A}$ receptors in these cell lines. We previously demonstrated that A1A1v cells endogenously express 5-HT$_{1A}$ receptors (Dai et al, 2008). However, in the brain tissue preparation, the antibody detected a ~75 kD, 55 kD and several small bands, but not the 42 kD band, suggesting that the post-translational modifications to 5-HT$_{1A}$ receptors in the brain tissue are different from that in the cell lines. The majority of 5-HT$_{1A}$ receptors in vivo is either in post-translationally modified forms or is degraded. Since the 55 kD band has the highest density and is specifically located in the membrane fraction, we measured this band in the study to determine the effects of EB and/or fluoxetine on the protein level of 5-HT$_{1A}$ receptors.

In saline-challenged rats, treatment for 2 days with fluoxetine alone, but not EB or EB plus fluoxetine, significantly increased the protein levels of 5-HT$_{1A}$ receptors in the PVN membrane fraction (Figure 2B, two-way ANOVA: main effect for fluoxetine $F_{(1,23)} = 8.05$, $P=0.009$). On the other hand, in the rats challenged with 8-OH-DPAT, 5-HT$_{1A}$ receptors in the PVN membrane were significantly reduced in the groups treated for 2 days with fluoxetine alone and EB plus fluoxetine (Figure 2B, two-way ANOVA showed a main effect for fluoxetine $F_{(1,16)} = 18.59$, $P=0.0005$).

Treatment with EB, but not fluoxetine, reduces Gαz protein in the DRM of PVN membrane

To determine the membrane location of Gαz proteins, we isolated DRM in the cortex with Triton X-100 treatment followed by sucrose gradient centrifugation. DRM fractions were identified by flotillin 1, a marker of DRM. As figure 3A shows, the Gαz antibody detected a ~35 kD and a ~33 kD band. Interestingly, the 35 kD band was mainly located in the DRM, whereas the 33 kD band was in the Triton X-100 soluble fractions. This result suggests that the 35 kD Gαz proteins may be the active form of Gαz proteins, because G-protein coupled receptors (GPCRs) are coupled with G protein in the DRMs in the plasma membrane. To rule out the possibility of non-selective bands detected by the Gαz antibody, we unilaterally injected recombinant adenovirus containing a Gαz antisense sequence (Gαz-antisense-Ad) into the hypothalamus as previously described (McAllister et al., 2012; Rossi et al., 2010). Five days after the injection, the rats were decapitated and the brain was sectioned using a cryostat. The virus infected region in the hypothalamus and the contra-lateral region were punched out for immunoblot assay. As shown in figure 3B, the Gαz antisense-Ad reduced both 35 kD and 33 kD bands, suggesting that both bands are Gαz proteins.

Interestingly, acute treatment with 8-OH-DPAT for 15 min significantly increased the ratio of the 35 to 33 kD Gαz proteins in the membrane of PVN, which resulted from a reduction in the 33 kD Gαz proteins (Fig. 3C, one-way ANOVA: for 35/33 kD Gαz, $F_{(1,13)} =29.485$, $P<0.05$; for 35 kD Gαz, $F_{(1,5)} =0.59$, $P=0.477$; for 33 kD Gαz, $F_{(1,8)} =46.35$, $P<0.05$). On the other hand, treatment with EB for 2 days significantly reduced the ratio of the 35/33 kD Gαz proteins (two-way ANOVA: main effect for EB $F_{(1,22)}=15.44$, $P<0.05$) which resulted from a reduction of 35 kD Gαz proteins (Fig 3D, two-way ANOVA: main effect for EB

$F_{(1,21)}=1023$, $P<0.05$). Fluoxetine treatment for 2 days did not alter Gαz protein levels in the membrane fraction of the PVN compared to vehicle-treated controls.

Acute treatment with 8-OH-DPAT increased sumoylation of Gαz protein in the DRM

To determine the post-translational modification of Gαz proteins, we found that Gαz protein can be sumoylated, especially those in the DMR (Figure 3E and 3F). Using Triton X-100 treated sucrose fractions of the cortex tissue and immunoprecipitation with a SUMO-1antibody followed by blotting with a Gαz antibody, we detected a 35kD sumoylated Gαz protein in the DRM fractions (Fig. 3E). Conversely, using immunoprecipitation with Gαz antibody followed by blotting with SUMO1 antibody, we also detected a 35 kD sumoylated Gαz protein band (Fig.3F). When the blot was subsequently examined with a Gαz protein antibody, two bands, 35 kD and 33 kD, were detected (Fig. 3F), confirming that the band detected by the SUMO1 antibody is Gαz protein. Treatment with 8-OH-DPAT (200 μg/kg, sc) for 15 min increased the sumoylated Gαz protein in the cortex (Fig. 3E) and the hypothalamus without the PVN (Fig.3G, t-test: $t_{(9)}=4.67$, $P<0.05$).

Treatment with EB reduced the protein level of GPR30 in the membrane of PVN

To determine the membrane location of GPR30, we examined GPR30 in the Triton X-100 treated sucrose fractions of cortex membrane preparation using immunoblot. As figure 4A shows, the majority of GPR30 (~32 kD) was located the DRM. However, a higher molecular mass band (~47 kD) was detected in the Triton X-100 soluble fractions (Fig 4A).

In the comparison with the vehicle-treated group (saline/oil pretreated group), injection of EB for 2 days significantly reduced GPR30 levels in the PVN membrane fraction (two-way ANOVA showed a significant effect for EB in saline-challenged groups: ($F_{(1,25)}=6.34$, $P=0.019$). However, post hoc tests did not detect additional significant differences among the groups (Figure 4B). These data suggest that treatment with EB, but not fluoxetine, reduced GPR30 expression in the PVN membrane.

Subcellular distribution of Gαz protein and GPR30 in the rat hippocampus

Although we determined the localization of GPR30 and Gαz proteins in the DRM, the distribution of these proteins in the subcellular organelle is still unclear. Thus, we conducted a gradient centrifugation of rat hippocampus to separate subcellular organelle based on their density. Consistent with the observation from Triton X-100 treated brain membrane, the 35 kD Gαz proteins were located in the plasma membrane, as marked by Na$^+$/K$^+$ ATPase and flotillin-1 (Fig 5). On the other hand, the 33 kD Gαz protein was mainly co-localized with the fractions containing cytosol and endosome markers, LDH and EEA1, respectively. Similarly, the GPR30 (~ 32 kD) was co-localized with plasma membrane markers, Na$^+$/K$^+$ ATPase and flotillin-1, which is consistent with the results shown in Triton X-100 treatment study. On the other hand, a 47 kD GPR30 protein band was co-localized with trans-Golgi network, ER and Golgi-ER intermediate compartment markers, TGN 38, calreticulin and LMAN-1. Furthermore, a small band was detected in these same fractions. In addition, a ~40 kD GPR30 band was detected in the cytosolic fractions (Fig 5).

Discussion

One of the challenges for improving the effectiveness of SSRIs is to identify adjuvant treatment to hasten the onset of therapeutic effects. Desensitization of 5-HT$_{1A}$ receptors, especially in the PVN, is related to the therapeutic effects of SSRIs (Gomez-Gil et al., 2010; Polter and Li, 2010). Thus, acceleration of the desensitization of 5-HT$_{1A}$ receptor signaling may hasten the therapeutic effects of these drugs. In the present study, we demonstrated that

treatment with estradiol accelerates fluoxetine-induced desensitization of 5-HT_{1A} receptor signaling in the PVN. Furthermore, we identified several molecular mechanisms that could contribute to desensitization response caused by fluoxetine, EB and the combination of both fluoxetine and EB.

Reductions in the magnitude of hormone responses to 8-OH-DPAT indicate a desensitization of the 5-HT_{1A} receptor signaling in the PVN (Carrasco and Van de Kar, 2003; Van de Kar and Blair, 1999). In the present study, we used this approach to assess the function of 5-HT_{1A} receptors in the PVN (Fig.1). Treatment with fluoxetine or EB for 2 days produced at most a partial desensitization of 5-HT_{1A} receptors in the PVN. Treatment with EB for 7 days produced no further reduction in the response. However, administration of EB and fluoxetine for 2 days produced a nearly full desensitization of 5-HT_{1A} receptors, suggesting a synergistic effect between EB and fluoxetine. These results are consistent with the studies by Estrada-Camarena et al.(Estrada-Camarena et al., 2006), in which they found that treatment with estradiol accelerated fluoxetine-induced antidepressant-like behaviors measured by the forced swim test. The effects of EB and fluoxetine were blocked by co-injection with a 5-HT_{1A} receptor antagonist (Estrada-Camarena et al., 2006). These results demonstrate that administration of EB can accelerate the anti-depressant-like effects of SSRIs via 5-HT_{1A} receptor signaling as measured via either behavioral or neuroendocrine indices. Although the function of 5-HT_{1A} receptors differ among brain regions and it is unlikely that all of the 5-HT_{1A} receptors in the brain will respond similarly to EB, studies have demonstrated that treatment with EB reduces 5-HT_{1A} receptor signalling in the hypothalamus, hippocampus, dorsal raphe and cortex (Lu and Bethea, 2002; Mize et al., 2003; Sanchez et al., 2011), suggesting that estradiol treatment may induce desensitization of 5-HT_{1A} receptors not only in the PVN, but also in other brain regions. Furthermore, as a GPCR, the subcellular distribution, G proteins coupling and the response to agonist stimulation of 5-HT_{1A} receptors in different brains should be similar. Therefore, the results observed in the present study impact our understanding estradiol-induced desensitization of 5-HT_{1A} receptors in other brain regions.

In the present study, we found that treatment with fluoxetine alone for 2 days increased 5-HT_{1A} receptor protein in the PVN membrane, which was prevented by the EB plus fluoxetine treatment. The up-regulation of 5-HT_{1A} receptors could be a transient response to the SSRI treatment. SSRIs block 5-HT reuptake and thus increase the 5-HT concentration in the synaptic cleft, which stimulates 5-HT_{1A} autoreceptors and consequently reduces firing rate of serotonin neurons. As a result, 5-HT release is reduced, which could induce the up-regulation of postsynaptic 5-HT_{1A} receptors. Since the 5-HT release will be increased once the 5-HT_{1A} autoreceptors are desensitized, the up-regulation of 5-HT_{1A} receptors can be reversed. This transient increase of 5-HT_{1A} receptors could contribute to the delayed desensitization of 5-HT_{1A} receptor signaling. The fluoxetine-induced up-regulation of 5-HT_{1A} receptors is negated by treatment with EB plus fluoxetine, likely contributing to the more rapid desensitization. Unlike in the saline-challenged rats, the 5-HT_{1A} receptor protein levels were significantly reduced in rats treated with either fluoxetine alone or EB plus fluoxetine treatment after acute 8-OH-DPAT challenge (Figure 2B). Evidence has shown that acute stimulation of 5-HT_{1A} receptors causes an internalization of the receptors (Fichter et al., 2010), resulting in a reduction of 5-HT_{1A} receptors in the membrane fraction. The differential effects of stimulation (in 8-OH-DPAT-challenged rats) on 5-HT_{1A} receptor levels in the membrane fraction of the saline-pretreated rats and the rats pre-treated with fluoxetine suggest that the 5-HT_{1A} receptors in the fluoxetine-treated rats are more susceptible to internalization. On the other hand, unlike the other studies that reported a reduction in the density of 5-HT_{1A} receptor binding sites using 3H-8-OH-DPAT binding following treatment with EB for up to one month (Lu and Bethea, 2002; Mize et al., 2003; Sanchez et al., 2011), we did not detect an EB-induced reduction of 5-HT_{1A} receptor levels.

This could be due to the immunoblot assay approach used in the present study, which measures the protein levels of 5-HT$_{1A}$ receptors, including active and inactive receptors, whereas 5-HT$_{1A}$ receptor agonist binding only measures the active 5-HT$_{1A}$ receptors or the duration of treatment with EB.

The DRM of plasma membrane, including lipid rafts and calveolae, is a planar domain in the membrane containing high concentrations of cholesterol and glycosphingolipid (Allen et al., 2007; Chini and Parenti, 2004). The DRM is resistant to solubilization with non-ionic detergent, such as Triton X-100. Although it is believed that DRMs are located in the plasma membrane, recent studies demonstrated that DRMs are also located in other membrane organelles, such as mitochondria, ER and Golgi (Simons and Gerl, 2010). While the DRM plays a role in assembling GPCR signaling pathways, components in the DRM dynamically move in and out of the DMR based on the activity of GPCR signaling. Disrupting the DRM reduces the binding capacity of 5-HT1A receptors (Pucadyil and Chattopadhyay, 2004), suggesting that the DRM is important in the functioning of 5-HT$_{1A}$ receptors.

Studies have demonstrated that several G proteins, such as Gαi$_{1/2}$, Gαo, Gαq$_{/11}$ and Gαs, are located in the DRM (Allen et al., 2009; Lents et al., 2009; Sugama et al., 2007; Yuyama et al., 2007). In the present study, we demonstrated that Gαz proteins are located in the DRM (Fig.3A). Interestingly, we detected two forms of Gαz proteins with 35 and 33 kD mass. The 35 kD Gαz proteins are mainly located in the DRM, whereas the 33 kD Gαz proteins are Triton X-100 soluble (Fig.3A) and located in the cytosol and endosome (Fig.5). These data suggest that the 35 kD Gαz proteins are the form of Gαz proteins bound to GPCR and may be modified by palmitoylation and myristoylation as data showed that these modifications are necessary for Gαi protein to be located in the DRM (Moffett et al., 2000). Indeed, Gαz proteins have been shown to be palmitoylated (Tu et al., 1997). Furthermore, we found, for the first time, that the 35 kD Gαz proteins can be sumoylated in the DRM and the sumoylated Gαz proteins are increased after acute treatment with 8-OH-DPAT (Fig. 3). It is possible that the sumoylation is a trigger to inactivate Gαz proteins and move them out of the DRM.

Our present results show that acute treatment with 8-OH-DPAT significantly reduced the 33 kD Gαz proteins, which could result from increased degradation of Gαz proteins (Fig. 3C). In contrast, treatment with EB for 2 days significantly reduced the levels of the 35 kD Gαz proteins, but not 33 kD Gαz proteins (Fig. 3D). The 35 kD Gαz proteins are located in the DRM, where the protein may be coupled to 5-HT$_{1A}$ receptors. Reduction of these Gαz proteins may contribute to EB-induced desensitization of 5-HT$_{1A}$ receptors. These results suggest that the EB-induced alterations in the Gαz proteins may be mediated by different mechanisms from that induced by acute treatment of 8-OH-DPAT. Our previous results showed that inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) blocked the SSRI-induced reduction in Gαz protein and desensitization of 5-HT$_{1A}$ receptors (unpublished observations). These data suggest that activation of the MEK pathway can induce reductions in Gαz protein, which were necessary for SSRI-induced desensitization of 5-HT$_{1A}$ receptor signaling in the PVN. Interestingly, GPR30 stimulation also results in increased activity of the MEK pathway (Filardo et al., 2000). Thus, it is possible that estradiol-induced desensitization of 5-HT$_{1A}$ receptors is mediated by activation of MEK pathway through stimulation of GPR30. The increased activity of MEK pathway could result in a reduction of Gαz protein and consequently desensitization of 5-HT$_{1A}$ receptors.

Our previous studies demonstrated that EB-induced desensitization of 5-HT$_{1A}$ receptors is mediated by GPR30 (Xu et al., 2009). Although our previous studies have demonstrated that ERβ does not mediate the EB-induced desensitization of 5-HT$_{1A}$ receptors in the PVN, we

cannot completely rule out the involvement of ERα in this EB-induced effect. Although further studies are necessary to investigate the role ERα, GPR30 stimulation was both necessary and sufficient for the desensitization response. GPR30 is a membrane-associated estrogen receptor. A study using immunocytochemistry demonstrated that stimulation of GPR30 with EB triggers the translocation of the receptors from the cell surface to the trans-Golgi network, where GPR30 are degraded (Cheng et al., 2011). In the present study, we further confirmed that the majority of unmodified GPR30 (~ 32 kD) are located in the DRM of the plasma membrane (Fig. 4A and 5), whereas a modified GPR30 (~47 kD) is Triton X-100 soluble (Fig 4A) and is co-localized with TGN and ER markers, TGN38 and calreticulin, respectively (Fig 5). These data are consistent with the observation by Cheng et al. (Cheng et al., 2011). They found that ubiquinated GPR30 are located in the TGN and degraded in the proteasome. It is possible that the 47 kD GPR30 band detected in our study is ubiquinated GPR30, although further confirmation is needed. Furthermore, a small band (~15 kD) was observed in the TGN and ER fractions, suggesting that GPR30 may be degraded in these organelles (Fig 5). We also detected a ~40 kD band with the GPR30 antibody, which is co-localized with cytosol and endosomal markers. Further studies are required to identify this isoform of GPR30. Since the unmodified GPR30 is the active form of the receptor, we measured this form of GPR30 in the present study. As shown in Fig 4B, we found that treatment with EB for 2 days reduced protein levels of GPR30 in the PVN, suggesting an EB-induced down-regulation of GPR30. This may contribute to the ability of EB to produce only a partial desensitization of 5-HT$_{1A}$ receptors with 2 days of EB treatment, but no further desensitization with longer treatments. Furthermore, selective stimulation of GPR30 has also been shown to have anti-depressant-like effects as demonstrated in a tail-suspension test (Dennis et al., 2009).

In conclusion, the present studies demonstrate, for the first time, that 2-day treatment with EB accelerates fluoxetine-induced desensitization of 5-HT$_{1A}$ receptor signaling in the PVN. From these studies, we found two new mechanisms by which EB could accelerate SSRI-induced desensitization of 5-HT$_{1A}$ receptor signaling. First, EB prevents the fluoxetine-induced increase in 5-HT$_{1A}$ receptor levels. Secondly, EB decreases Gαz proteins levels in the DRM which may contribute to the EB-induced desensitization of 5-HT$_{1A}$ receptors. The reduction in the active Gαz may result from sumoylation of Gαz and could possibly be mediated via GPR30-induced increases in activity of the MEK pathway. Based on our present results and the literature, we can hypothesize that SSRIs and EB-induced desensitization of 5-HT$_{1A}$ receptors are mediated by different mechanisms. SSRI-induced desensitization of 5-HT$_{1A}$ receptors is mediated by an increase in the 5-HT concentration in the synaptic cleft, which further stimulates 5-HT$_{1A}$ receptors and consequently results in internalization and un-coupling of 5-HT$_{1A}$ receptors to G-proteins. On the other hand, EB-induced desensitization is dependent on GPR30. GPR30 couples to Gαs to increase adenylyl cyclase activity, and, via Gβγ, it causes trans-activation of epidermal growth factor (EGF) receptors, which leads to activation of mitogen-activated protein kinase (MAPK) and phosphotidylinositol 3-kinase (PI3K) pathways (Filardo, 2002; Prossnitz et al., 2008). We previously demonstrated that the desensitization of 5-HT$_{1A}$ receptor signaling produced by EB was blocked by pertussis toxin (Xu et al., 2009), which suggests that the desensitization response is mediated by GPR30 activation of Gβγ signaling rather than Gαs, since Gαs signaling is not sensitive to pertussis toxin and Gβγ signaling is (Filardo et al., 2000; Xu et al., 2009). Increases in the activity of the MAPK and/or PI3 kinase pathway may alter expression and post-translational modifications of Gαi/o proteins and other members in the 5-HT$_{1A}$ receptor signaling pathway. As a result, G proteins and other components of the 5-HT$_{1A}$ receptor signaling pathway could be removed from active sites such as DRM, resulting in a reduction in signaling. The synergy between these mechanisms underlying SSRI- and EB-induced desensitization of 5-HT$_{1A}$ receptors results in a more rapid desensitization of 5-HT$_{1A}$ receptors. More studies are required to test this speculative

hypothesis. Understanding the mechanisms underlying the desensitization of 5-HT$_{1A}$ receptors will have a significant impact on development of better therapeutic approaches to treat depression. Our results provide fundamental evidence that EB accelerates the therapeutic effects of SSRIs on the hypothalamic-pituitary system and taken together with the accelerated anti-depressant-like behavioral effects of EB, strongly support the use of EB as an adjuvant for SSRI therapy.

Acknowledgments

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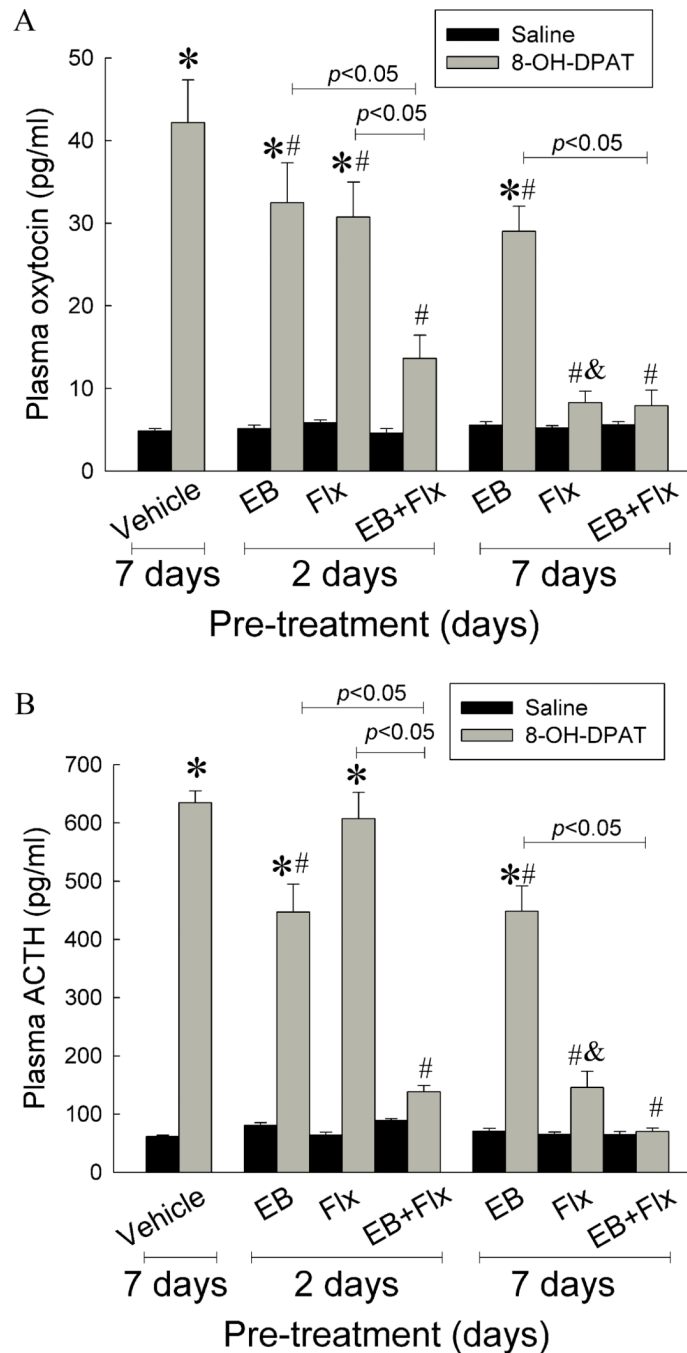
A

B

Figure 1. Effects of fluoxetine and/or EB treatment for 2 and 7 days on hormonal response to 8-OH-DPAT

Plasma levels of oxytocin (**A**) and ACTH (**B**) were measured 15 min after a saline or 8-OH-DPAT injection in ovariectomized rats pre-treated with fluoxetine and EB, alone or in combination, for 2 or 7 days. The data are presented as the mean ± SEM (n = 8–14). *: significantly different from saline-challenged group with same pre-treatment; #: significantly different from 8-OH-DPAT-challenged vehicle group; &: significantly different from 2 day pretreatment groups with same treatment. A line between groups indicates significant difference between the groups. All of the differences were determined by ANOVA and Student Newman-Keuls post-hoc tests. Vehicle: rats treated with saline and

oil for 7 days; EB: rats treated with saline and EB, Flx: rats treated with fluoxetine and oil and EB plus Flx: rats treated with EB plus fluoxetine.
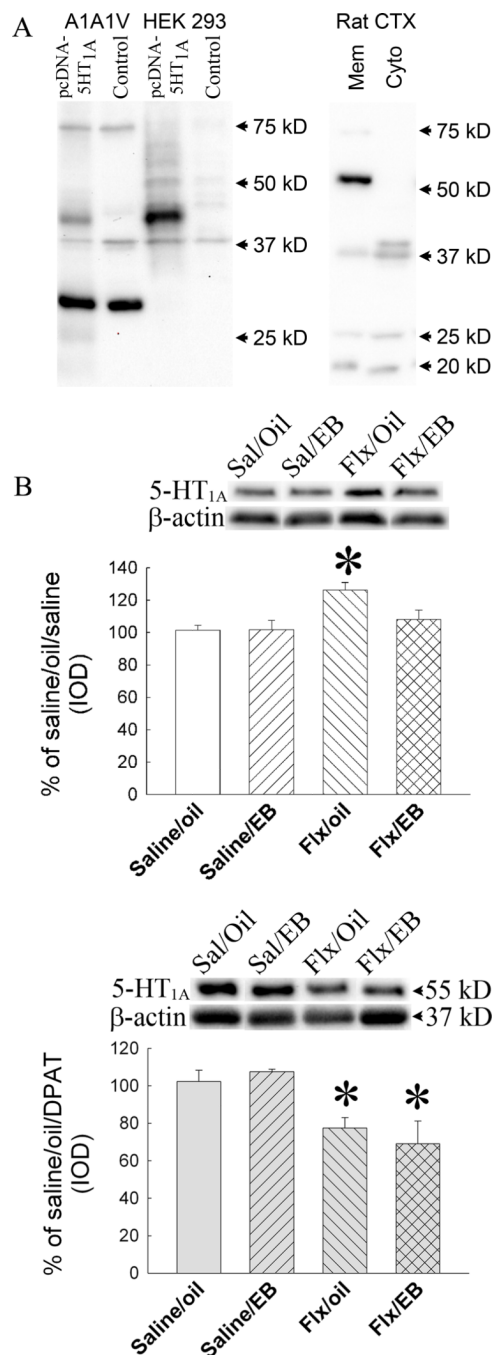
Figure 2. Effects of fluoxetine and/or EB treatment for 2 days on protein levels of 5-HT$_{1A}$ receptor in the PVN membrane of saline- or 8-OH-DPAT-challenged rats
A. Characterization of 5-HT$_{1A}$ receptor antibody: Left panel shows that the rabbit-anti-5-HT$_{1A}$ receptor antibody detected 5-HT$_{1A}$ receptors expressed in HEK 293 and A1A1V cells that were transfected with pcDNA-5HT$_{1A}$ receptors as well as endogenously expressed 5-HT$_{1A}$ receptors. Right panel shows the detection of 5-HT$_{1A}$ receptors in the cytosol and membrane of rat hippocampus. **B**: Immunoblot assay for protein levels of 5-HT$_{1A}$ receptors in the PVN membrane from fluoxetine and/or EB-treated rats challenged with saline (top) and 8-OH-DPAT (bottom). The data are presented as the mean ± SEM (n = 6–8). *: significantly different from saline/oil group with same challenge by Student Newman-Keuls

post-hoc test, $P < 0.05$. Saline/oil: rats treated with saline and oil; Saline/EB: rats treated with saline and EB; Flx/oil: rats treated with fluoxetine and oil and Flx/EB: rats treated with combined fluoxetine and EB.
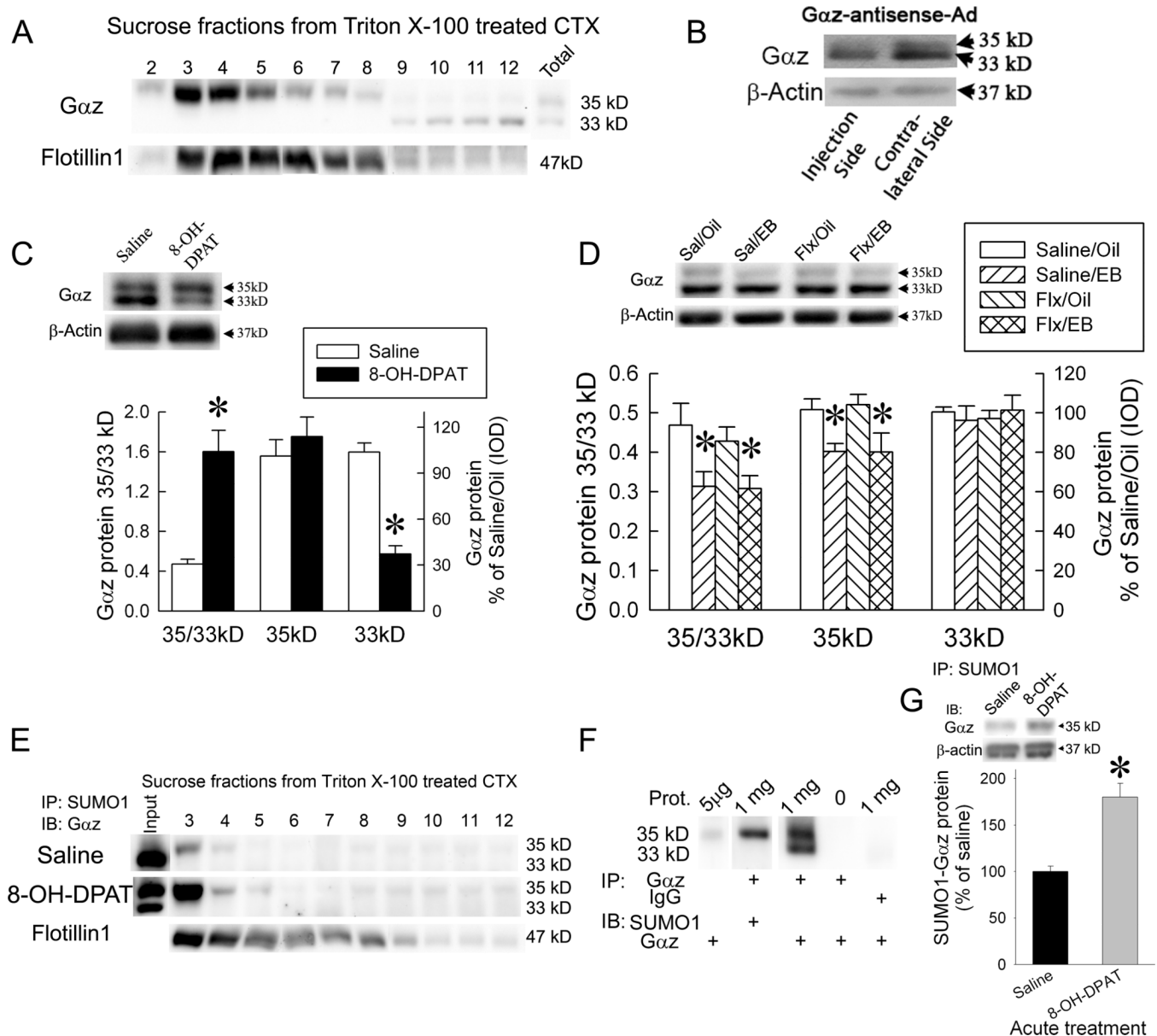
Figure 3. Effects of fluoxetine and/or EB treatment for 2 days and acute 8-OH-DPAT on the Gαz protein and sumoylated Gαz in the DRM

A: Immunoblot of sucrose fractions from Triton X-100 treated cortex membrane to determine the distribution of Gαz protein in the DRM. Fractions containing the DRM were identified by the DRM marker, flotillin; Total: Triton X-100-treated membrane preparation without sucrose gradient centrifugation. **B**: To verify that both the 35 and 33 kD bands detected by anti-Gαz antibody are Gαz proteins, we unilaterally injected recombinant adenovirus containing Gαz-antisense sequence (Ad-Gαz-AS) into the PVN. Immunoblotting of Gαz protein performed using tissue from the viral injection site and its contra-lateral side of the PVN, showed that injection of Ad-Gαz-AS reduces both 35 and 33 kD Gαz proteins. **C**: Effect of acute treatment with 8-OH-DPAT on the ratio of 35/33 kD Gαz protein (left y-axis) and protein level of Gαz protein (right y-axis). The data were presented as the mean ± SEM (n=4–5). *: significantly different from saline treated group by Student Newman-Keuls post hoc test, $P < 0.05$. **D**: Effect of EB and/or fluoxetine

treatment for 2 days on the ratio of 35/33 kD Gαz protein (left y-axis) and protein level of Gαz protein (right y-axis). The data are presented as the mean ± SEM (n = 5–6). *: significantly different from saline/oil group by Student-Newman-Keuls post-hoc test. **E and F**: Sumoylation of Gαz protein is verified by imunoprecipitation with anti-SUMO1 antibody (E) and anti-Gαz antibody (F). Flotillin 1 shown in (E) was prepared using 8-OH-DPAT-treated fractions. IP: immunoprecipitation, IB: Immunobloting. IgG: Rabbit IgG. Input: sample without IP. **G**: Sumoylated Gαz protein in the hypothalamus without PVN was increased by acute treatment with 8-OH-DPAT. The data were normalized to sumoylated β-actin and calculated as described in Methods. The data are presented as the mean ± SEM (n=5–6). *: significantly different from saline treated group by *t*-test, *P* < 0.05.
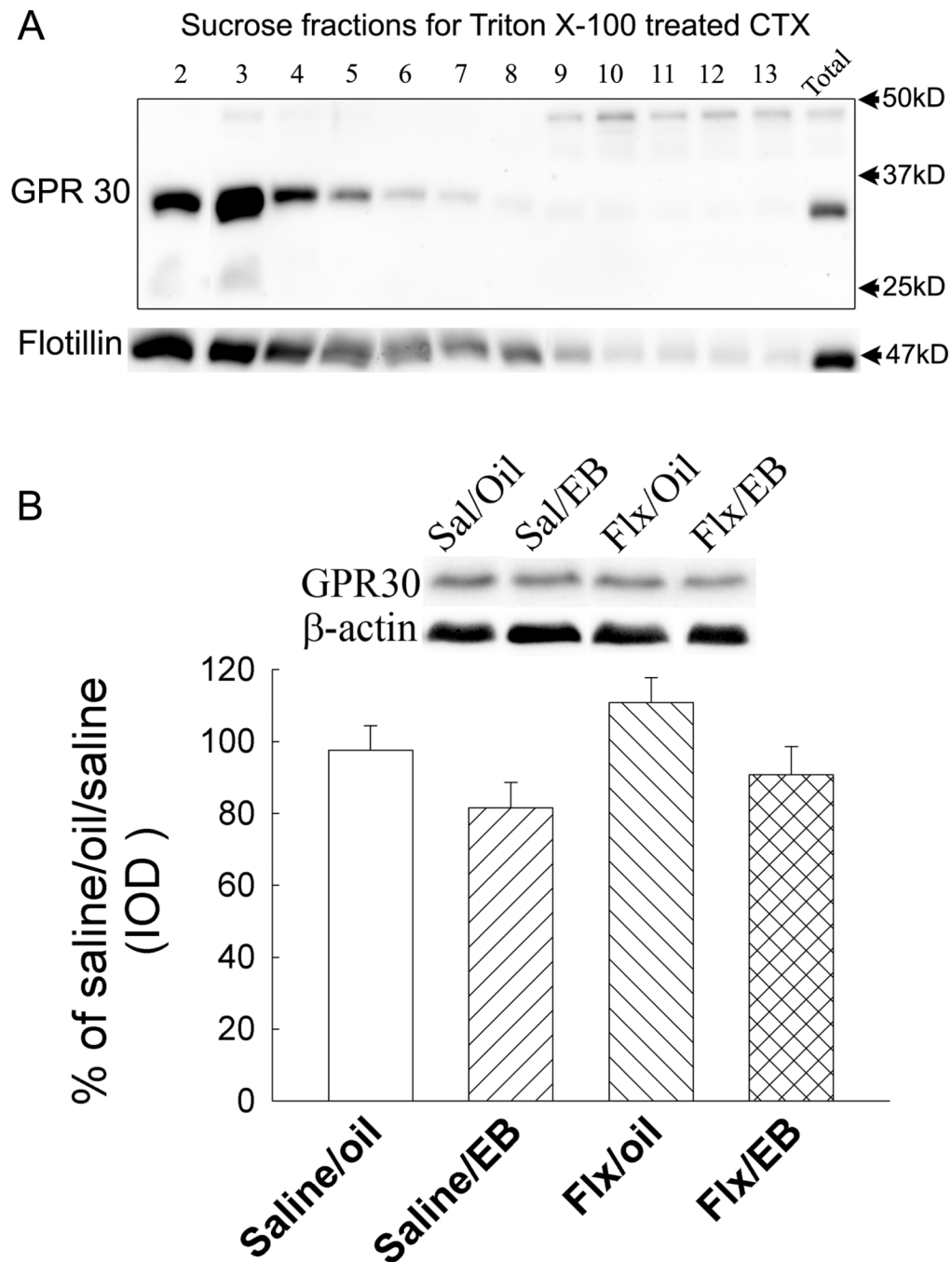
Figure 4. Effects of fluoxetine and/or EB treatment for 2 days on protein levels of active form of GPR30 in the membrane of the PVN

A: The majority of non-modified GPR30 (~32 kD is located in the DRM; Total: Triton X-100-treated membrane preparation without sucrose gradient centrifugation. **B**: Effect of EB and/or fluoxetine treatment for 2 days on the protein level of GPR30. The data are presented as the mean ± SEM (n = 5–6). Two-way ANOVA showed a significant difference on the effect of EB, although no significant difference between individual groups was detected with the Student Newman-Keuls post-hoc test.
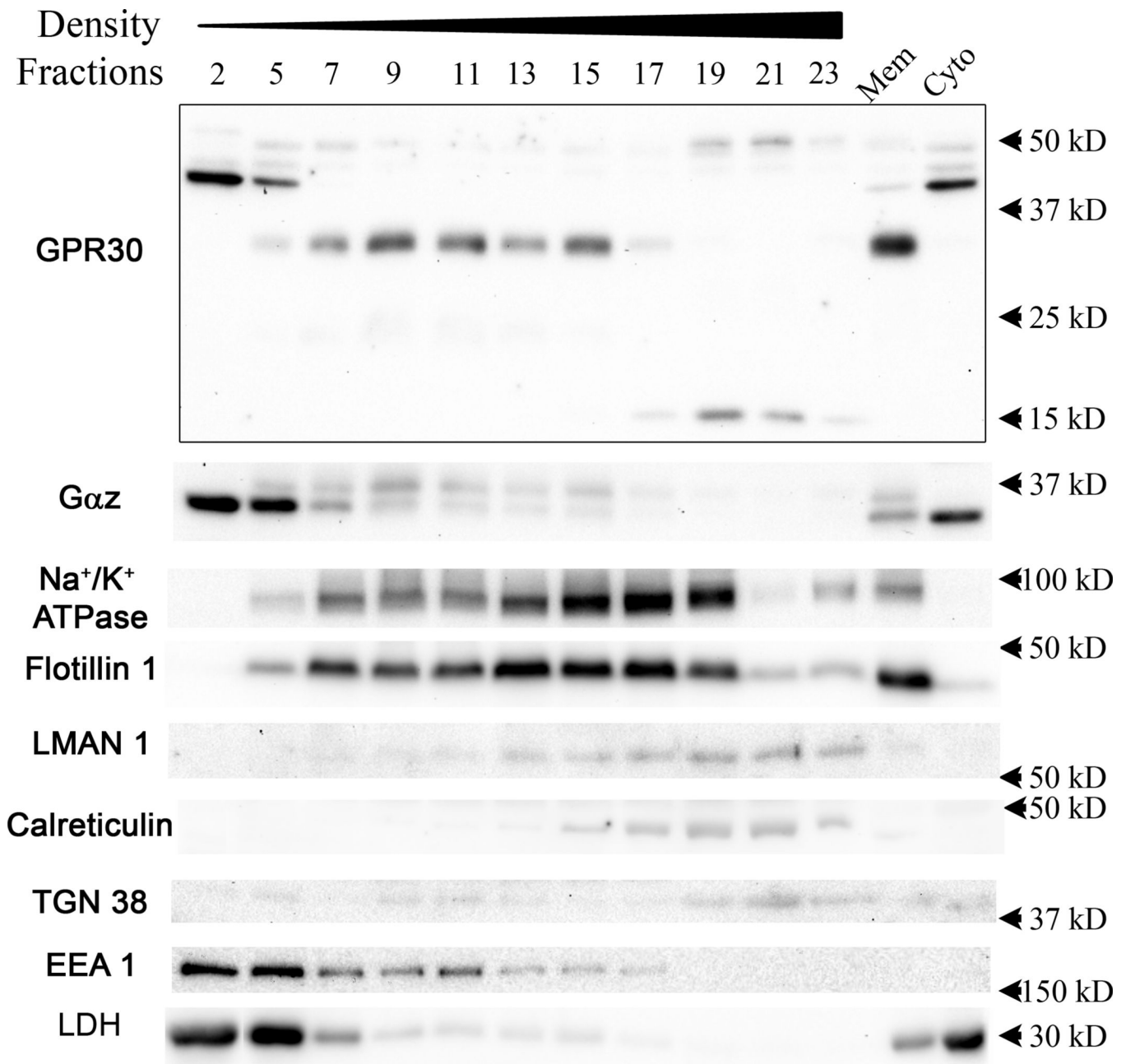
Figure 5. Subcellular distribution of Gαz protein and GPR30 in rat hippocampus
Immunoblots shows the co-localization of Gαz protein and GPR30 with plasma membrane
markers, Na⁺/K⁺ ATPase α1 and flotillin 1; ER marker, calreticulin; ER-Golgi intermediate
compartment marker, LMAN1 (Lectin, Mannose-binding 1, or ERGIC 53); trans-Golgi
network marker, TGM 38 (Trans-Golgi network protein2); endosome marker, EEA1(early
endosome antigen 1) and cytosol marker, LDH (lactate dehydrogenase). Mem and Cyto
indicate the total membrane fraction and cytosolic fraction of the cortex tissue prepared as
described previously (Creech et al., 2012).

Table 1

Antibodies used for immunoprecipitation and immunoblots

Antibodies	IP	Immunoblot	Source
Mouse-anti-SUMO-1 (D-11)	4 μg		Santa Cruz #sc-5308
Rabbit-anti-SUMO-1		1:1000	Enzo #BML-PW0505
Rabbit-anti-Gαz (1–20)	4 μg	1:2000	Santa Cruz #sc-388
Rabbit-anti-5HT$_{1A}$ receptor		1:500	gift from Dr. Scrogin
Rabbit-anti-GPR30		1:1000	Novus, # NLS4271
Mouse-anti-Flotillin-1		1:2000	BD #610820
Mouse-anti-Na$^+$/K$^+$ ATPase α1		1:2000	Santa Cruz #sc-21712
Rabbit-anti-calreticulin		1:2000	Abcam #Ab4
Rabbit-anti-LMAN1		1:500	Epitomic #T3357
Rabbit-anti-TGN38 (M290) *		1:1000	Santa Cruz #sc-33784
HRP conjugated LDH		1:4000	Abcam #Ab7639-1
Goat-anti-EEA1 (C-15)		1:1000	Santa Cruz #sc-6414
Mouse-anti-β-actin (C4)		1:20,000 (30–60 min)	MP #69100

Abcam: Abcam Inc. Cambridge, MA; BD: BD Biosciences San Jose, CA; Enzo: Enzo Life Sciences Inc. Farmingdale, NY; Epitomics: Epitomics Inc. Burlingame, CA; MP: MP Biomedicals, LLC, Solon, OH; Novus: Novus Biologicals Inc., Littleton, Co; Dr. Scrogin: Karie E Scrogin at Loyola University Chicago; Santa Cruz: Santa Cruz Biotechnology Inc., Santa Cruz, CA.

*
Wash with 2% milk in 1% Tween 20 TBS.