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Role of SAP97 in the regulation of 5-HT_{2A}R endocytosis and signaling

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Running Title: *SAP97 regulates 5-HT$_{2A}$R activity*

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Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; β_1AR, β_1-adrenergic receptor; CRFR, corticotropin receptor; ERK1/2, extracellular regulated protein kinase 1/2; FL, FLAG; GFP, green fluorescence protein; GPCR, G protein-coupled receptor; htt, huntingtin; IP, inositol phosphate; NMDAR, N-methyl-D-aspartate receptor; PDZ, PSD95/Disc Large/Zona Occludens; rLuc, renilla luciferase; 5-HT, serotonin; 5-HT$_{2A}$R, serotonin 2A receptor;5-HT$_{2C}$R, serotonin 2C receptor; shRNA, single hair pin RNA; TP receptor, thromboxane A2 receptor; YFP yellow fluorescence protein

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ABSTRACT

Serotonin (5-HT) interacts with a wide variety of 5-HT receptors (5-HTR) of which 5-HT_{2A}R plays an important target for antidepressant and atypical antipsychotic drugs. The carboxyl-terminal tail of 5-HT_{2A}R encodes a motif that mediates interactions with PSD-95/Discs Large/Zona Occludens 1 (PDZ) domain containing proteins. In the present study, we found that 5-HT_{2A}R interacts with synapse-associated protein 97 (SAP97; also known as DLG1) by co-immunoprecipitation in human embryonic 293 (HEK 293) cells and cortical brain lysates. We find that 5-HT_{2A}R expression results in the recruitment of SAP97 from the cytosol to the plasma membrane and that this recruitment is dependent upon an intact 5-HT_{2A}R PDZ binding motif. We also show that 5-HT_{2A}R interacts with SAP97 using bioluminescence energy transfer and that overexpression of SAP97 retards 5-HT_{2A}R endocytosis, while single hair pin RNA knockdown facilitates 5-HT_{2A}R internalization. The knockdown of SAP97 in HEK 293 cells results in a reduction in the maximum efficacy for 5-HT_{2A}R-stimulated inositol phosphate formation and that the deletion of the 5-HT_{2A}R PDZ motif also impairs 5-HT_{2A}R signaling. Similar to what has been observed for the corticotropin releasing factor receptor 1 (CRFR1), SAP97 expression is essential for 5-HT_{2A}R-stimulated ERK1/2 phosphorylation by a PDZ interaction-independent mechanism. Moreover, we find that SAP97 is not responsible for CRFR1-mediated sensitization of 5-HT_{2A}R signaling. Taken together, our studies show that SAP97 plays a conserved role in regulating 5-HT_{2A}R endocytosis and ERK1/2 signaling, but plays a novel role in regulating 5-HT_{2A}R G protein coupling.

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INTRODUCTION:

Serotonin (5-HT) is a monoamine neurotransmitter that regulates a wide range of higher neurological functions including the regulation of mood, sleep, and appetite (Hale et al., 2012). The majority of serotonergic neurons in the brain project outwards from the raphe nuclei towards a multitude of brain regions, including many structures within the limbic system (Bobillier et al., 1976). Once released, serotonin activates a large subset of 5-HT receptors (5-HT_{1-7}R), all of which, with exception to 5-HT_3R, are G protein-coupled receptors (GPCRs) (Barnes, 2011). The 5-HT_{2A}R and 5-HT_{2C}R GPCRs are currently primary molecular targets for drugs used in the treatment of mood and behavioral disorders, such as: anxiety disorders, depression, schizophrenia, obsessive-compulsive disorder, and eating disorders (Gray and Roth, 2001; Roth and Xia., 2004; Catapano and Manji, 2007). The involvement of these specific 5-HT_2Rs in depression is highlighted by the examination of post-mortem human brain tissue from depressed individuals and victims of suicide (Catapano and Manji, 2007). The brains of these subjects are found to have significantly greater expression of both 5-HT_{2A}R and 5-HT_{2C}R when compared to their control counterparts. Untreated schizophrenics show a similar increase in 5-HT_{2A}R expression in the brain (Fribourg et al., 2011). Additionally, current antidepressants and atypical (second generation) anti-psychotics have demonstrated the ability to down-regulate 5-HT_{2A}R expression in the cortical brain (Catapano and Manji, 2007; Fribourg et al., 2011), suggesting an importance for 5-HT_{2A}R in the symptoms of depression and psychoses. In particular, pharmacological studies and knockout mice have demonstrated that 5-HT_{2A}R and 5-HT_{2C}R contribute to anxiety and are pharmacological targets for the treatment of anxiety (Weisstaub et al., 2006).

Although many studies have investigated the pharmacological regulation of 5-HT_{2A}R and 5-HT_{2C}R in psychiatric disorders, there are a limited number of studies that have focused on the contribution of C-terminal 5-HT_{2A}R and 5-HT_{2C}R PSD-95/Disc Large/Zona Occludens (PDZ)

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binding motif interactions with PDZ domain-containing proteins to 5-HT_2R-related disorders (Abbas et al., 2009; Jones et al., 2009; Magalhaes et al. 2010; Pichon et al., 2010; Wattiez et al., 2013). For example, PSD-95 regulates the actions of atypical antipsychotics on 5-HT_2Rs and kalirin-7 mediates 5-HT_2R-dependent modulation of spine morphology (Abbas et al., 2009; Jones et al., 2009). In addition, disruption of 5-HT_{2A}R PDZ protein interactions enhances SSRI efficacy in neuropathic pain and alleviates mechanical sensitivity to inflammation (Pichon et al., 2010; Wattiez et al., 2013). Thus, the emerging understanding of the importance of PDZ domain-containing proteins in the regulation of 5-HT_{2A}R activity will be essential for understanding of how the intracellular trafficking of 5-HT_{2A}R may contribute to the regulation of agonist-stimulated 5-HT_{2A}R cellular responses (Backstrom et al., 2000; Xia et al., 2003a; Xia et al., 2003b; Gavarini et al., 2006; Jones et al., 2009; Magalhaes et al. 2010; Pichon et al., 2010; Wattiez et al., 2013).

The 5-HT_{2A}R primarily signals via the activation $G\alpha_{q/11}$-mediated signaling pathways, leading to PLC activation and the accumulation of inositol phosphate (IP) and diacylglycerol resulting in the release of intracellular Ca^{2+} stores and the activation of protein kinase C. However, alternate signaling pathways have been identified, including the extracellular regulated protein kinase 1/2 (ERK1/2) signalling pathway that can be activated by both G protein-dependent and - independent mechanisms (Raymond et al., 2001; Magalhaes et al., 2012). Recently, studies from our laboratory have demonstrated that the activation of the corticotropin releasing factor receptor 1 (CRFR1) enhances subsequent $5HT_{2A/C}$R-mediated IP_3 signaling via a mechanism that is dependent upon PDZ protein-interactions with both receptors (Magalhaes et al., 2010). This suggests that one or more PDZ domain-containing proteins interact with both CRFR1 and $5HT_{2A}$R to positively modulate $5HT_{2A}$R signaling.

Previously, SAP97 has been demonstrated to interact with both 5-HT_{2A}R and 5-HT_{2C}R (Becamel et al., 2002; Becamel et al., 2002). We have shown that SAP97 interacts with CRFR1

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to negatively regulate receptor endocytosis without affecting inositol phosphate signaling (Dunn et al.,2013). Although SAP97 is essential for CRF-mediated ERK1/2 signaling, it regulates CRFR1 signaling independently of its receptor interactions (Dunn et al., 2013). Therefore, in the present study, we have tested the hypothesis that $5HT_{2A}R$ signaling and trafficking may also be modulated as a consequence of the interaction with SAP97 with the receptor. We find that similar to what we reported for CRFR1, SAP97 is involved in the regulation of $5HT_{2A}R$ endocytosis and ERK1/2 activation. However, unlike what was previously observed for CRFR1 signaling, SAP97 expression is required for $5HT_{2A}R$ G protein coupling, but is not exclusively involved in the observed CRFR1 pretreatment-dependent enhancement of $5HT_{2A}R$ signaling.

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MATERIALS AND METHODS:

Materials:

ECL Western blotting detection reagents were purchased from GE Healthcare and BioRad. The Dowex 1-X8 (formate form) resin with 200-400 mesh was purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin (BSA) was obtained from BioShop Canada Inc. (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was from BioRad (Mississauga, ON). Rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-402) and rabbit anti-p44/42 MAPK antibodies were obtained from Cell Signaling Technology (Pickering, Ontario, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen. Mouse anti-SAP97 antibody was obtained from Assay Designs/Enzo Life Sciences (Farmingdale, NY). Alexa Fluor 647 anti-rabbit IgG and Zenon antibody were purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti-FLAG antibody and all other biochemical reagents were purchased from Sigma.

Plasmid Constructs:

FLAG-tagged $5HT_{2A}R$ and $5HT_{2A}R$-ΔSCV constructs were described previously (Magalhaes et al., 2010). The YFP-SAP97 (rat isoform 2) construct and SAP97 shRNA were described previously and graciously provided by Dr. Suleiman W. Bahouth (Neuroscience Institute, University of Tennessee Health Sciences Center) (Gardner et al., 2006; Dunn et al., 2013). The YFP-SAP97 (rat isoform 2) construct was subcloned into peGFP-N1. A FLAG-tagged 5-$HT_{2A}R$ *renilla luciferase* (rLuc) fusion protein (FL-5-$HT_{2A}R$-x22-SCV) was custom synthesized by GenScript (Piscataway, NJ) to insert rLuc into the 5-$HT_{2A}R$ C-terminal tail 22 amino acids upstream of the PDZ binding motif. The construct was digested with HindIII/EcoR1 from a pU57 plasmid and subcloned into a pcDNA3.1 plasmid expression vector digested with the same restriction enzymes.

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***Cell Culture and Transfection*:**

Human embryonic kidney (HEK 293) cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were seeded on 10-cm dishes at 70–80% density 24 h prior to transfection. Transfection was performed using a modified calcium phosphate method, as described previously (Caron and Ferguson, 2004). Transfections were performed with 1 μg of each construct, with exception that 3 μg of plasmid cDNA was used for all shRNA constructs. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and suspended with trypsin, 0.25% EDTA. All experiments were conducted 48 hours after the initial transfection, with the exception of transfections involving SAP97 shRNA, which were conducted 72 hours after initial transfection to optimize the knockdown of endogenous SAP97, as confirmed by Western blotting.

***Co-immunoprecipitation*:**

Transfected HEK293 cells were seeded onto 10-cm dishes the day before the experiment. Cells were serum-starved for 1 hour in (Hanks buffered saline solution (HBSS), and dishes were treated with either HBSS alone or with 10 μM 5HT agonist in HBSS for 30 min at 37 °C. Cells were subsequently lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μg/ml leupeptin, and 5μg/ml aprotinin) for 20 minutes on a rocking platform at 4°C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 x *g* for 15 min at 4°C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 400 μg of protein was incubated overnight at 4 °C with FLAG-immunoprecipitation beads from Sigma. After incubation, beads were washed three times with

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cold lysis buffer and incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted to identify co-immunoprecipitated GFP-SAP97 (rabbit anti-GFP, 1:1000). An additional Western blot was performed to examine FLAG-$5HT_{2A}$R/FLAG-$5HT_{2A}$R-ΔSCR (rabbit anti-FLAG, 1:1000), and GFP-SAP97 (rabbit anti-GFP, 1:1000) protein expression. For the co-immunoprecipitation of endogenous proteins from cortical extracts, adult mouse brains were employed. Tissue was dissected and homogenized on ice in lysis buffer containing protease inhibitors. The particulate fraction was removed by centrifugation, and 2 mg of supernatant protein was incubated with 5 μl/sample of either goat polyclonal $5HT_{2A}$R or FLAG antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and protein G Sepharose beads overnight at 4°C. Afterwards, the beads were washed three times with lysis buffer and proteins were eluted in 3x SDS-PAGE loading buffer by warming the samples at 55°C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes for immunoblotting with antibodies described in the *Figure Legends.*

Live Cell Confocal Microscopy:

Following transfection, HEK 293 cells were re-seeded onto 35 mm glass bottom confocal dishes. Cells were serum-starved for 1h at 37°C in HBSS and then labeled with rabbit anti-FLAG antibody (1:200) and Zenon Alexa Fluor 647 rabbit IgG1 labeling kit (Invitrogen) at 4°C for 30 min. The cells were washed with HBSS and warmed to 37°C for live imaging using a heated stage. Confocal microscopy was performed on a Zeiss LSM-510 META laser scanning confocal microscope using a Zeiss 63x, 1.3 NA, oil immersion lens. Co-localization studies were performed using dual excitation (488 and 633 nm) and emission (band pass 505–550 nm and long pass 650 nm for YFP/GFP and Alexa Fluor 647, respectively) filter sets. The specificity of

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labeling and absence of signal crossover were established by examination of single-labeled samples.

Bioluminescence Resonance Energy Transfer:

HEK 293 cells were transfected with 20 ng of plasmid cDNA encoding 5-HT_{2A}R-rLuc-x22-SCV and increasing ratios of plasmid cDNA encoding either YFP-SAP97 or YFP-huntingtin (htt) in 96 well plates. Coelenterazine, was added to each well (5 uM final. Light emission was measured from the luciferase donor and YFP acceptor at 460 nm and 535 nm wavelengths, respectively. The BRET ratio was defined as [(emission at 535 nm)-(emission at 460 nm) X Cf]/(emission at 460 nm) where Cf corresponded to (emission at 535 nm/emission at 460 nm) for the control, Rluc expressed alone.

***Receptor Endocytosis*:**

Following transfection, HEK 293 cells were re-seeded into 12-well plates. Cells were serum-starved for 1h at 37°C in HBSS and then stimulated with or without 10 μM 5HT in HBSS at 37°C for the times indicated in the figure legends. Cells were washed with cold HBSS and treated with mouse anti-FLAG antibody (1:1000) for 45 min on ice. Cells were washed with cold HBSS and additionally treated with Alexa Fluor 647 donkey anti-mouse IgG (Invitrogen) (1:1000) for 45 min on ice. Cells were washed with cold PBS and treated with 5mM EDTA in PBS for 5 min on ice. Newly suspended HEK 293 cells were then transferred to flow cytometry tubes containing 4% formaldehyde in PBS. Samples were run on a FACSCalibur cytometer using BD CellQuest Pro software until 10,000 cells were counted. The geometric mean of fluorescence was determined using FlowJo analysis software, with less fluorescence corresponding to less 5-HT_{2A}R on the membrane.

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Measurement of inositol phosphate formation:

HEK 293 cells were transiently transfected with the cDNAs as described in the *Figure Legends*. 48 h post-transfection cells were incubated overnight in inositol-free DMEM with 100 μCi/ml *myo*-[^{3}H]-Inositol. For all experiments cells were incubated for 1 h in warm HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM NaHCO3, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, pH 7.4) and were then incubated with 10 mM LiCl alone for 10 min followed by increasing doses of 5-HT in LiCl for 30 min. Cells were placed on ice and the reaction was stopped with 500 μl of perchloric acid and was neutralized with 400 μl of 0.72 M KOH, 0.6 M $KHCO_3$. Total cellular [^{3}H]-inositol incorporation was determined in 50 μl of cell lysate. Total inositol phosphate was purified by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin and [^{3}H]-inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

***ERK Phosphorylation*:**

Following transfection, HEK 293 cells were re-seeded into 6-well plates. Cells were serum starved for 1h at 37°C in HBSS and then stimulated with 10 μM 5HT agonist for the duration of the described time points. Cells were lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1mM AEBSF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin) for 20 min on a rocking platform at 4°C. Samples were collected into 1.5 mL Eppendorf tubes and centrifuged at 15,000 x *g* for 15 minutes at 4 °C to pellet insoluble material. A Bronsted-Lowry protein assay was performed, and 50 μg of protein was incubated overnight at room temperature in 3x SDS Loading Buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for ERK1/2 (rabbit anti-p44/42 MAPK, 1:1000), phospho-ERK1/2 (rabbit anti-phospho-p44/42 MAPK, 1:1000), SAP97 (mouse anti-SAP97, 1:1000), and

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FLAG-5HT$_{2A}$R expression (rabbit anti-FLAG, 1:1000), followed by a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:10,000) or anti-mouse antibody (1:10,000) where appropriate. Proteins were detected using chemiluminescence with the enhanced chemiluminescence kit from GE Healthcare.

***Statistical Analysis*:**

Densitometric data were normalized first for protein expression, and the maximum value was set to 100, with all other values displayed as the percentage thereof. One-way analysis of variance test was performed to determine significance, followed by a post hoc Tukey multiple comparison test to determine which means were significantly different ($p < 0.05$) from one another.

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RESULTS:

SAP97 is co-immunoprecipitated with 5-HT$_{2A}$R in a PDZ-binding motif-dependent manner: Previously, we demonstrated that the class I carboxyl-terminal tail CRFR1 PDZ binding motif was essential for CRFR1-mediated sensitization of 5-HT$_{2A}$R signaling and that the PDZ domain-containing protein SAP97 interacted with CRFR1 to regulate its endocytosis and signaling (Magalhaes et al., 2010; Dunn et al., 2013). Therefore, we tested whether SAP97 might also be co-immunoprecipitated with 5-HT$_{2A}$R in a PDZ motif-dependent manner. We found that green fluorescent protein (GFP-SAP97) fusion protein was co-immunoprecipitated with a FLAG (FL) epitope-tagged 5-HT$_{2A}$R (FL-5-HT$_{2A}$R) from HEK 293 cells, but that agonist stimulation with 10 μM 5-HT did not affect the association of GFP-SAP97 with FL-5-HT$_{2A}$R (Fig. 1A). This interaction was dependent on an intact PDZ binding motif at the end of the carboxyl-terminal tail, as the deletion of the last three critical amino acid residues (ΔSCV) of the 5-HT$_{2A}$R carboxyl-terminal tail prevent GFP-SAP97 co-immunoprecipitation with the resulting FL-5-HT$_{2A}$R-ΔSCV mutant (Fig. 1A). We found that endogenous SAP97 could also be co-immunoprecipitated with endogenous 5-HT$_{2A}$R from cortical brain lysates (Fig.1 B). Thus, SAP97 interacts with 5-HT$_{2A}$R via the 5-HT$_{2A}$R carboxyl-terminal PDZ binding motif.

SAP97 colocalization with 5HT$_{2A}$R at the plasma membrane is dependent on an intact 5HT$_{2A}$R PDZ-binding motif: GFP-SAP97 when expressed alone in HEK 293 cells was diffusely localized throughout the cytoplasm and was not localized to the plasma membrane (Fig. 2A). However, when GFP-SAP97 was co-expressed with FL-5-HT$_{2A}$R in HEK293 cells, GFP-SAP97 immunofluorescence redistributed to the plasma membrane, where it colocalized with Alexa Fluor 647 labeled FL-5-HT$_{2A}$R (Fig. 2B). In contrast, deletion of the 5-HT$_{2A}$R carboxyl-terminal PDZ binding motif (ΔSCV) resulted in a FL-5-HT$_{2A}$R-ΔSCV mutant that was unable to promote the redistribution of GFP-SAP97 from the cytosol to the plasma membrane

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(Fig. 2C). To further establish whether SAP97 interacts with 5-HT_{2A}R, we prepared a FL-5-HT_{2A}R renilla luciferase (rLuc) fusion protein, where rLuc was inserted in frame into the 5-HT_{2A}R carboxyl-terminal tail 22 amino acid residues upstream of the SCV PDZ binding motif (FL-5-HT_{2A}R-rLuc-x22-SCV). We found that this construct was expressed at the cell surface of HEK 293 cells (Fig. 3A) and was coupled to the stimulation of inositol phosphate formation (Fig. 3B). The EC_{50} for FL-5-HT_{2A}R mediated IP formation was 8.6 nM and the EC_{50} for FL-5-HT_{2A}R-rLuc-x22-SCV mediated IP formation was 9.3 nM. Although the maximum efficacy for FL-5-HT_{2A}R-rLuc-x22-SCV stimulated IP formation was attenuated when compared with the maximum efficacy for FL-5-HT_{2A}R stimulated IP formation, this was the consequence of reduced cell surface FL-5-HT_{2A}R-rLuc-x22-SCV expression (data not shown). When HEK 293 cells were transfected with a constant amount of FL-5-HT_{2A}R-rLuc-x22-SCV plasmid cDNA (20 ng) along with increasing ratios of plasmid cDNA expressing YFP-SAP97 we observed an increasing, but saturable level of bioluminescence resonance energy transfer (BRET) (Fig. 3C). In contrast, co-expression of FL-5-HT_{2A}R-rLuc-x22-SCV with increasing rations of wild-type YFP-htt did not result in an increased BRET ratio (Fig. 3D). Thus, the immunofluorscence subcellular localization data, co-immunoprecipitation data, and BRET data all indicated that direct SAP97 interactions with 5-HT_{2A}R were PDZ binding motif dependent.

SAP97 antagonizes the internalization of 5HT_{2A}R: We previously demonstrated that SAP97 contributed to the regulation of agonist-stimulated CRFR1 internalization by attenuating the endocytosis of the receptor (Dunn et al., 2013). Therefore, we tested whether the overexpression of GFP-SAP97 or knockdown of endogenous SAP97 expression in HEK 293 cells would alter the agonist-stimulated internalization of FL-5-HT_{2A}R. In HEK 293 cells expressing FL-5-HT_{2A}R the overexpression of SAP97 reduced agonist-stimulated (30 min, 10 μM 5-HT) internalization of the receptor and SAP97 shRNA expression resulted in an increase in FL-5-HT_{2A}R endocytosis, as measured by a loss of cell surface FL-5-HT_{2A}R

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immunofluorescence (Fig.4A). In contrast, deletion of the FL-5-HT_{2A}R PDZ binding motif resulted in a FL-5-HT_{2A}R-ΔSCV mutant that was impaired in its internalization, when compared to the wild-type receptor and neither GFP-SAP97 overexpression nor SAP97 shRNA expression altered the extent of FL-5-HT_{2A}R-ΔSCV endocytosis (Fig. 4A). In addition, the deletion of the SCV motif, SAP97 overexpression and SAP97 knockdown had no effect on 5-HT_{2A}R cell surface expression (Fig. 4B). Taken together, similar to what was observed for the CRFR1 (Dunn et al., 2013), FL-5-HT_{2A}R internalization was modulated by SAP97 expression, but normal internalization of the receptor was dependent upon an intact PDZ binding motif. This observation suggested that either some other PDZ protein influenced the normal endocytosis of the receptor or the SCV motif itself might contribute directly to the regulation of 5-HT_{2A}R endocytosis. For example the SCV motif might serve as a site for G protein-coupled receptor kinase phosphorylation.

SAP97 regulation of 5HT_{2A}R-mediated IP formation: We previously found that SAP97 did not contribute to the regulation of CRFR1-mediated cAMP signaling (Dunn et al., 2013). However, a structurally similar PDZ containing protein, PSD95, was previously demonstrated to enhance 5HT_{2A}R-mediated inositol phosphate (IP) signaling (Xia et al., 2003b). Therefore, we tested whether either the overexpression of GFP-SAP97 or SAP97 shRNA treatment altered 5-HT-stimulated IP formation in FL-5-HT_{2A}R expressing HEK 293 cells. In HEK 293 cells transfected with FL-5-HT_{2A}R, overexpression of GFP-SAP97 did not alter IP formation in response to increasing concentrations of 5-HT (Fig. 5A). However, shRNA knockdown of endogenous SAP97 significantly attenuated the maximum efficacy for 5-HT-stimulated IP formation in cells transfected with FL-5-HT_{2A}R, when compared to cells treated with scrambled shRNA (Fig. 5A). In contrast, neither GFP-SAP97 overexpression nor SAP97 shRNA treatment altered the dose response for 5-HT-stimulated IP formation in cells expressing FL-5-HT_{2A}R-

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ΔSCV (Fig. 5B). Interestingly, when the dose response curves for 5-HT-stimulated IP formation in FL-5-HT_{2A}R and FL-5-HT_{2A}R-ΔSCV were compared, the maximum efficacy for FL-5-HT_{2A}R-ΔSCV-stimulated IP formation was found to be significantly attenuated when compared to wild-type FL-5-HT_{2A}R (Fig. 5C). These results indicated that endogenous SAP97 interactions with the 5-HT_{2A}R PDZ binding motif contribute to the regulation of 5-HT_{2A}R-mediated $G\alpha_{q/11}$ signaling.

SAP97 regulation of 5HT_{2A}R-mediated ERK1/2 phosphorylation is PDZ binding motif-independent: We recently demonstrated that SAP97 regulated CRFR1-mediated ERK1/2 phosphorylation in a manner that was independent of SAP97 interactions with CRFR1 PDZ binding motif (Dunn et al., 2013). Therefore, we tested the effect of GFP-SAP97 overexpression and shRNA knockdown on ERK1/2 phosphorylation in response to 10 μM 5-HT stimulation of the 5-HT_{2A}R for 0, 5 and 10 min. We found the overexpression of GFP-SAP97 did not significantly alter 5-HT-stimulated ERK1/2 phosphorylation in response to the activation of the 5-HT_{2A}R, but SAP97 shRNA treatment significantly attenuated 5-HT_{2A}R-mediated ERK1/2 phosphorylation (Fig. 6A). Similar to what we observed for a CRFR1 mutant receptor that lacked its carboxyl-terminal PDZ binding motif, ERK1/2 phosphorylation in response to the activation of the 5-HT_{2A}R-ΔSCV mutant was also unaffected by GFP-SAP97 overexpression, but was still significantly attenuated following the knockdown of SAP97 in response to SAP97 shRNA treatment (Fig. 6B). Taken together these results support a generalized role for SAP97 in the regulation of GPCR-mediated ERK1/2 phosphorylation that does not require PDZ domain interactions with GPCRs.

SAP97 is not exclusively involved in CRFR1-mediated enhancement of 5HT_{2A}R-mediated IP formation: Our primary rationale for assessing the role of SAP97 in the regulation of CRFR1

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and 5-HT_{2A}R signaling and endocytosis was to determine whether SAP97 was the PDZ protein underlying CRFR1-mediated sensitization of 5-HT_{2A}R signaling (Magalhaes et al., 2010). Therefore, we tested the effect of SAP97 shRNA-mediated knockdown on CRFR1-stimulated enhancement of 5-HT_{2A}R signaling. In HEK 293 cells transfected with CRFR1, 5-HT_{2A}R and scrambled shRNA, pretreatment with 500 nM CRF for 30 min resulted in an increase in the maximum efficacy for 5-HT-stimulated IP formation, when compared to cells that were not CRF pretreated (Fig. 7). As demonstrated in Fig. 5A, SAP97 shRNA treatment reduced the maximum efficacy for 5-HT-stimulated IP formation, when compared to 5-HT-stimulated IP responses in cells transfected with CRFR1, 5-HT_{2A}R and scrambled shRNA (Fig. 7). However, SAP97 shRNA down-regulation of SAP97 expression did not block the CRF pretreatment-induced increase in the maximum efficacy for 5-HT-stimulated IP formation in CRFR1 and 5-HT_{2A}R expressing cells (Fig. 7). Taken together, these results indicated that, although SAP97 contributed to the regulation of 5-HT_{2A}R-mediated IP formation, it did not exclusively contribute to the CRFR1-mediated sensitization of 5-HT_{2A}R-signaling previously reported by Magalhaes and colleagues (2010).

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DISCUSSION:

We previously demonstrated that two GPCRs linked to the manifestation of mood disorders and psychiatric disease, CRFR1 and $5HT_{2A}R$, exhibit crosstalk that is dependent upon PDZ-binding motif interactions with an unknown PDZ protein that results in the heterologous sensitization of $5HT_{2A}R$ signaling (Magalhaes et al., 2010). This prompted us to screen for PDZ domain-containing proteins that may interact with both CRFR1 and $5HT_{2A}R$ (Dunn et al., 2013) and which may also function as novel regulators of both CRFR1 and $5HT_{2A}R$ trafficking and signaling. We show here, that similar to what we previously reported for CRFR1 (Dunn et al., 2013), SAP97 functions to antagonize $5HT_{2A}R$ endocytosis. Thus, SAP97 appears to play a generalized and functionally consistent role in regulating the endocytosis of both the CRFR1 and $5HT_{2A}R$. Although SAP97 expression is essential for both CRFR1- and $5HT_{2A}R$-mediated activation of ERK1/2 phosphorylation, the role of SAP97 in regulating GPCR-dependent ERK1/2 signaling is independent of PDZ domain interactions with the receptors. Moreover, we find that the loss of SAP97 expression impairs $5HT_{2A}R$-stimulated IP signaling, but that SAP97 is not a PDZ domain containing protein that is exclusively required for CRFR1-dependent heterologous sensitization of $5HT_{2A}R$ signaling (Magalhaes et al., 2010).

SAP97 has previously been reported to interact with several neurotransmitter receptors including: the β_1-adrenergic receptor (β_1AR), CRFR1, somatostatin receptor subtype 1, kainite receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR) and was identified as a $5\text{-}HT_{2A}R$ interacting protein in a proteomic screen (Leonard et al., 1998; Bassand et al., 1999; Metha et al., 2001; Sans et al., 2001; Rumbaugh et al., 2003; Bécamel et al., 2004; He et al., 2006; Gardner et al., 2007; Cai et al., 2008; Dunn et al., 2013). Interestingly, each of these receptors have a class I PDZ-binding motif on the distal region of the carboxyl terminal tail. SAP97 plays a role in regulating either the cell surface localization, endocytosis, signaling, compartmentalization or recycling of each of these receptors proteins. For example, SAP97 regulates the cell surface expression of the

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AMPAR via its association with the AMPAR GluR1 subunit in the secretory pathway, but SAP97 is not involved in the NMDAR-mediated internalization of the receptor (Sans et al., 2001; Rumbaugh et al., 2003). SAP97 is also involved in the subcellular localization of kanaite receptors (Metha et al., 2001) and in the case of the NMDAR, SAP97 modulates NMDAR-mediated CaMKII phosphorylation and NMDAR synaptic localization (Gardoni et al., 2003). It is also reported that SAP97 interactions may mediate somatostatin receptor type 1-dependent regulation of synaptic growth cone dynamics (Cai et al., 2008). We have shown previously (Dunn et al., 2013) and in the present study that SAP97 interactions also contribute to the antagonism of agonist-induced endocytosis of both CRFR1 and 5-HT_{2A}R. Taken together, it appears that SAP97 may have a general role in promoting the membrane localization of transmembrane receptors that specifically encode class I PDZ-binding motifs. Future studies will be required to understand how PDZ proteins regulate the functional activity of GPCRs *in vivo* and contribute to the physiological regulation of GPCR-modulated animal behaviours and pathophysiological GPCR signaling.

Our previous studies have shown that SAP97 does not contribute to the regulation of $G\alpha_s$-mediated cAMP signaling in response to CRFR1 activation (Dunn et al., 2013). In contrast, our current study now suggests that SAP97 expression facilitates the ability of the 5-HT_{2A}R to stimulate IP formation via the activation of $G\alpha_{q/11}$. Previous studies have demonstrated that NHERF1 and PSD95 contribute to the regulation of IP signaling via the thromboxane A2 receptor (TP receptor) and 5-HT_{2A}R, respectively (Rochdi et al., 2002; Xia et al., 2003b). The association of PSD95 with 5-HT_{2A}R enhances 5-HT_{2A}R signal transduction similar to what we have observed for the structurally related PDZ protein SAP97 (Xia et al., 2003b). In contrast, activation of the TP receptor increases NHERF1 interactions with $G\alpha_{q/11}$, which in turn functions to impair $G\alpha_{q/11}$/TP receptor interactions that are required for the activation of phospholipase Cβ1-mediated IP formation (Rochdi et al., 2002). This suggests that PDZ domain containing

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proteins may function to both positively and negatively regulate the interface between GPCRs and heterotrimeric G proteins such as $G\alpha_{q/11}$. Future studies will be required to determine the mechanism by which PSD95 and SAP97 function to enhance 5-HT_{2A}R signaling and whether this mechanism is either complimentary to or antagonistic to the means by which NHERF1 antagonizes TP receptor signaling.

We previously demonstrated that CRF-mediated ERK1/2 phosphorylation is dependent upon the endogenous expression of SAP97 in both HEK293 and AtT20 cells (Dunn et al., 2013). This role for SAP97 in the regulation of ERK1/2 signaling is independent of the class I CRFR1 PDZ-binding motif, and SAP97 expression is required for CRFR2-mediated ERK1/2 phosphorylation, a receptor which, despite ~60% sequence homology with CRFR1, does not contain a class I PDZ-binding motif (Dunn et al., 2013). Our studies with 5-HT_{2A}R indicate that SAP97 plays a generalized role in regulating GPCR-dependent ERK1/2 signaling. Previous studies have demonstrated the ability of p38 MAPK family proteins to interact with and subsequently phosphorylate SAP97 following cellular stress responses resulting in disrupted SAP97 interactions with the actin cytoskeleton (Sabio et al., 2005). The fact that SAP97 is a substrate for p38 MAPK suggests that it may also serve as a scaffold for the assembly of ERK signaling complexes. This provides a potential molecular mechanism whereby SAP97 might regulate ERK1/2 signaling downstream of SAP97/GPCR.

Of central importance to this study was to assess whether SAP97 represents the PDZ protein that is required for CRFR1-mediated sensitization of 5-HT_{2A}R signaling. Although SAP97 shRNA-dependent knockdown of SAP97 protein expression significantly attenuates $5HT_{2A}$R-mediated IP signaling, CRF pretreatment in the absence of SAP97 expression still evokes heterologous sensitization of 5-HT_{2A}R-mediated IP signaling. This suggests that SAP97 is either not involved in the crosstalk between CRFR1 and $5HT_{2A}$R, or that other PDZ proteins are also required and/or compensate for the loss of SAP97 expression.

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In summary, in the current study we have characterized the role of the PDZ domain-containing protein SAP97 as a novel regulator of $5HT_{2A}R$ trafficking and signaling and confirm a receptor-independent role for SAP97 in regulating ERK1/2 signaling. PDZ domain containing proteins of the PSD95/SAP97 family have an established link to the regulation and treatment of mood disorders and psychiatric disease that may implicate $5HT_{2A}R$ signaling (Gray and Roth, 2001; Roth et al., 2004; Catapano and Manji, 2007). The efficacy of second-generation atypical antipsychotics appears to be dependent upon their ability to down regulate $5HT_{2A}R$-mediated signaling, while up-regulating metabotropic-glutamate receptor 2 (mGluR2) signaling (Fribourg et al., 2011). In the current study, we achieved down-regulation of $5HT_{2A}R$ signaling by preventing the interaction of SAP97 with $5HT_{2A}R$, thereby providing a novel strategy for manipulating $5HT_{2A}R$ function. Furthermore, the structurally similar PSD95 has been documented as an essential protein in atypical antipsychotic action on $5HT_{2A}R$ and $5HT_{2C}R$ (Abbas et al., 2009), suggesting the relationship between SAP97 and $5HT_{2A}R$ may be similarly important in the treatment of mood disorders. Further understanding of how PDZ proteins regulate CRFR1 and $5HT_{2A}R$ function will be essential for understanding their contribution to the pathological cell signaling associated with psychiatric diseases and the development of new strategies to treat these diseases.

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Authorship Contributions:

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Conducted experiments: Henry A. Dunn., Cornelia Walther, George Y. Yuan, Fabiana. A. Caetano, and Christina M. Godin

Contributed new reagents or analytic tools: Fabiana. A. Caetano., and Christina M. Godin

Performed data analysis: Henry A. Dunn and Stephen S. G. Ferguson

Wrote or contributed to the writing of the manuscript: Henry A. Dunn and Stephen S. G. Ferguson

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Footnotes:

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FIGURE LEGENDS:

Figure 1: **SAP97 co-immunoprecipitates with 5-HT$_{2A}$R in a PDZ-binding motif-dependent and agonist-independent manner:** **(A)** Representative immunoblot of GFP-SAP97 co-immunoprecipitated with FL-5-HT$_{2A}$R but not FL-5-HT$_{2A}$R-ΔSCV, which lacks the PDZ-binding motif. Transient transfections with 1μg of plasmid cDNA for each construct were performed in HEK 293 cells as labelled. Samples were run using SDS-PAGE and immunoblotted with rabbit anti-GFP. **(B)** Effect of 5HT treatment was quantified using densitometry and had no significant effect on the amount of GFP-SAP97 co-immunoprecipitated with FL-5HT$_{2A}$R. Data are representative of the mean ± SEM of six independent experiments. **(C)** Representative immunoblot of endogenous SAP97 co-immunoprecipitated with 5HT2AR antibody from adult mouse cortex. Data are representative of three independent experiments.

Figure 2: **GFP-SAP97 co-localizes at the membrane with FL-5HT$_{2A}$R in a PDZ-binding motif-dependent manner: (A)** Representative confocal microscopy image demonstrating the subcellular localization of GFP-SAP97 (green) when expressed alone HEK 293 cells. **(B)** Representative confocal image demonstrating the colocalization of FLAG-tagged 5HT$_{2A}$R (red) labelled with Zenon Alexa Fluor 633-conjugated mouse anti-FLAG antibody GFP-SAP97 (green). **(C)** Representative confocal image demonstrating the colocalization of FLAG-tagged 5HT$_{2A}$R-ΔSCV (red) labelled with Zenon Alexa Fluor 633-conjugated mouse anti-FLAG antibody GFP-SAP97 (green). HEK 293 cells were transfected with 3 μg of receptor and 1 μg of GFP-SAP97 plasmid cDNA, respectively. Data are representative of over 30 cells.

Figure 3: **YFP-SAP97 directly interacts with 5HT$_{2A}$R-rLuc and FL-5HT$_{2A}$R-rLuc-x22-SCV: (A)** Representative confocal microscopy image demonstrating surface expression of FL-5HT$_{2A}$R (green) and **(B)** FL-5HT$_{2A}$R-rLuc-x22-SCV (green) with mouse anti-flag followed by secondary

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mouse Alexa Fluor 488. **(C)** Dose response curves for 5-HT-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min in HEK 293 cells transfected with 1 μg of plasmid cDNA expressing FL-5-HT_{2A}R or FL-5-HT_{2A}R-rLuc-x22-SCV. The EC_{50} for FL-5-HT_{2A}R mediated IP formation was 8.6 nM and the EC_{50} for FL-5-HT_{2A}R-rLuc-x22-SCV mediated IP formation was 9.3 nM. The data are representative of the mean ± SEM of three independent experiments. **(D)** HEK293 cells were transfected with 20 ng of plasmid cDNA expressing FL-5-HT_{2A}R-rLuc-x22-SCV cDNA with increasing ratios of plasmid cDNA expressing either YFP-SAP97 or YFP-Htt. Total cDNA was normalized with pcDNA3.1. The data represent the mean ± SEM of six independent experiments.

Figure 4: **Role of SAP97 in the internalization of $5HT_{2A}$R:** HEK 293 cells were transfected with 1 μg of plasmid cDNA expressing either FL-$5HT_{2A}$R or FL-$5HT_{2A}$R-ΔSCV along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA. **(A)** Shown is the loss of cell surface receptor following the treatment of cells with 10 μM 5-HT for 30 min, as assessed by flow cytometry. **(B)** Shown is the relative cell surface expression of FL-5-HT_{2A}R and FL-$5HT_{2A}$R-ΔSCV in cells transfected with control shRNA, SAP97 shRNA or GFP-SAP97. The data represents the mean ± SEM of four independent experiments.

Figure 5: **SAP97 plays an integral role in $5HT_{2A}$R-mediated IP_3-signaling. (A)** Dose response curves for 5-HT_{2A}R-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min in cells transfected with 1 μg of plasmid cDNA expressing FL-$5HT_{2A}$R along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA. **(B)** Dose response curves for 5-HT_{2A}R-ΔSCV-mediated IP formation in response to treatment with

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increasing concentrations of 5-HT for 30 min in cells transfected with 1 μg of plasmid cDNA expressing FL-5HT_{2A}R-ΔSCV along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA. **(C)** Comparison of the dose response curves for 5-HT_{2A}R- and 5-HT_{2A}R-ΔSCV-mediated IP formation in response to treatment with increasing concentrations of 5-HT for 30 min. Cells were transfected with 1 μg of plasmid cDNA expressing either FL-5-HT_{2A}R or FL-5-HT_{2A}R-ΔSCV. The data represent the mean ± SEM of six independent experiments.

Figure 6: Endogenous SAP97 is required for 5HT_{2A}R-mediated ERK1/2 phosphorylation independent of PDZ-binding motif interactions: **(A)** Representative immunoblot showing ERK1/2 phosphorylation in response 10 μM 5-HT treatment for 0, 5, 10 min in HEK 293 cells transfected with 1 μg of plasmid cDNA encoding FL-5-HT_{2A}R along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid cDNA expressing SAP97 shRNA.. Shown in the panels below are corresponding immunoblots for total ERK1/2, FL-5-HT_{2A}R, GFP-SAP97, and endogenous SAP97 protein expression. Immunoblot of GFP-SAP97 overexpression is shown at a different exposure (as indicated by box) than the exposure for endogenous SAP97 treated with scrambled and SAP97 shRNA. **(B)** Densitometric analysis of ERK1/2 phosphorylation in response to 10 μM 5HT treatment for 0, 5, and 10 min in HEK 293 cells transfected with FLAG-5HT_{2A}R and either scrambled shRNA, GFP-SAP97, or SAP97 shRNA. The data represent the mean ± SEM of four independent experiments. **(C)** Representative immunoblot showing ERK1/2 phosphorylation in response 10 μM 5-HT treatment for 0, 5, 10 min in HEK 293 cells transfected with 1 μg of plasmid cDNA encoding FL-5-HT_{2A}R-ΔSCV along with either 3 μg of plasmid cDNA expressing scrambled shRNA (SCR), 1 μg of plasmid cDNA expressing GFP-SAP97 or 3 μg of plasmid

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cDNA expressing SAP97 shRNA.. Shown in the panels below are corresponding immunoblots for total ERK1/2, FL-5-HT_{2A}R-ΔSCV, GFP-SAP97, and endogenous SAP97 protein expression. Immunoblot of GFP-SAP97 overexpression is shown at a different exposure (as indicated by box) than the exposure for endogenous SAP97 treated with scrambled and SAP97 shRNA. **(D)** Densitometric analysis of ERK1/2 phosphorylation in response to 10 μM 5HT treatment for 0, 5, and 10 min in HEK 293 cells transfected with FLAG-5HT_{2A}R-ΔSCV and either scrambled shRNA, GFP-SAP97, or SAP97 shRNA. The data represent the mean ± SEM of four independent experiments.

Figure 7: SAP97 shRNA knockdown does not prevent CRFR1-mediated enhancement of 5HT_{2A}R-mediated signaling: 5-HT stimulated dose response curves for inositol phosphate (IP) formation in HEK 293 cells that are transfected with 1μg of plasmid cDNA encoding HA-CRFR1 and 1 μg of plasmid cDNA encoding FL-5HT_{2A}R along with 3 μg of plasmid cDNA encoding either scrambled shRNA (SCR) or SAP97 shRNA. Transfected HEK 293 cells were treated either with or without 500 nM CRF for 30 min prior to being treated with increasing concentrations of 5-HT for 30 min. The data represent the mean ± SEM of 5 independent experiments.

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Figure 1

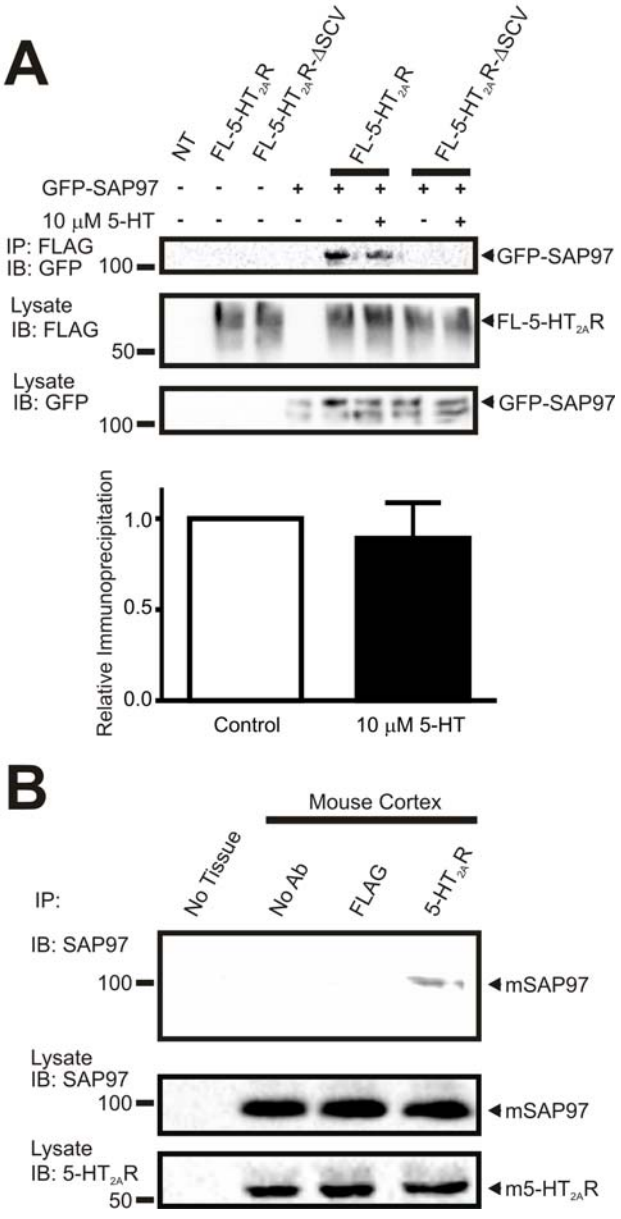

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Figure 2

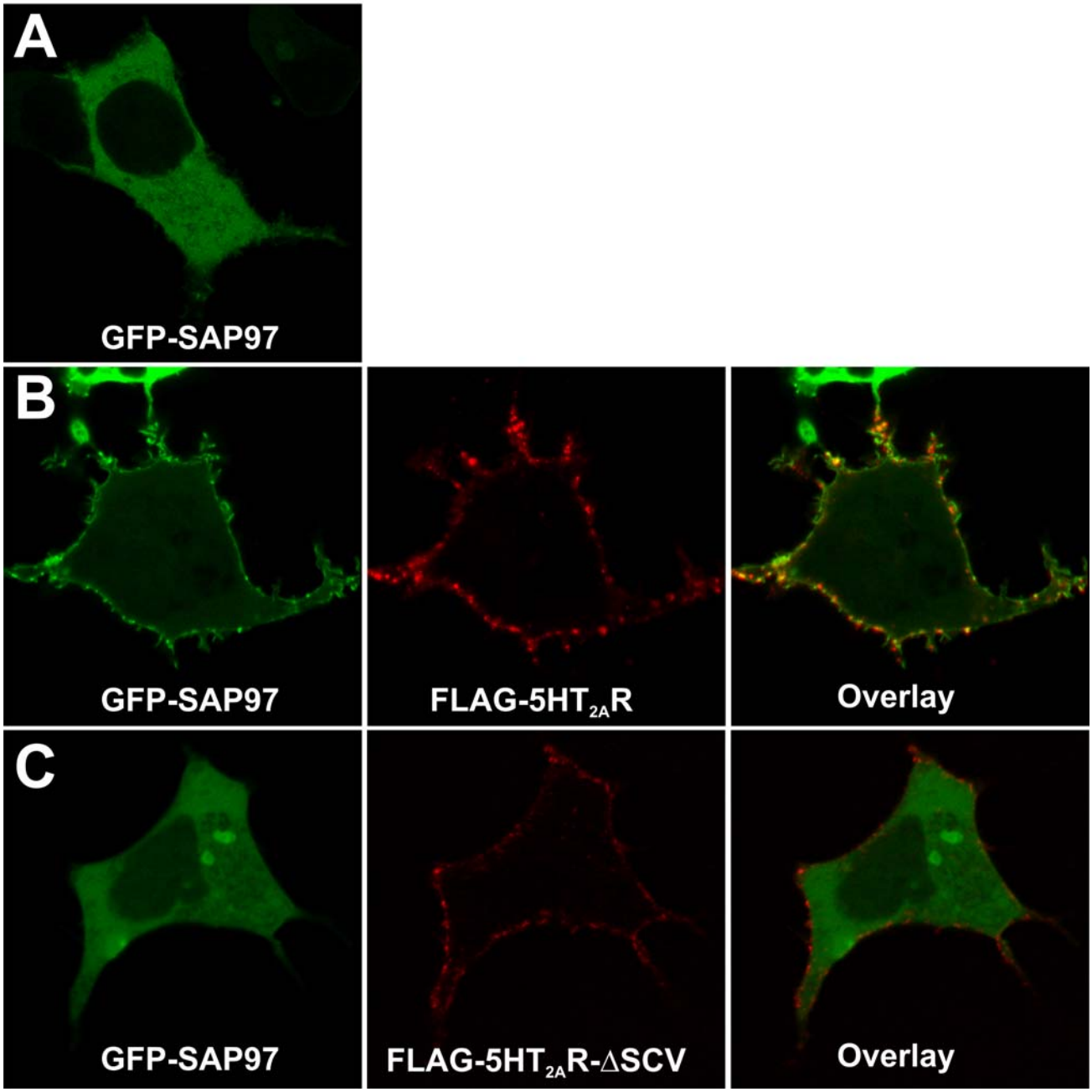

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Figure 3

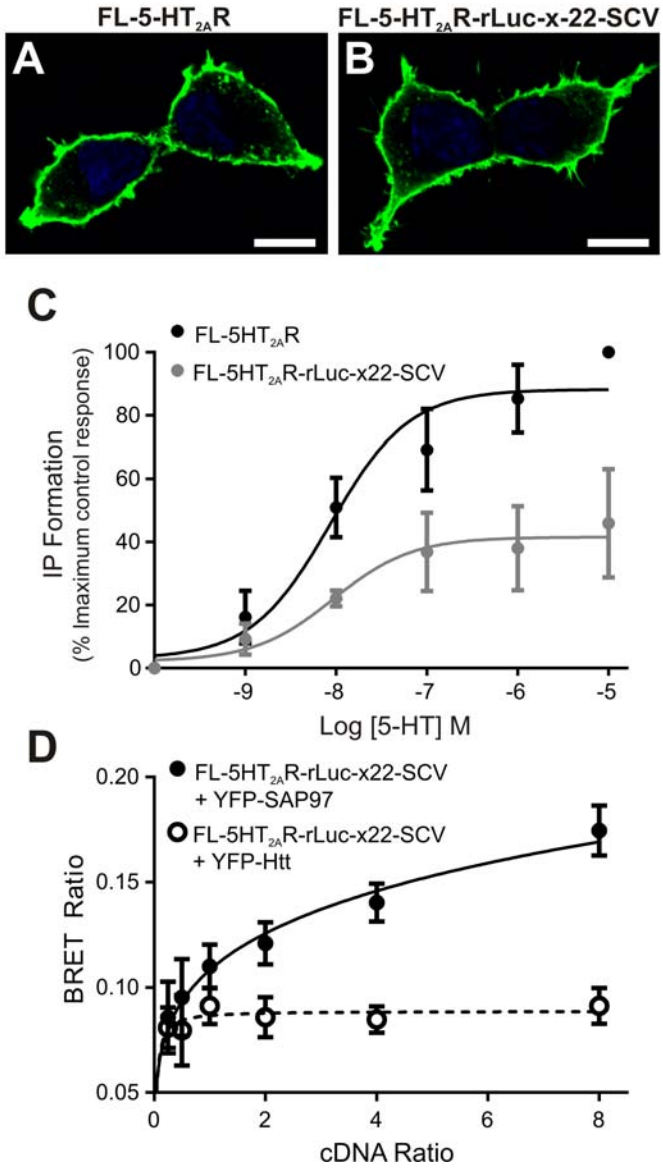

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Figure 4

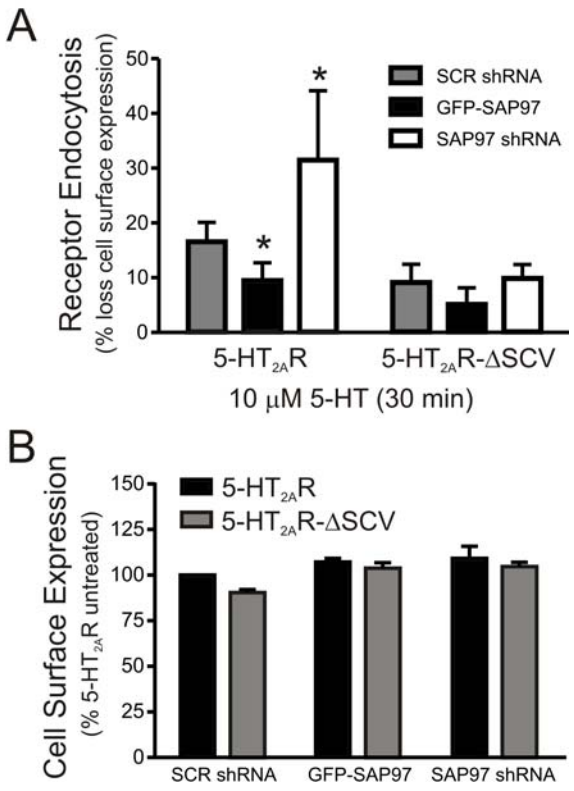

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Figure 5

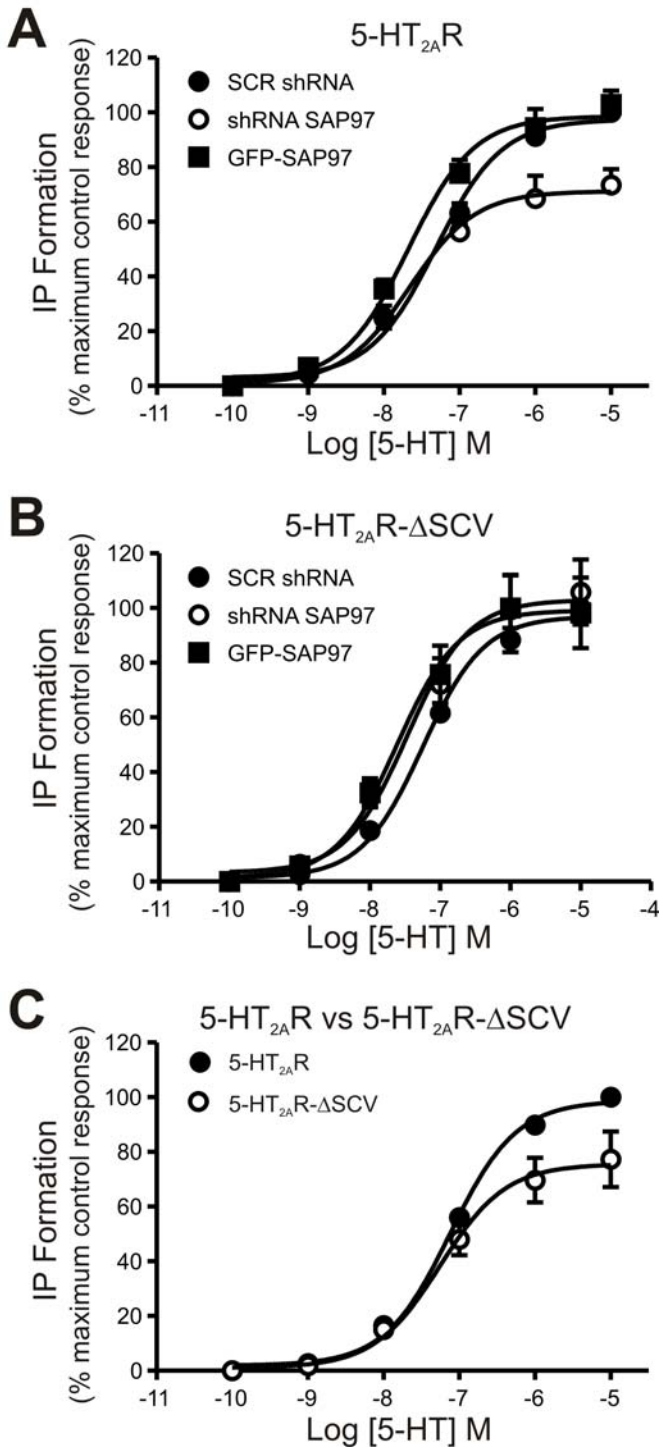

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Figure 6

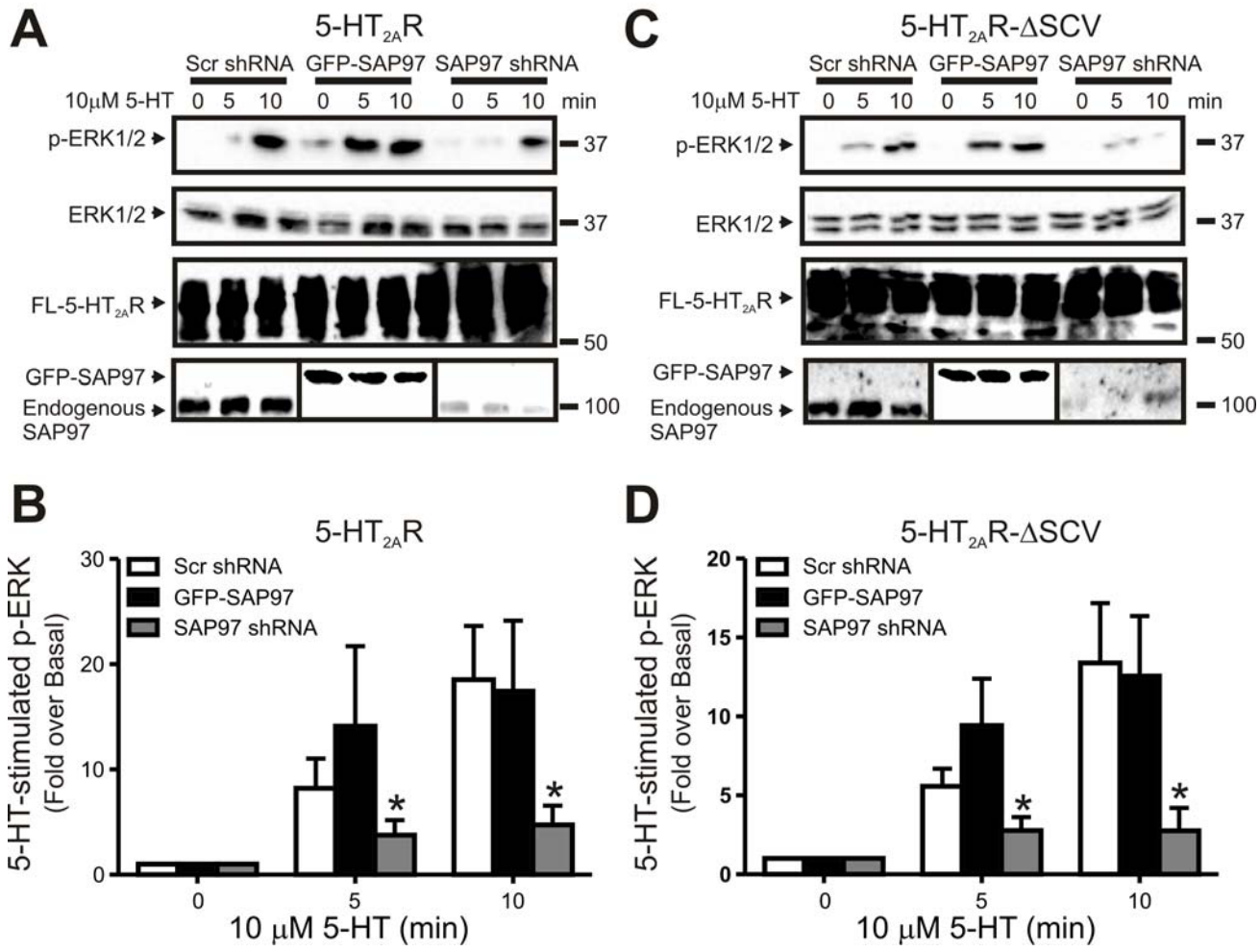

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Figure 7