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Impact of Paroxetine on Proximal β -adrenergic Receptor Signaling

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Abstract

β -adrenergic receptors (β AR) regulate numerous functions throughout the body, however G protein-coupled receptor kinase (GRK)-dependent desensitization of β AR has long been recognized as a maladaptive process in the progression of various disease states. Thus, the development of small molecule inhibitors of GRKs for the study of these processes and as potential therapeutics has been at the forefront of recent research efforts. Via structural and biochemical analyses, the selective serotonin reuptake inhibitor (SSRI) paroxetine was identified as a GRK2 inhibitor that enhances β AR-dependent cardiomyocyte and cardiac contractility and reverses cardiac dysfunction and myocardial β AR expression in mouse models of heart failure. Despite these functional outcomes, consistent with diminished β AR desensitization, the proximal β AR signaling mechanisms sensitive to paroxetine have not been reported. In this study, we aimed to determine whether paroxetine prevents classic β AR desensitization-related signaling mechanisms at a molecular level. Therefore, via immunoblotting, radioligand binding, fluorescence resonance energy transfer (FRET) and microscopy assays, we have performed an assessment of the effect of paroxetine on proximal β AR signaling responses. Indeed, paroxetine treatment inhibited ligand-induced β 2AR phosphorylation in a concentration-dependent manner. Additionally, for both β 1AR and β 2AR, paroxetine decreased ligand-induced β arrestin2 recruitment and subsequent receptor internalization. Thus, paroxetine inhibits β AR desensitization mechanisms consistent with GRK2 inhibition and provides a useful pharmacological tool for studying these proximal GPCR signaling responses.

Keywords

Paroxetine; β -adrenergic receptor; G protein-coupled receptor kinase; β -arrestin

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Disclosures

None.

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1. Introduction

β -adrenergic receptor (β AR) signaling regulates a large repertoire of functions throughout the body, including cardiac contractility, vascular relaxation, immune cell function and central effects^{1–3}. β AR desensitization has long been recognized as a contributing factor to the progression of various disease states and is largely mediated via regulation by G protein-coupled receptor kinase (GRK)-dependent processes^{4, 5}. Upon β AR stimulation, GRK-dependent phosphorylation of the C-terminus of the receptor increases the recruitment of β arrestins (β arr), multifunctional scaffolding proteins that sterically interdict the association between β AR and active G proteins, as well as engage receptor internalization, mechanisms that act to arrest G protein-dependent β AR signaling and desensitize the receptor to prolonged stimulation. Since these classical desensitization mechanisms have been shown to lead to dysfunctional β AR signaling over time, contributing to maladaptive remodeling during heart failure in particular, the development of small molecule inhibitors of GRKs for the study of these processes and as potential therapeutics has been at the forefront of recent research efforts⁶.

Via structural and biochemical analyses, the selective serotonin reuptake inhibitor (SSRI) paroxetine was recently identified as a GRK2 inhibitor, whereas its potency for inhibition of other GRK family members was shown to be much weaker⁷. Functionally, paroxetine was demonstrated to enhance β AR-dependent cardiomyocyte and cardiac contractility, as well as reverse cardiac dysfunction and myocardial β AR expression in mouse models of heart failure, while another SSRI that lacks GRK inhibitory capacity (fluoxetine) did not^{7, 8}. These results are consistent with reduced β AR desensitization in response to GRK2 inhibition and highlighted the applicability of paroxetine for studying the impact of GRK2 inhibition in functional cells and in vivo models. However, despite these functional outcomes the proximal β AR signaling mechanisms sensitive to paroxetine have not been reported. Therefore, in this study we aimed to determine whether paroxetine indeed prevents GRK-dependent β AR signaling processes, including β AR phosphorylation, β arr recruitment and receptor internalization.

2. Material and Methods

2.1 Materials

All reagents were attained from Fisher Scientific unless otherwise denoted.

2.2 Cell culture and infection with adenovirus

U2OS cells and Flag- β 1AR HEK 293 cells⁹ were grown at 37°C in MEM (Cellgro Corning) supplemented with 10% fetal bovine serum (Gemini Bio-products) and 1% penicillin/streptomycin/amphotericin B (Gemini Bio-products). U2OS cells were infected with adenoviruses encoding Flag- β 2AR-mCFP, Flag- β 1AR-mCFP or β arr2-mYFP at MOIs as indicated for 24 h, after which the cells were used for experiments outlined below.

2.3 Detection of β 2AR phosphorylation using western blotting

U2OS cells were seeded in 6-well plates and infected with Flag- β 2AR-mCFP adenovirus at a multiplicity of infection (MOI) of 25 for 24 h. The cells were stimulated with increasing concentrations of isoproterenol for 5 min. To evaluate paroxetine or fluoxetine dose-dependent β 2AR phosphorylation inhibition, cells were pretreated with paroxetine or fluoxetine for 30 min at increasing concentrations prior to isoproterenol stimulation for 5 min at 10 nM. Media were removed and cells were washed on ice three times with cold phosphate-buffered saline (Cellgro). Cells were lysed on ice with 120 μ l RIPA buffer (10 mM deoxycholate, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, 10 mM NaF, 1 HALT protease inhibitor cocktail (Thermo Scientific), and 1 phosphatase inhibitor cocktail (Millipore)), scraped, and nutated at 4°C for 30 min. Lysed cell debris were separated from protein lysate by centrifugation at $15000 \times g$ for 20 min. Lysates were run on SDS/10% PAGE gels and transferred to Immobilon-PSQ polyvinylidene fluoride 0.2 mm pore size membranes (Millipore), which were then incubated with phospho-Ser355/356 β 2AR antibody (1:200, Santa Cruz Biotechnology), mouse monoclonal M2 Flag antibody (1:10000, Sigma) and GAPDH antibody (1:1000, Cell Signaling Technology). Membranes were subsequently incubated with appropriate anti-rabbit or anti-mouse IRDye (680 or 800)-labelled antibodies and detected using the LiCOR Biosciences Odyssey system. Phosphorylation data are calculated as phospho- β 2AR/Flag- β 2AR expression/GAPDH loading control.

2.4 Radioligand binding analysis

To determine the effect of paroxetine or fluoxetine on membrane β 2AR density, U2OS cells were grown to confluency in 10 cm plates and underwent 45 min treatment of 30 μ M of paroxetine or fluoxetine. Media were removed and cells were washed on ice three times with cold PBS (Cellgro). Membrane preparations from U2OS cells were prepared by homogenization in ice-cold lysis buffer (25 mM Tris pH 7.4, 5 mM EDTA, 1 μ g/mL Aprotinin, 1 μ g/mL Leupeptin) and centrifuged at $1000 \times g$ for 5 min at 4°C. The supernatant was centrifuged at $30,000 \times g$ and the crude membrane pellet underwent resuspension in lysis buffer containing 10% glycerol and stored at -80°C until use. Membrane preparations (25 μ g of protein) were incubated with 200 pM ^{125}I -cyandopindolol (Perkin Elmer) in binding buffer (75 mM Tris pH 7.4, 2 mM EDTA, 12.5 mM MgCl_2 , 1 μ g/mL Aprotinin, 1 μ g/mL Leupeptin). Incubations were performed in the presence or absence of propranolol (10 μ M) to determine non-specific binding. The reactions were performed in a 250 μ L volume and allowed to equilibrate at 37°C for 1 h prior to filtering the membranes through a Whatman glass fiber filter (Whatman GF/C, Brandel Inc). Each filter was washed 5 times with 5 mL of ice-cold wash buffer (10 mM Tris pH 7.4, 10 mM EDTA) to remove unbound drug. The amount of total and non-specific radiolabel bound to cell membranes was determined in a gamma counter. Each assay was performed in duplicate from at least three independent experiments per condition and receptor density was normalized to mg of membrane protein.

2.5 Fluorescence resonance energy transfer (FRET) detection

U2OS cells were seeded in either 96 well plates or 35 mm glass-bottom dishes (MatTek Corporation) coated with 10 µg/ml fibronectin. For β arr2 recruitment assays the cells were infected with adenoviruses encoding β arr2-mYFP (MOI of 10) and either Flag- β 1AR-mCFP (MOI of 10) or Flag- β 2AR-mCFP (MOI of 20). The cells underwent pretreatment with vehicle, paroxetine or fluoxetine (30 µM each) for 45 min, following which they were rinsed and media replaced with imaging buffer (HBSS buffer (Cellgro), 0.2% bovine serum albumin, 20 mM HEPES) and stimulated with buffer or ISO (100 nM). FRET detection was performed using either a M1000 multimode plate reader (Tecan) for 96 well plates or a Leica DMI4000B inverted microscope with a Leica DFC365 FX 1.4-megapixel monochrome digital camera for 35 mm plates. Cyan fluorescent protein (CFPex/CFPem), yellow fluorescent protein (YFPex/em) and FRET (CFPex/YFPem) readings were measured every 60 seconds (plate reader) or 3.6 seconds (microscope). Quantification of FRET (corrected FRET (cFRET) = FRET – (CFP*CFP bleedthrough) – (YFP*YFP bleedthrough)) were expressed as a percentage of total CFP emission (%FRET = cFRET/[cFRET + CFP]). Microscope bleedthrough values were: 36% (CFP) and 13% (YFP); plate reader bleedthrough values were: 40% (CFP) and 2% (YFP).

2.6 Detection of β AR internalization using fluorescence microscopy

U2OS cells or HEK 293 cells stably expressing Flag- β 1AR were grown on 10 mm glass coverslips coated with 10 µg/ml fibronectin. U2OS cells were infected with Flag- β 2AR-mCFP adenovirus at a MOI of 5 for 24 h. The cells underwent pretreatment with vehicle, paroxetine or fluoxetine (30 µM each for 45 min), followed by stimulation with vehicle or isoproterenol (300 nM for 20 min). After treatment, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with ice-cold 0.2% Triton X-100 for 5 min, rinsed with phosphate-buffered saline and blocked with 1% bovine serum albumin for 1 h. Incubation with anti-Flag mouse monoclonal M2 antibody (1:10000, Sigma) for 20 min in 1% bovine serum albumin was followed by Alexa Fluor® 568-conjugated goat anti-mouse IgG (1:1000, ThermoFisher Scientific) for 1 h. U2OS cells were counter-stained with NucBlue Reagent (Molecular Probes) to visualize nuclei. Samples were mounted on slides and visualized using a Carl Zeiss Axio Observer Z1 fluorescence microscope with a Hamamatsu ORCA-flash 4.0 CMOS camera at a magnification of 63X. β AR internalization was imaged within 4 fields of view per coverslip (containing ~1–3 cells per field) and calculated as number of internalized Flag- β AR puncta within each cell/respective cell surface area (analyzed via ImageJ) and normalized to the maximal internalization induced by ISO.

2.7 Statistics

Data are presented as mean $\pm$ standard error of the mean (S.E.M.). Statistical analyses were performed using a one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison post-test using Prism 5.01 software (GraphPad Software). Each treatment condition was performed in a minimum of 3 independent cell preparations, as indicated.

3. Results

3.1 Paroxetine inhibits agonist-induced β 2AR phosphorylation

GRKs are well-established mediators of β 2AR phosphorylation following agonist binding^{10, 11}. It was previously reported that paroxetine selectively inhibits the kinase activity of GRK2 at concentrations between 1–100 μ M in biochemical studies and required high concentrations in whole cells to inhibit thyrotropin-releasing hormone receptor (TRHR) phosphorylation⁷. Therefore, we initially sought to determine whether paroxetine can inhibit β 2AR phosphorylation in a concentration-dependent manner. Flag- β 2AR-mCFP-expressing U2OS cells were stimulated with increasing concentrations of isoproterenol (ISO) for 5 min and β 2AR phosphorylation was assessed via immunoblot analysis, wherein ISO-induced β 2AR phosphorylation occurred in a concentration-dependent manner (Fig. 1A), with an EC_{50} of 11.0 ± 3.3 nM (Fig. 1B). Thus, using a sub-maximal response-inducing concentration of ISO (10 nM), we tested the impact of paroxetine pretreatment on β 2AR phosphorylation, in comparison to the SSRI fluoxetine, previously shown not to inhibit GRK2⁸, used as a negative control. Pretreatment with paroxetine, but not fluoxetine, concentration-dependently inhibited ISO-induced β 2AR phosphorylation (Fig. 1C) with an IC_{50} of 5.9 ± 2.8 μ M (Fig. 1D). We next sought to determine whether the ability of paroxetine to reduce agonist-induced β 2AR phosphorylation is in fact mediated by changes in receptor membrane density during the pretreatment period. Therefore, we assessed the impact of pretreatment of U2OS cells with 30 μ M of either paroxetine or fluoxetine on β 2AR membrane density via radioligand binding analysis with ¹²⁵I-cyandopindolol, which revealed no significant alterations in membrane β 2AR expression (Fig. 1E). Thus, paroxetine inhibits agonist-induced β 2AR phosphorylation, the most proximal GRK-dependent regulatory response to receptor stimulation, independent of changes in receptor expression at the plasma membrane.

3.2 Paroxetine inhibits agonist-induced β arr2 recruitment to β 2AR

β arr recruitment to ligand-activated GPCRs plays a well-documented role in receptor desensitization processes^{12, 13}. Since GRK-mediated phosphorylation enhances β arr recruitment to GPCRs after agonist binding and paroxetine pretreatment inhibited agonist-induced β 2AR phosphorylation, we next sought to determine the impact of paroxetine on the recruitment of β arr to β 2AR via FRET analysis. Initially, U2OS cells expressing mCFP-tagged β 2AR and mYFP-tagged β arr2 were treated with increasing concentrations of ISO and the FRET response monitored over time (Fig. 2A), where an increase in FRET indicates β arr2 recruitment⁹. ISO rapidly and concentration-dependently increased β arr2 recruitment to β 2AR with an EC_{50} of 230 ± 37 nM (Fig. 2B). Subsequently, we tested the impact of pretreatment with either 30 μ M paroxetine or fluoxetine on β arr2 recruitment to β 2AR following ISO stimulation using a sub-maximal concentration of 100 nM, where the ISO-mediated increase in β 2AR- β arr FRET was almost completely abrogated by pretreatment with paroxetine (Fig. 2C and D). Of note, fluoxetine induced an intermediate effect on β arr2 recruitment to β 2AR after ISO stimulation.

3.3 Paroxetine prevents agonist-induced β 2AR internalization

Following ligand stimulation, GRK-dependent β arr recruitment to β 2AR induces its internalization and sequestration away from the plasma membrane as a form of desensitization^{12, 13}. Since paroxetine reduced β arr2 recruitment to β 2AR following ISO stimulation, we next tested whether β 2AR internalization was similarly impacted. U2OS cells expressing Flag- β 2AR-mCFP were stimulated with ISO for 20 min in the presence or absence of 30 μ M paroxetine or fluoxetine and receptor internalization assessed via fluorescence microscopy. As expected, ISO stimulation induced a robust β 2AR internalization response as demonstrated by the presence of multiple puncta (Fig. 3A). As predicted by the reduction in β arr2 recruitment observed above, paroxetine pretreatment completely attenuated the β 2AR internalization response to ISO (Fig. 3B). Although fluoxetine was observed above to exert effects on β arr2 recruitment to β 2AR, albeit to a lesser extent than paroxetine, fluoxetine did not inhibit ISO-induced β 2AR internalization.

3.4 Paroxetine also inhibits proximal β 1AR-dependent signaling processes

Paroxetine has been used as a pharmacologic agent for the purpose of enhancing cardiomyocyte contractility and reversing cardiac remodeling after injury, processes in which β 1AR signaling plays a predominant role². Thus, it is important to determine whether, beyond effects on β 2AR, paroxetine also disrupts proximal β 1AR signaling classically associated with GKR2-mediated desensitization. Initially, we assessed the β arr2 recruitment response to increasing concentrations of ISO over time in U2OS cells expressing mCFP-tagged β 1AR and mYFP-tagged β arr2 (Fig. 4A). FRET between β 1AR and β arr2 was increased in a concentration-dependent manner, with an EC₅₀ of 104 $\pm$ 21 nM (Fig. 4B). Subsequently, cells were pretreated with 30 μ M paroxetine or fluoxetine prior to stimulation with a sub-maximal concentration of ISO (100 nM), wherein ISO-stimulated β arr2 recruitment to β 1AR was ablated by paroxetine pretreatment, while fluoxetine had no effect on this process (Fig. 4C and D). Finally, we tested the impact of paroxetine on ISO-induced β 1AR internalization using HEK 293 cells stably expressing flag-tagged β 1AR and treated with ISO with or without paroxetine or fluoxetine pretreatment. As observed with β 2AR internalization, ISO-induced β 1AR internalization was completely abrogated by paroxetine pretreatment, and was unaffected by fluoxetine (Figure 5A and B). Therefore, the effects of paroxetine on the proximal signaling responses of both β 1AR and β 2AR are consistent with inhibition of GRK2.

4. Discussion

GRK2 activity has increasingly been shown to contribute to the progression of numerous diseases and organ dysfunction^{5, 6}. For instance, a large body of work over the last two decades has identified GRK2 as a major regulator of cardiac dysfunction, wherein genetic ablation or inhibition of GRK2 is sufficient to protect the heart from ischemic insult, prevent adverse cardiac remodeling during chronic HF and prevent β AR desensitization to enhance cardiomyocyte contractility^{4, 14–17}. Thus the identification and validation of small molecules inhibitors of GRK2 remains an important priority and has led to the discovery of a number of several types of antagonists, including RNA aptamers, peptides and various small molecules with distinct chemical and structural properties⁶, though generally with poor

potency, selectivity or physicochemical properties unfit for therapeutics. However, the Food and Drug Administration-approved SSRI paroxetine was identified using a high throughput aptamer displacement assay to bind directly to GRK2 to inhibit its ability to phosphorylate rhodopsin, tubulin and thyrotropin-releasing hormone receptor, while increasing β AR-stimulated cardiomyocyte (in vitro) and cardiac (in vivo) contractility⁷. Subsequently, paroxetine has been shown to reverse cardiac dysfunction induced by either ischemia⁸ or high fat diet¹⁸, inhibit T cell activation¹⁹ and decrease fibroblast cytokine production²⁰. Despite the increasing use of paroxetine in studies in which its effects are attributed to inhibition of receptor desensitization processes classically engaged by GRK2, such as β arr recruitment and GPCR internalization, no studies have confirmed whether it actually attenuates these responses. The mechanistic impact of paroxetine on proximal β AR signaling is of particular interest in light of the aforementioned studies which detailed its beneficial effects on cardiac structure and function following injury^{8, 18}, suggestive of a GRK2 inhibition-mediated reversal of β AR desensitization. Thus, using a variety of molecular approaches we have determined that, consistent with GRK2 inhibition, paroxetine indeed attenuates proximal β AR signaling processes, including β arr recruitment, receptor phosphorylation and internalization.

An unexpected result observed in our study was the impact of the maximal concentration of fluoxetine on β 2AR phosphorylation and β arr2 recruitment to β 2AR following ISO stimulation. Although fluoxetine did not block ISO-mediated β 2AR phosphorylation and β arr2 recruitment as effectively as paroxetine, a moderate level of inhibition was achieved. Fluoxetine was previously shown not to impact β AR-attributable effects, including cardiac contractility and post-ischemic cardiac remodeling in vivo at concentrations equimolar to paroxetine⁸, thus providing an in-class SSRI negative control for testing against paroxetine's additional GRK2 inhibitory effects. Since β AR-mediated effects on cardiomyocyte contractility and maladaptive cardiac remodeling following injury are attributed to β 1AR signaling in particular², and β 1AR signaling was unaffected by fluoxetine in our study, it is possible that the effects of fluoxetine on GRK-dependent β 2AR signaling we observed are not functionally relevant in vivo, at least in the heart. Alternatively, it has been demonstrated that different cell types express unique cohorts of GRK isoforms such that, beyond GRK2, β 2AR has been shown to be predominantly sensitive to GRK6 in HEK 293 cells and to GRK3 in U2OS cells^{10, 11}. Thus, although fluoxetine did not inhibit GRK-mediated phosphorylation activity in vitro or downstream functional effects on cardiac contractility in vivo^{7, 8}, whether it has affinity for and can partially inhibit GRK3 in U2OS cells has not been established. As such, the applicability of using fluoxetine as a negative control for paroxetine-mediated effects may need to be validated on a cell-by-cell basis. Regardless, and despite its proximal effects on β 2AR phosphorylation and β arr2 recruitment in U2OS cells, ultimately fluoxetine did not inhibit ligand-stimulated β 2AR internalization, suggesting that even if high concentrations of fluoxetine can negatively impact proximal GRK-dependent β 2AR signaling responses, the threshold for inhibiting subsequent downstream processes including receptor internalization has not been achieved. In all, our results suggest that while the use of fluoxetine as a negative control for comparative studies with paroxetine should be validated in a receptor- and cell type-dependent manner, its lack of effect on proximal β 1AR signaling responses are consistent with its previous use in cardiac studies.

An important observation in our study is the lack of effect of paroxetine pretreatment alone on β 2AR membrane expression, which suggests that paroxetine, and pharmacologic inhibition of GRK2 in general, does not directly induce changes in receptor dynamics. Rather, the decreased ligand-induced β -arrestin recruitment and receptor internalization following stimulation would be attributable to the lack of GRK2-mediated receptor phosphorylation. This fits with the known requirement for GRK2 to be recruited to the receptor after ligand stimulation²¹, thus its inhibition prior to β 2AR stimulation would not be expected to alter membrane-localized desensitization signaling dynamics. Although there are no reports directly comparing the impact of paroxetine on receptor phosphorylation amongst distinct GPCRs, in terms of relative efficacy paroxetine inhibited ligand-induced β 2AR phosphorylation in our study with an IC_{50} of $\sim 6 \mu M$, compared to an IC_{50} of $\sim 30 \mu M$ for the TRHR previously reported⁷, possibly suggesting more reliance upon GRK2 for β 2AR versus TRHR phosphorylation. Of note, a recent study showed that β 2AR phosphorylation induced by hypoxic stress was also decreased by the GRK2 inhibitor 182200²², however the concentration of inhibitor 182200 required to partially block the response was $125 \mu M$, as compared to the $10\text{--}30 \mu M$ range of paroxetine sufficient to almost completely abrogate β 2AR phosphorylation in our study. More in line with our study, Lowe *et al.* demonstrated a similar impact of $30 \mu M$ Takeda Cmpd 101 on ligand-induced μ -opioid receptor phosphorylation, β arr2 recruitment and receptor internalization²³. Thus, paroxetine appears to have similar, or better, potency for GRK2 inhibition compared with other commercially-available small molecule antagonists.

5. Conclusions

In summary, our results confirm that paroxetine blocks proximal signaling effects at β ARs including receptor phosphorylation, β arr recruitment and internalization, providing a mechanistic link between previous structural/biochemical studies identifying paroxetine as a GRK2 inhibitor^{7, 24, 25} and subsequent functional/pathophysiologic studies showcasing its effectiveness at reversing cardiac dysfunction^{8, 18}. Thus, paroxetine inhibits β AR desensitization mechanisms consistent with GRK2 inhibition and provides a useful pharmacological tool for studying these facets of receptor regulation. This is highly relevant as efforts continue to develop next generation small molecule compounds with higher potency and efficacy toward GRK2 inhibition based on the structural properties of paroxetine²⁶.

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Abbreviations

β AR	β -adrenergic receptor
β arr	β -arrestin
EC_{50}	half-maximal effective concentration

FRET	fluorescence resonance energy transfer
GRK	G protein-coupled receptor
IC₅₀	half-maximal inhibitory concentration
ISO	isoproterenol
mCFP	monomeric cyan fluorescent protein
mYFP	monomeric yellow fluorescent protein
SSRI	selective serotonin reuptake inhibitor
TRHR	thyrotropin-releasing hormone receptor

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Highlights

- The SSRI paroxetine was previously identified as a GRK2 inhibitor
- We define the impact of paroxetine on proximal β AR signaling for the first time
- Paroxetine blocks agonist-induced β 2AR phosphorylation
- Paroxetine abolishes agonist-mediated β arr recruitment and β AR internalization
- The SSRI fluoxetine has partial effects on β 2AR, but no impact on β 1AR, signaling

Figure 1. Paroxetine inhibits agonist-induced β 2AR phosphorylation

A) Ad-Flag- β 2AR-mCFP-transfected U2OS cells were stimulated with increasing concentrations of ISO for 5 min, following which the cells were processed for western blotting analysis. ISO concentration-dependently increased phosphorylation of β 2AR (P- β 2AR). B) Concentration-response curve of the results from (A), wherein % P- β 2AR responses were normalized by both total FLAG and GAPDH intensities, reveals an EC_{50} of 11.0 ± 3.3 nM. C) Flag- β 2AR-transfected U2OS cells were pretreated with increasing concentrations of paroxetine (upper panels) or fluoxetine (lower panels) for 30 min followed by stimulation with 10 nM ISO for 5 min. Western blot analysis shows concentration-dependent inhibition of ISO-induced P- β 2AR by paroxetine but not fluoxetine. D) Inhibitory concentration-response curves of the results from (C), yielding an IC_{50} of 5.9 ± 2.8 μ M for paroxetine, while an IC_{50} for fluoxetine inhibition of ISO-induced P- β 2AR could not be attained from the data. E) Pretreatment of U2OS cells with paroxetine or fluoxetine (30 μ M each) did not alter β AR density as assessed by [125 I]-CYP binding analysis. ns = not significant, One-way ANOVA. The data are represented by the mean $\pm$ S.E of 3 (E) or 4 (B, D) independent experiments each.

Figure 2. Paroxetine inhibits agonist-induced β arr2 recruitment to β 2AR

U2OS cells were infected with Ad- β arr2-mYFP in conjunction with Ad-Flag- β 2AR-mCFP.

Live cell fluorescence resonance energy transfer (FRET) in the cells was measured in response to increasing concentrations of ISO over time (A), revealing an EC_{50} of 230 ± 37 nM (B). Subsequently, the cells were stimulated with 100 nM ISO in the presence or absence of 30 μ M of paroxetine or fluoxetine (C). Pretreatment with paroxetine significantly reduced β Arrestin2 recruitment to β 2AR (D), while fluoxetine pretreatment reduced β Arrestin2 recruitment to β 2AR to a lesser extent. The data are represented by the mean $\pm$ S.E of 4 independent experiments. *** $P < 0.001$, One-way ANOVA.

Figure 3. Paroxetine prevents agonist-induced β 2AR internalization

A) U2OS cells were infected with Ad-FLAG- β 2AR-mCFP and pretreated with vehicle, paroxetine or fluoxetine (30 μ M each) prior to stimulation with ISO (300 nM) for 20 min to induce β 2AR internalization as visualized by the formation of intracellular puncta. Scale bar = 20 μ m. Images shown are representative of 3 independent experiments at 63X and inset boxes show magnified images at a ratio of 1:1.67. B) Quantified β 2AR internalization results. Data represent the mean $\pm$ S.E. from 3 independent experiments. ns = not significant, *** $P < 0.001$, One-way ANOVA.

Figure 4. Paroxetine inhibits agonist-induced β arr2 recruitment to β 1AR

A) U2OS cells were infected with Ad- β arr2-mYFP in conjunction with Ad-Flag- β 1AR-mCFP. Live cell FRET in the cells was measured in response to increasing concentrations of ISO over time (A), revealing an EC₅₀ of 104 ± 21 nM (B). Subsequently, live cell FRET in the cells was measured via whole-field measurements at 20X magnification in response to ISO (100 nM, given at 30 sec) over time in the presence or absence of 30 μ M paroxetine or fluoxetine (C). Pretreatment with paroxetine significantly reduced β arr2 recruitment to β 1AR, while fluoxetine pretreatment had no significant impact (D). The data are represented by the mean ± S.E of 4 independent experiments. *** P < 0.001, One-way ANOVA.

Figure 5. Paroxetine prevents agonist-induced β 1AR internalization

A) HEK 293 cells stably expressing Flag- β 1AR were pretreated with vehicle, paroxetine or fluoxetine (30 μ M each) prior to stimulation with ISO (300 nM) for 20 min to induce β 1AR internalization as visualized by the formation of intracellular puncta. Scale bar = 20 μ m. Images shown are representative of 3 independent experiments at 63X and magnified at a ratio of 1:1.67. B) Quantified β 1AR internalization results. Data represent the mean $\pm$ S.E. from 3 independent experiments. ns = not significant, *** $P < 0.001$, One-way ANOVA.