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Dynamic modulation of FGFR1–5-HT1A heteroreceptor complexes. Agonist treatment enhances participation of FGFR1 and 5-HT1A homodimers and recruitment of β-arrestin2

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ABSTRACT

New findings show that neurotrophic and antidepressant effects of 5-HT in brain can, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal and raphe FGFR1–5-HT1A heteroreceptor complexes enhancing the FGFR1 signaling. The dynamic agonist modulation of the FGFR1–5-HT1A heteroreceptor complexes and their recruitment of β-arrestin is now determined in cellular models with focus on its impact on 5-HT1AR and FGFR1 homodimerization in the heteroreceptor complexes based on BRET^2 assays. The findings show that coagonist treatment with 8-OH-DPAT and FGF2 but not treatment with the 5-HT1A agonist alone markedly increases the BRETmax values and significantly reduces the BRET50 values of 5HT1A homodimerization. The effects of FGF2 or FGF20 with or without the 5-HT1A agonist were also studied on the FGFR1 homodimerization of the heteroreceptor complexes. FGF2 produced a marked and rapid increase in FGFR1 homodimerization which partially declined over a 10 min period. Cotreatment with FGF2 and 5-HT1A agonist blocked this decline in FGFR1 homodimerization. Furthermore, FGF2 alone produced a small increase in the BRET^2 signal from the 5-HT1A-β-arrestin2 receptor–protein complex which was additive to the marked effect of 8-OH-DPAT alone. Taken together, the participation of 5-HT1A and FGFR1 homodimers and recruitment of β-arrestin2 was demonstrated in the FGFR1–5-HT1A heteroreceptor complexes upon agonist treatments.

1. Introduction

In 1999 the concept was introduced that the growth-promoting activity of many G protein coupled receptors (GPCRs) involves activation of receptor tyrosine kinases (RTKs) and their downstream signaling cascades [1]. Many observations led to the emergence of the so called 'transactivation' concept, which refers to the activation of RTKs by GPCR ligands and links GPCR signaling to the mitogen activated protein kinase signaling cascade [1–4]. A bidirectional cross-communication between RTKs and GPCRs appears to exist involving e.g. heterotrimeric G proteins and β-arrestins [5–7]. Recently evidence was obtained that integration of neurotrophic factor and transmitter signaling can take place in RTK-GPCR heteroreceptor complexes at the level of the plasma membrane [3,8–10].

New findings show that neurotrophic and antidepressant effects of 5-HT in brain can, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal FGFR1–5-HT1A heteroreceptor complexes enhancing the FGFR1 signaling [9]. The dynamic modulation of these heteroreceptor complexes has been continued in the current study using the BRET^2 assay in HEK293T cells. The participation of 5-HT1A and FGFR1 homodimers and recruitment of β-arrestin2 is demonstrated upon agonist treatment.

2. Materials and methods

Detailed descriptions are available in Supplementary material on: chemicals and reagents; receptor constructs; cell culture,

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transfection and immunofluorescence microscopy; membrane preparation and ligand binding assay.

2.1. Quantitative BRET^2 saturation assay

Forty-eight hours after transfection HEK293T cells with constant (0,5 μg) or increasing amounts of cDNA of Receptor-Rluc8 (5-HT1A or FGFR1) and Receptor-GFP^2 (5-HT1A or FGFR1) respectively were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 μg of protein) were distributed in duplicates into 96-well microplates (either black clear-bottomed or white opaque, Corning 3651 or 3600) for fluorescence and luminescence determinations. The total fluorescence of cell suspensions was quantified and then divided by the background (mock-transfected cells) in a POLARstar Optima plate-reader (BMG Lab-technologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm, and 10 nm bandwidth emission filter corresponding to 510 nm. And total bioluminescence was determined on samples incubated for 10 min with 5 μM h-coelenterazine (Molecular Probes, Eugene, OR, USA). The background values for total luminescence were negligible and subtracted from sample values. For BRET^2 measurement, coelenterazine-400a also known as *DeepBlue*™ C substrate (VWR, Sweden) was added at a final concentration of 5 μM, and readings were performed 10 s after each injection using the POLARstar Optima plate-reader (BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings [410 nm (with 80 nm bandwidth) and 515 nm (with 30 nm bandwidth)]. The BRET^2 ratio is defined as previously described [11,12]. For determining the effects of each receptor agonist and the combined treatment, transfected HEK293T cells were incubated for 10 min at 25 °C in the absence or presence of the indicated agonist concentration prior to performing BRET^2 analysis.

2.2. BRET^2 competition assay

Forty-eight hours after transfection, HEK293T cells transiently transfected with constant amounts (0.5 μg) of plasmids encoding for FGFR1^{Rluc8} and 5-HT1A^{GFP2} and increasing amounts (0.1–8 μg) of plasmids encoding for wild-type FGFR1 or 5-HT1A and the mock pcDNA3.1+; respectively. The energy transfer was determined as described for the BRET^2 saturation assay. For further details in titration of donor and acceptor fusion proteins curves see Supplementary materials.

2.3. Monitoring FGFR1 activation using BRET^{2}assay

For concentration–response and kinetic BRET^2 experiments, HEK293T cells were transiently transfected at a constant ratio (1:2:1) of FGFR1^{Rluc8}/FGFR1^{GFP2}/5HT1A in presence of heparin (0.5 μM). Cells were treated with the indicated FGF ligand concentration, in presence or absence of 5-HT1A agonist, or vehicle for 2 min before BRET^2 measurement. FGF ligand-promoted BRET^2 was calculated by subtracting the BRET^2 ratio obtained in the absence of the FGF ligand from that obtained in the presence of the FGF ligand. In kinetic measurements, coelenterazine-400a was injected simultaneously with FGF ligand (30 ng/ml) and 8-OH-DPAT, and reading were then collected 2 s after each injection. In each experiment, the specificities of FGFR1/FGFR1 interactions were assessed by comparison with cells expressing FGFR1^{GFP2} alone. Also as a negative control was used cells individually expressing FGFR1^{Rluc8} mixed prior to exposition to coelenterazine-400a with cells individually expressing FGFR1^{GFP2}.

2.4. Monitoring receptor-interacting proteins (β-arrestin2) using BRET^{2}assay

For dose–response and kinetic BRET^2 experiments, HEK293T cells were transiently transfected at a constant ratio (1:1:2) of FGFR1/5HT1A^{Rluc8}/β-arrestin2^{GFP2} and/or (1:2) for FGFR1^{Rluc8}-β-arrestin2^{GFP2}. Agonist-promoted BRET^2 was calculated by subtracting the BRET^2 ratio obtained in the absence of agonist addition from the one obtained in the presence of an agonist. In the case of kinetic measurements, coelenterazine-400a was injected simultaneously with the agonist, and reading were then collected 2 s after each injection. In each experiment, the specificities of Receptor–β-arrestin2 interactions were assessed by comparison with cells expressing Receptor^{GFP2} alone.

2.5. Statistical analysis

The number of samples (n) in each experimental condition is indicated in Figure legends. All data were analyzed using the commercial program GraphPad PRISM 4.0 (GraphPad Software, USA). When two experimental conditions were compared, statistical analysis was performed using an unpaired t test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. BRET isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided BRETmax and BRET50 values. The correlation between fluorescence or luminescence and receptor density was analyzed by a linear regression curve fitting with the same software.

3. Results

The existence of FGFR1–5-HT1A heteroreceptor complexes and their agonist regulation by 8-OH-DPAT and/or FGF2 [9] was demonstrated previously. However, the dynamic modulation of these heteroreceptor complexes was not previously described.

3.1. Effects of combined treatment with the 5-HT1A agonist 8-OH-DPAT and FGF2 on 5-HT1A receptor homodimerization in HEK cells containing FGFR1–5-HT1A heteroreceptor complexes

HEK293T-27 cells were transiently co-transfected with a constant amount of FGFR1 (1 μg cDNA), 5HT1A-Rluc8 (1 μg cDNA), and increasing concentrations of 5-HT1A-GFP2 (0.25–5 μg cDNA) and then treated with the 5-HT1A agonist 8-OH-DPAT and FGF2 or with 8-OH-DPAT alone. A highly significant increase in the quantitative BRET^2 signal was found for the 5-HT1A homodimer when analyzed in the presence of FGF2 (50 ng/ml) and 8-OH-DPAT (100 nM), which gives BRETmax value of 33.78 ± 0.97 (means ± S.E.M., mBU) (Fig. 1A). 8-OH-DPAT (100 nM) alone failed to change the BRETmax value. The combined treatment with FGF2 and 8-OH-DPAT also produced a significant reduction of the BRET50 value indicating an increase in the affinity for the interaction between the two protomers (Fig. 1B).

3.2. Effects of combined and single treatment with 8-OH-DPAT and FGF2 on FGFR1 homodimerization in HEK cells containing FGFR1–5-HT1A heteroreceptor complexes

We have previously demonstrated with BRET^2 analysis that FGF2 increases the formation of FGFR1 homodimers [13]. In the current study the modulatory effect of 5-HT1A agonist 8-OH-DPAT was studied on the FGF2 induced FGFR1/FGFR1 homodimer formation by means of BRET^2 analysis. HEK293T cells were transiently

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