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Evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT system

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ABSTRACT

The ascending midbrain 5-HT neurons known to contain 5-HT1A autoreceptors may be dysregulated in depression due to a reduced trophic support. With *in situ* proximity ligation assay (PLA) and supported by co-location of the FGFR1 and 5-HT1A immunoreactivities in midbrain raphe 5-HT cells, evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes were obtained in the dorsal and median raphe nuclei of the Sprague–Dawley rat. Their existence in the rat medullary raphe RN33B cell cultures was also established. After combined FGF-2 and 8-OH-DPAT treatment, a marked and significant increase in PLA positive clusters was found in the RN33B cells. Similar results were reached upon coactivation by agonists in HEK293T cells using the Fluorescent Resonance Energy Transfer (FRET) technique resulting in increased FRETmax and reduced FRET50 values. The heteroreceptor complex formation was dependent on TMV of the 5-HT1A receptor since it was blocked by incubation with TMV but not with TMII. Taken together, the 5-HT1A autoreceptors by being recruited into a FGFR1–5-HT1A heteroreceptor complex in the midbrain raphe 5-HT nerve cells may develop a novel function, namely a trophic role in many midbrain 5-HT neuron systems originating from the dorsal and medianus raphe nuclei.

1. Introduction

There is evidence for the existence of direct (heteroreceptor complexes) and/or indirect G protein coupled receptor (GPCR)–receptor tyrosine kinase (RTK) interactions [1–5]. Even in the absence of neurotrophic factor binding to the RTK they can lead to transactivation of RTKs and produce effects on cell proliferation, differentiation and neuronal plasticity [6].

We recently presented evidence for the existence of FGFR1–5-HT1A heteroreceptor complexes in the rat hippocampus with a partial characterization of their interface [7]. The findings demonstrated their enhancing role in hippocampal plasticity which was proposed to play a role in reversing the depression-induced atrophy of hippocampal neurons [7].

Evidence is presented in the current work for the existence of FGFR1–5-HT1A heteroreceptor complexes in midbrain raphe 5-HT nerve cells. This demonstration makes it likely that antidepressant drugs by increasing extracellular 5-HT levels in the midbrain raphe nuclei, not only increases 5-HT1A autoreceptor signaling but also produce an enhanced activation of the FGFR1 receptor protomer in this heteroreceptor complex leading to increased trophism of the 5-HT nerve cells. In line with this hypothesis, extended treatment with the selective 5-HT re-uptake inhibitor zimelidine caused increases in 5-HT immunofluorescence in the dorsal raphe cells [8].

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2. Materials and methods

2.1. Plasmid constructs

The cDNA encoding the human 5-HT1A and FGFR1 without its stop codon was subcloned in $pGFP^2$-N1 (Perkin-Elmer, Madrid, Spain) and pEYFP-N1 (Clontech, Heidelberg, Germany) using standard molecular biology and PCR techniques [9,10].

2.2. Cell culture and transfection

HEK293T or RN33B cells (a CNS-derived neuronal precursor cell line [11]) (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) foetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2. For transfection, cells were plated in 6-well dishes at a concentration of 1×10^6 cells/well or in 75 cm^2 flasks and cultured overnight before transfection. Cells were transiently transfected (cDNA molar ratio 1:1) using linear PolyEthylenImine reagent (PEI) (Polysciences Inc., Warrenton, PA, USA).

2.3. Double immunolabeling histochemistry

Adult age-matched male Sprague–Dawley rats ($n = 3$) were anesthetized and perfused intracardially with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, post-fixed by immersion overnight in 4% paraformaldehyde in PBS and coronal sections (20 μm) were cut on a vibratome and processed for free-floating immunohistochemistry. Sections were permeabilized with buffer A containing 0.2% Triton X-100 for 5 min, and then preincubated in a blocking buffer containing 0.3% (wt/vol) triton and 4% (wt/vol) bovine serum albumin. After 1 h at room temperature, sections were labeled with the indicated primary antibodies for 1 h, extensively washed, and stained with the indicated fluorescence labeled secondary antibodies. Samples were rinsed and visualized employing a Leica SP2 confocal microscope. The primary antibodies used were as follows: rabbit polyclonal antiserum against 5-HT1A receptor (ab44635) (5 μg/ml; Abcam, Stockholm, Sweden) and mouse monoclonal [M2F12] (ab829) antibodies against FGFR1 (5 μg/ml; Abcam, Stockholm, Sweden). The secondary antibodies used were as follows: Alexa Fluor 488-conjugated goat anti-mouse IgG (1:2000; Invitrogen, Stockholm, Sweden), Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:2000; Invitrogen, Stockholm, Sweden).

2.4. In situ proximity ligation assay

In situ proximity ligation assay (in situ PLA) was performed as described previously [12]. Free-floating formalin fixed brain sections ($n = 3$, male Sprague–Dawley rats) and RN33B cell cultures were employed using the following primary antibodies: rabbit monoclonal anti-5HT1A (VTG Biosciences) and mouse monoclonal anti-FGFR1 (Abcam). Control experiments employed only one primary antibody or cells transfected with cDNAs encoding only one type of receptor. The PLA signal was visualized and quantified by using a confocal microscope Leica TCS-SL confocal microscope (Leica, USA) and the Duolink Image Tool software.

2.5. Transmembrane peptide treatment

A series of peptides, representing each of the predicted TM segments for the human 5-HT1A (UniProt identifier number: P08908) were synthesized by VTG Biosciences (Stockholm, Sweden) by using 9-fluorenylmethoxycarbonyl chemistry and purified by means of high-performance liquid chromatography (HPLC) analysis (reverse phase C4 column) to ⩾98% purity. TM-I peptide consisted of residues 37–62 (VITSLLLGTLIFCAVLGNACVVAAIA); TM-II peptide of residues 74–98 (LIGSLAVTDLMVSVLVLPMAALYQV); TM-III peptide of residues 110–132 (DLFIALDVLCCTSSILHLCAIAL); TM-IV peptide of residues 153–178 (AAALISLTWLIGFLISIPP MLGWRTP); TM-V peptide of residues 192–217 (DHGYTIYST FGAFYIPLLLMLVLYGR); TM-VI peptide of residues 346–367 (TLGIIMGTFILCWLPFFIVALV); and TM-VII peptide of residues 379–403 (TLLGAIINWLGYSNSLLNPVIYAYF). At the C-terminal juxtamembrane sequence of each TM peptide was introduced the tribasic sequence (RKR) as found in many membrane proteins in order to reduce possible artifact formation through disulfide bridges and to ensure incorporation into the plasma membrane of cells as demonstrated previously [7]. Immediately before use, the peptides were solubilized in dimethyl sulfoxide (DMSO) and diluted in the corresponding cell culture medium to yield a final concentration of 1% DMSO. We verified that, for each tested concentration of DMSO alone, no effect on cell viability was observed. Cells were incubated with the above mentioned peptides at 37 °C for 2 h prior to performing FRET analysis or *in situ* PLA assays. Incorporation of the peptide into cellular membranes under these conditions was checked with a rhodamine-labeled TM-I peptide.

2.6. FRET experiments

Forty-eight hours after transfection HEK293T cells with constant (1 μg) or increasing amounts of cDNA of $FGFR1^{GFP2}$ and $5\text{-}HT1A^{YFP}$ or $5\text{-}HT2A^{YFP}$ respectively were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (40 μg of total protein) were distributed in duplicates into 96-well microplates and GFP^2 and YFP fluorescence were measured with a POLARstar Optima plate reader (BMG Labtech, Offenburg, Germany), using excitation filters at 410 nm (10-nm bandwidth) and 485 nm (12-nm bandwidth), as well as emission filters corresponding to 510 nm (10-nm bandwidth), and 530 nm (10-nm bandwidth), respectively. Mock-transfected cells were used for background subtraction. FRET signals were collected using 410/10 nm excitation and 530/10 nm emission filters. Removal of acceptor bleed-through, the correction of acceptor fluorescence intensity changes and the calculations of the normalized FRET values (n-FRET) were carried out as previously described [13].

2.7. Data 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. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. FRET isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided FRETmax and FRET50 values.

3. Results

3.1. Existence of FGFR1–5-HT1A heteroreceptor complexes in the dorsal and median raphe nuclei, in raphe RN33B cells and in HEK293T cells

We used three different approaches, *in situ* PLA, FRET technology, supplemented with double immunolabeling procedures.