- SUPPLEMENTARY MATERIALS, METHODS AND FIGURES -

Enhancement of the FGFR1 signaling in the FGFR1-5-HT1A heteroreceptor complex in midbrain raphe 5-HT neuron systems. Relevance for neuroplasticity and depression

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1.1 Chemicals, reagents and Drug Administration.

(S)-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride ((S)-WAY 100135 dihydrochloride), 2-[(1,2-Dihydro-2-oxo-3*H*-indol 3-yl idene)methyl]-4-methyl-1*H*-pyrrole-3-propanoic acid (SU 5402), *N*-[2-[[4 (Diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-*d*]pyrimidin-7-yl]-*N*' (1,1dimethylethyl)urea (PD173074) and (±)-7-Hydroxy 2-dipropylaminotetralin hydrobromide (8-OH-DPAT hydrobromide) were purchased from Tocris Cookson Inc. FGF-2 was purchased from Cell Signalling Technology (Stockholm, Sweden). Dulbecco's modified Eagle's medium, penicillin/streptomycin, and fetal bovine serum was purchased from Invitrogen. Restriction enzymes were from New England Biolabs (Stockholm, Sweden), and coelenterazine 400 was obtained from MRW (Stockholm, Sweden). The injected amounts (i.c.v.) were 8-OH-DPAT (60□g) and FGF-2, (50ng) unless otherwise indicated. Phospho-FGF Receptor 1 (Tyr653/654) (55H2) mouse monoclonal antibody was purchased from Cell Signalling Technology (Stockholm, Sweden). Rabbit polyclonal antiserum against 5-HT1A receptor (ab44635), mouse monoclonal [M2F12] FGFR1 (ab829) antibodies were obtained from Abcam (Stockholm, Sweden). Rabbit monoclonal

anti-5-HT1A (vtg544) antibody was obtained from VTG Biosciences (Stockholm, Sweden). Monoclonal mouse anti-phospho-ERK1/2 and rabbit monoclonal anti-5-HT antibodies were from Sigma-Aldrich (Stockholm, Sweden). Mouse anti-NeuN monoclonal antibody was purchased from Millipore (Stockholm, Sweden). Secondary infrared probe-labelled rabbit-anti-mouse antibodies were from LI-COR Biosciences (Stockholm, Sweden). Alexa Fluor 488-conjugated goat anti-mouse IgG and the Alexa Fluor 546-conjugated goat anti-rabbit IgG were from Invitrogene (Stockholm, Sweden).

1.2 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.

1.3 Cell culture, transfection, RNA interference.

HEK293T or RN33B cells (a CNS-derived neuronal precursor cell line) (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 $75cm^2$ 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). siRNAs against rat FGFR1 and 5-HT1A were from Dharmacon/Thermo Scientific (Stockholm, Sweden). Non-silencing siRNA Alexa Fluor 488 was from Qiagen (Cat No 1022563) and used as a negative control. Cells were plated the day before transfection in 12-well culture dishes coated with poly-D-lysine (BD Biocoat, USA) and then transfected with the corresponding SMARTpool siRNA kit (Dharmacon).

1.4 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. 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 artefact formation through disulfide bridges and to ensure incorporation into the plasma membrane of cells as has been demonstrated previously [12,16]. Immediately before use, the peptides were solubilised 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 two hours prior to performing FRET analysis or signalling assays. Incorporation of the peptide into cellular membranes under these conditions was checked with a rhodamine-labeled TM-I peptide.

1.5 5-HT Immunocytochemistry. RN33B cells were treated under different pharmacological conditions as indicated and grown for an additional 48-56 hour (in the case of TM peptide incubation, neurons where incubated 2 hours before pharmacological treatment). RN33B cells were stained with rabbit anti-5-HT monoclonal antibody (5 µg/ml; Sigma-Aldrich) and mouse anti-NeuN monoclonal antibody (5 µg/ml; Millipore). 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). Each image was acquired with a Leica SP2 confocal microscope (40×) and consisted of one image as a single z-scan image. Acquisition of microscopy images and 5-HT immunoreactivity semi-quantifications were performed by investigators

blind to the experimental condition. 5-HT immunoreactivity semi-quantification is expressed as intensity of 5-HT immunoreactivity per field area or per NeuN positive cell). Typically, images of 200-300 cells per condition were captured for each experiment and four independent experiments were performed. The application settings were adjusted at the beginning of analysis and kept the same for all images in the experiment.

1.6 *Animals and drugs.* Male Sprague–Dawley rats, 8 weeks old, weighing 310–350 g obtained from Charles River Laboratories were used for all experiments. The animals were housed under a 12-h light/dark cycle under constant environmental conditions, with ambient temperature of 21 ± 2 °C, relative humidity of 40-50% and with food and water available *ad libitum*. The number of rats in each treated group was three and the total number of rats used in this study was 36. All efforts were made to minimize the number of animals used and their suffering. All experimental procedures were performed in accordance with the European Communities Council Directive (86/609/EEC) and (2010/63/EU).

1.7 Surgical preparation. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p., Nembutal injection, Dainippon Pharmaceutical) and placed in a stereotaxic frame. Body temperature was maintained at 37°C and cannula for i.c.v. injection of samples was implanted into the right lateral ventricle according to coordinates obtained from Paxinos and Watson (anterior posterior (AP) co-ordinates referred to bregma, lateral (L) co-ordinates to the midsagittal suture line, and ventral coordinates (V) to the surface of the skull. AP, -1.4 mm; L, ±2.2 mm and V, -4.3 mm (injection site). Permanent guide cannula was maintained in place by dental cement anchored by stainless screws fixed to the skull and fitted with an obturator. Five days after surgery, the obturators were removed and a 28-gauge injector cannula was inserted extending 1.5 mm below the tip of the guides. The microinjection cannula was connected via PE-20 tubing to a Hamilton syringe mounted on

a syringe pump (Harvard Apparatus). Rats were microinjected with recombinant human FGF-2 (50ng), 8-OH-DPAT (0.2 micromoles), combined treatment with TM-II synthetic peptide (0.4micromoles), combined treatment with TM-V synthetic peptide (0.4micromoles), and treatment with artificial cerebrospinal fluid (aCSF) for a control group. Fresh solutions were prepared immediately before the injections by dissolving the peptides/agonists aCSF (composition of the aCSF solution was: 120 mM NaCl, 20 mM NaH_2CO_3, 2 mM KCl, 0.5 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.8 mM $MgCl_2$, 0.5 mM Na_2SO_4 and 6.8 mM d-glucose). Total volumes were infused (FGF-2: 2µl; 8-OH-DPAT: 3µl; TM synthetic peptides: 3µl) in freely moving animals at 2.0 µl/min and injection cannula were left in place for another minute to prevent backflow of the solution. The animals were killed 6h, 12h or 24 h after the infusions.

1.8 Western blot analysis in animal tissue preparation. Raphe midline area of the midbrain tissue was homogenized in cold RIPA buffer (50mM TrisHCl pH7.4, 150mM NaCl, 1%triton, 0.1%SDS and H_2O), protease inhibitor cocktail (Sigma-Aldrich, cat. num P8340), and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, cat. num P5726), left on ice for 30 min. and then centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was collected and stored at −20°C and an aliquot taken for protein determination by the BCA method. The supernatant samples (50 µg per lane) and molecular weight biotinylated markers (RPN2107, GE Healthcare Europe GmbH, Milan Italy), were run on 10% polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane (RPN303E, Hybond-C-extra, GE Healthcare Europe GmbH, Milan Italy). The membranes were incubated for 1h in blocking buffer : 1x TBS, 0.1%Tween-20, 5% w/v nonfat dry milk. Following three washings for 5 min. with TBS/T, the membranes were incubated with gentle shaking overnight at -4°C with a specific antibody in primary antibody dilution buffer: 1x TBS, 0.1%Tween-20, 5% BSA. For detection of pERK1/2 and pFGFR the following antibodies

were used: rabbit anti-Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (9101 Cell Signaling) diluted 1:1000 and mouse anti-p-FGFR (Tyr653/654) antibody (Cell Signaling 55H2) diluted 1:1000. Following three washing for 5 min with TBS/T the membranes were incubated for 1 h at room temperature with anti-rabbit IgG horseradish peroxidase-conjugated diluted 1:8000 (Sc 2054, Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and goat anti-mouse IgG horseradish peroxidase-conjugated diluted 1:7000 (sc-2005 Santa Cruz Biotechnology). Immuno-complexes were visualized with chemiluminescence reagent (RPN2108, GE Healthcare Europe GmbH, Milan Italy) according to the manufacturer's instructions. The Hyperfilm (ECL-films 28906837, GE Healthcare Europe GmbH, Milan Italy) were developed using Kodak developer and fixer (catalog No. 1900984 and 1902485, Kodak GBX, Eastman Kodak)., and the densitometric evaluation of bands was performed by measuring the optical density (O.D.) using the Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-20011) or Image Gauge V4.0 from Fujifilm.

FIGURES

Supplementary Figure 1. FGFR1-5-HT1A heteroreceptor complexes role in 5-HT synthesis and storage in RN33B cells. **(A)** Effects of TM peptide incubation on 5-HT immunoreactivity in RN33B cell cultures after coactivation with 8-OH-DPAT and FGF-2 which produces a marked increase of 5-HT immunoreactivity. Red colour was used as a marker for 5-HT storage and NeuN immunoreactivity (green colour) as a marker for neuronal nuclei. RN33B brain-derived immortalized cells were treated for 48 hours without (Control) or with FGF-2 (50ng/ml) and 8-OH-DPAT (100nM). Treatment with TM peptides (0.4μM) involved TM-II and TM-V as indicated. 5-HT immunoreactivity was visualized by immunofluorescent labelling with 5-HT. 5-HT immunoreactivity was

determined in sample fields of a constant size divided by the number of NeuN nuclei. Representative images of 5-HT/NeuN-stained RN33B cells under different conditions are presented. Scale bar represents 20 μm. The quantification is seen in **(B)** where the data are presented as mean ± s.e.m, n=4. The combined group is significantly different from 8-OH-DPAT and FGF-2 alone groups ($^{+++}P<0.001$); the mean 5-HT immunoreactivity value of the group treated with TM peptide V is highly significantly reduced versus the combined treated group ($^{***}P<0.001$). Also the FGF-2 treated alone group is significantly different from the 8-OH-DPAT alone group ($^{\#\#}P<0.01$). The following concentrations were used: 8-OH-DPAT, 100nM; FGF-2, 50ng/ml and each TM peptide, 0.4μM. 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.

Supplementary Figure 1