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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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ABSTRACT

New findings show existence of FGFR1-5-HT1A heteroreceptor complexes in 5-HT nerve cells of the dorsal and median raphe nuclei of the rat midbrain and hippocampus. Synergistic receptor–receptor interactions in these receptor complexes indicated their enhancing role in hippocampal plasticity. The existence of FGFR1-5-HT1A heteroreceptor complexes also in midbrain raphe 5-HT nerve cells open up the possibility that antidepressant drugs by increasing extracellular 5-HT levels can cause an activation of the FGF-2/FGFR1 mechanism in these nerve cells as well. Therefore, the agonist modulation of the FGFR1-5-HT1A heteroreceptor complexes and their specific role is now determined in rat medullary raphe RN33B cells and in the caudal midline raphe area of the midbrain rich in 5-HT nerve cells. The combined i.c.v. treatment with FGF-2 and the 5-HT1A agonist 8-OHDPAT synergistically increased FGFR1 and ERK1/2 phosphorylation in the raphe midline area of the midbrain and in the RN33B cells. Cotreatment with FGF2 and the 5-HT1A agonist induced RN33B cell differentiation as seen from development of an increased number and length of extensions per cell and their increased 5-HT immunoreactivity. These signaling and differentiation events were dependent on the receptor interface since they were blocked by incubation with TMV but not by TMII of the 5-HT1A receptor. Taken together, the 5-HT1A autoreceptors by being part of a FGFR1-5-HT1A heteroreceptor complex in the midbrain raphe 5-HT nerve cells appears to have also a trophic role in the central 5-HT neuron systems besides playing a key role in reducing the firing of these neurons.

1. Introduction

There is evidence for the existence of FGFR1-5-HT1A heteroreceptor complexes in the rat hippocampus with a partial characterization of their interface [1–4] and in midbrain raphe 5-HT nerve cells [5]. TMV and TMVII of the 5-HT1A were shown to be part of the interface of this heteroreceptor complex based on BRET^2 and FRET competition assays in cellular models. A significant impact of the FGFR1-5-HT1A heteroreceptor complex on hippocampal plasticity was obtained in the studies on primary hippocampal cultures. The 5-HT1A agonist produced a rapid and concentration dependent

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transactivation of FGFR1 as seen from its increased phosphorylation. Furthermore, a rapid, specific and marked synergistic increase in ERK1/2 phosphorylation was observed upon cotreatment with FGF-2 and 8-OH-DPAT. It was found to be dependent on the receptor interface in incubation experiments with TMV and TMVII [1]. A major result was the evidence obtained that these transactivation and signaling events in the FGFR1-5-HT1A heteroreceptor complexes were linked to rapid and marked synergistic increases of neurite density (24 h), protrusions and growth cone (1 h) development in the hippocampal cultures. The findings demonstrated their enhancing role in hippocampal plasticity which was considered to play a role in reversing the depression-induced atrophy of hippocampal neurons [1].

The findings on the hippocampal FGFR1-5-HT1A heteroreceptor complexes [1] opened up the possibility that antidepressant drugs, including serotonin selective reuptake inhibitors (SSRI) [6] and 5-HT1A agonists, can cause an activation of the FGFR1 protomer signaling also in the FGFR1-5-HT1A heteroreceptor complex of midbrain raphe 5-HT nerve cells [5]. In line with this hypothesis, extended treatment with the SSRI drug zimelidine caused increases in 5-HT immunofluorescence in the dorsal raphe [7].

Evidence is presented in the current work for a major role of FGFR1-5-HT1A heteroreceptor complexes in trophic mechanisms of mesencephalic raphe 5-HT nerve cells. Upon coactivation of the receptor protomers they produce a substantial enhancement of plasticity in stable cell lines of medullary raphe 5-HT nerve cells with synergistic increases in the FGFR1 and ERK1/2 phosphorylation levels. In support of these results in vivo findings demonstrated that combined intraventricular injections of FGF-2 and 8-OH-DPAT in the rat produced also synergistic and prolonged increases in the FGFR1 and ERK1/2 phosphorylation levels in the raphe midline area of the midbrain. Taken together, a new role of the 5-HT autoreceptors is proposed in midbrain raphe 5-HT nerve cell bodies, namely one of neurotrophism through transactivation of FGFR1 when recruited into the discovered FGFR1-5-HT1A heteroreceptor complexes as observed both in *in vivo* and *in vitro* experiments.

2. Materials and methods

For chemicals, reagents, drug administration, plasmid constructs, cell culture, transfection, RNA interference, transmembrane peptide treatment, 5-HT immunocytochemistry, animals, surgical preparation and western blot analysis in animal tissue preparations see Supplementary Materials and Methods.

2.1. Small interface interfering peptides

In preliminary BRET/FRET [5,8] experiments we observed that the most important TM interface interaction between the FGFR1-5-HT1A heteroreceptor complexes corresponds to the TM-V and potentially also TM-VII. BRET/FRET competition experiments with synthetic peptides of TMII and TMV of the 5-HT1A receptor were performed where TMV incubation resulted in a significant loss of the 5HT1A capacity to interact with FGFR1, but incubations with TM-I, -II, -III, and -VI failed to block the BRET/FRET signal. Herein, TM-II is therefore employed as a negative small interface interfering peptide and TM-V as a positive blocker of the formation of the FGFR1-5-HT1A complexes.

2.2. In cell western phosphorylation assay

Non differentiated and in some cases differentiated RN33B cells were treated with the indicated concentration and incubation times of each 5-HT1A and FGFR1 agonist or antagonist and then fixed in a final concentration of 4% paraformaldehyde. After fixing, the cells were permeabilised by washing 5 times (0.1% triton-X100 in PBS), blocked for 90 min at room temperature in LI-COR Odyssey Blocking Buffer® and then incubated overnight at 4 °C with primary monoclonal mouse anti-phospho-ERK1/2 antibody (Sigma–Aldrich, Stockholm, Sweden) (diluted 1/10,000) or primary monoclonal mouse anti-phospho-FGFR1 antibody (Cell Signaling, Stockholm, Sweden) (diluted 1/1000). Then, after extensive wash, cells were incubated in the dark with secondary infrared probe-labeled rabbit-anti-mouse antibodies (diluted 1/1500) and normalization stains (DRAQ5, Sapphire700) for 1 h at room temperature, washed and scanned by the Odyssey infrared scanner (LI-COR Biosciences).

2.3. RN33B cell differentiation

Sterile poly-D-lysine–coated cover slips were used to grow RN33B cells at a density of 2×10^5 cells per 10 cm^2. Agonists were added to non-differentiated cells during 48 h after which the medium was changed and the same ligands were added to fresh nonsupplemented medium and incubation was continued for 48 h at 37 °C. Cells were then fixed with 4% cold paraformaldehyde (wt/vol), the cover slips were mounted on slides and the cells were analyzed. Brightfield images were obtained using an Olympus IX70 with a Q-Fire CCD camera. Average number of extensions per 1000 cells was determined as well as the mean extension lengths from measurements of 100 cells which were quantified per condition in three independent experiments; length was converted into micrometer and analyzed for statistical significance.

2.4. Studies on RN33B cell differentiated prior to agonist treatment

At a confluence of 80%, cells were transferred to complete serum free B16 medium (0.5% BSA, Sigma Aldrich, Stockholm, Sweden) and differentiated by shifting the temperature from 33 °C (two days incubation) to 39 °C (two days) and 5% CO_2 in room air.

2.5. Data analysis

The number of samples (n) in each experimental condition is indicated in Figure legends. All data were analyzed using the commercial program Prism 4.0 (GraphPad, San Diego, CA). 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.

3. Results

3.1. FGFR1 transactivation and ERK1/2 phosphorylation in RN33B cells

The 5-HT1A agonist modulation of the FGFR1 agonist-response in RN33B cells is displayed in Figs. 1 and 2. Both the 5-HT1A and FGFR1 agonists produced a rise of FGFR1 phosphorylation with combined treatment resulting in a synergistic action (Fig. 1A and B). The 8-OH-DPAT induced enhancement of FGFR1 transactivation was counteracted by both 5-HT1A (S-WAY-10013) and FGFR1 (PD173074) antagonists.

The consequences of the 5-HT1A induced increase in FGFR1 phosphorylation on its signaling were studied by analysis of single and combined FGF-2 and 8-OH-DPAT treatment on ERK1/2 phosphorylation (Fig. 2A). The synergistic increase of pERK1/2 by combined FGF-2 and 5-HT1A agonist treatment was blocked by the

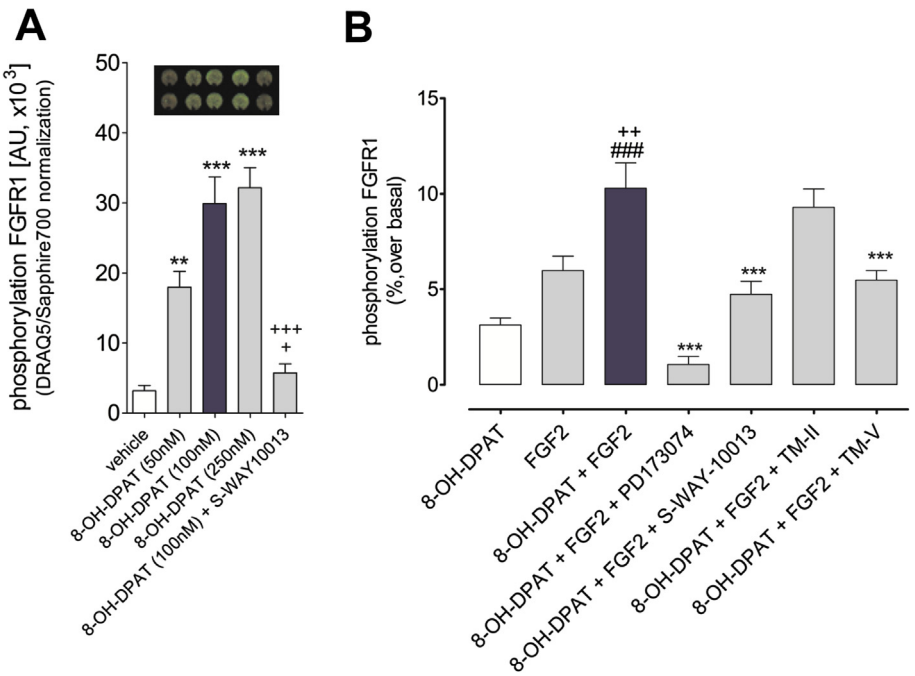

Fig. 1. FGFR1-5-HT1A receptor–receptor interactions involving FGFR1 transactivation in RN33B cells. Synergistic activation of the FGFR1 is induced by coactivation of 5-HT1A and FGFR1 in raphe RN33B cells. **(A)** An *in cell western* assay was used to measure 8-OH-DPAT concentration response effects of FGFR1 phosphorylation in RN33B cells co-expressing 5-HT1A and FGFR1 receptors. Similar experiments were carried out without stimulation (vehicle) and in presence of the selective 5-HT1A antagonist S-WAY10013 (1 μM). The data represent the mean ± S.E.M.; $n = 3$ in quadruplicates. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. 8-OH-DPAT (50 nM), (100 nM) and (250 nM) groups are significantly different compared to vehicle (**: $P < 0.01$, ***: $P < 0.001$, ***: $P < 0.001$ respectively); combined 8-OH-DPAT + S-WAY10013 group is significantly different compared to 8-OH-DPAT (50 nM), (100 nM) and (250 nM) groups (+: $P < 0.05$, +++: $P < 0.001$ respectively) **(B)** The synergistic increase of FGFR1 phosphorylation by cotreatment is blocked by the 5-HT1A TM peptide V. The following concentrations were used: 8-OH-DPAT (100 nM), FGF-2 (5 ng/ml), FGFR1 inhibitor PD173074 (60 nM), S-WAY10013 (1 μM) and each TM peptide (0.4 μM)). Combined treatment group is significantly different from 8-0H-DPAT and FGF-2 alone (###: $P < 0.001$ and ++: $P < 0.01$, respectively); the combined treatment groups with TM peptide V, 5-HT1A antagonist and FGFR1 inhibitor, respectively are significantly different from the combined treatment group alone (***: $P < 0.001$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test.

FGFR1 inhibitor and by the 5-HT1A antagonist and in siRNAfgfr1 or siRNA5ht1a transfected cells. Non-silencing siRNA control did not block this increase in the pERK1/2 (Fig. 2A).

The time course experiments of ERK1/2 phosphorylation are shown in Fig. 2B. Combined treatment with the 5-HT1A and FGFR1 agonists produced a synergistic increase of ERK1/2 phosphorylation starting already after 5 min and was still maintained after 120 min.

It is of substantial interest that the receptor interface of the FGFR1-5-HT1A receptor complex appeared to be crucial for this synergism since it was counteracted by incubation with TMV of the 5-HT1A receptor but not with TM-II (Figs. 1B and 2A). Thus, the 5-HT1A agonist induced synergistic activation of both FGFR1 transactivation and ERK1/2 phosphorylation appears to be dependent on the receptor interface of the FGFR1-5-HT1A heteroreceptor complex.

3.2. Differentiation of RN33B cells

To obtain a morphological correlate to the synergistic increase of pERK1/2 seen with FGF-2 and the 5-HT1A agonist cotreatment, effects of this combined treatment were studied on RN33B cell differentiation (number and length of neurites, and degree of 5-HT immunoreactivity). In Fig. 2A and B it is demonstrated that this combined treatment produced a highly significant increase in the number of extensions per 1000 RN33B cells after a 24 h-48 h incubation. The neuritic extensions were much longer and the cell somata more elongated (Fig. 3A and C). This increase was blocked not only by FGFR1 inhibitors and by the 5-HT1A antagonist (data not shown) but also by TMV, while TMII was ineffective.

Studies on potential effects of 5HT1A-FGFR1 heteroreceptor complexes on the serotonergic properties of RN33B cells showed that in absence of agonist treatment very few RN33B cells were 5-HT positive (Supplementary Fig. 1A). Semiquantitative image analysis showed that FGF-2 incubation caused an increase in the number of 5-HT-immunoreactive RN33B cells with high fluorescence intensity (Supplementary Fig. 1B). Robust increases in 5-HT immunoreactivity were only seen with combined treatment. RN33B cells cultured in presence of 8-OH-DPAT alone did not increase 5-HT immunoreactivity above control levels. In addition, no significant differences in the number of high-5-HT IR-expressing cells were detected from that seen with FGF-2 alone when cells were exposed to combined FGF-2 and 8-OH-DPAT treatment in the presence of synthetic peptide TM-V.

3.3. FGFR1 transactivation and ERK1/2 phosphorylation in the caudal midline area of the midbrain after in vivo treatment with FGF-2 and/or 8-OH-DPAT given i.c.v

In vivo results were obtained by exploring the effects of i.c.v. treatment with 8-OH-DPAT (200 nmoles) and/or FGF-2 (50 ng) on pFGFR1 and pERK1/2 levels in the rat caudal midline area of the midbrain containing the dorsal and median raphe nuclei (Fig. 4A and B). Upon combined i.c.v. treatment with 8-OH-DPAT and FGF-2 a synergistic and significant increase of phosphorylation of FGFR1 and ERK1/2 was observed at 6 h, 12 h and 24 h in the caudal midline area of the midbrain. As seen in Fig. 4A and B prior i.c.v. treatment

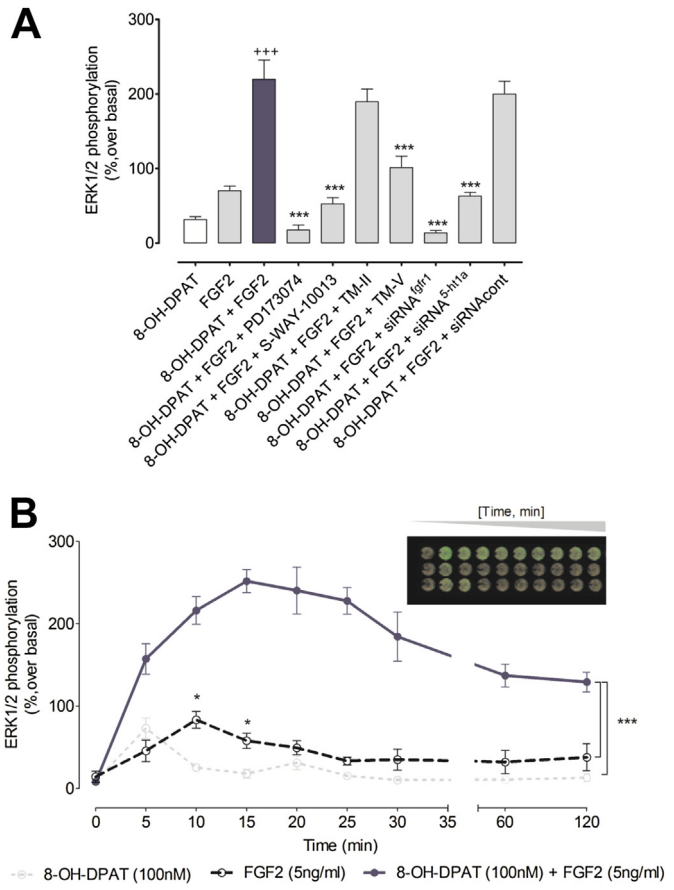

Fig. 2. FGFR1-5-HT1A receptor–receptor interactions involving ERK1/2 phosphorylation in RN33B cells. The synergistic activation of ERK1/2 phosphorylation is induced by coactivation of 5-HT1A and FGFR1 in raphe RN33B cells using the *in cell western* assay. **(A)** The synergistic increase of the ERK1/2 phosphorylation is blocked by the 5-HT1A TM peptide V, the siRNA^{fgfr1} and siRNA^{5ht1a}, but not by TM II and the non-silencing siRNA Alexa Fluor 488 (siRNAcont). The following concentrations were used, shown in parenthesis; 8-OH-DPAT (100 nM), FGF-2 (5 ng/ml), PD173074 (60 nM), S-WAY10013 (1 μM) and each TM peptide (0.4 μM)). Combined treatment group is significantly different from the 8-0H-DPAT and FGF-2 alone groups (+++: $P < 0.001$); the combined treatment groups with TM peptide V, 5-HT1A antagonist and FGFR1 inhibitor are significantly different from the combined treatment group alone (***: $P < 0.001$); the siRNA^{fgfr1} and siRNA^{5ht1a} groups showed a marked blocked of the synergistic effect of the combined treatment group (***: $P < 0.001$). Data are mean ± S.E.M., n = 5. **(B)** Time-course of ERK1/2 phosphorylation was significantly different in the range of 5–120 min between the combined treatment group and FGF-2 alone and 8-OH-DPAT alone (based on the area values under the curve, ***: $P < 0.001$) by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. With this analysis a significant difference between FGF-2 alone and 8-OH-DPAT alone was observed at 10–15 min (*: $P < 0.05$). Data are means ± S.E.M., n = 6.

(1 h) with TM V but not with TM II markedly counteracted the synergistic increase of the FGFR1 and ERK1/2 phosphorylation found after combined treatment with the FGF-2 and 8-OH-DPAT as seen in the 6–24 h time interval studied.

4. Discussion

Previous work showed the existence of FGFR1-5-HT1A heteroreceptor complexes in the hippocampus and the midbrain raphe of the rat brain and in cellular models by means of the PLA technique and FRET/BRET [2,5,8]. Also, using site-direct mutagenesis and small synthetic interface interfering peptides, we have provided a partial characterization of the FGFR1-5-HT1A heteroreceptor complexes interface [5,8]. In the current paper by means of the use of these selective synthetic small interfering peptides it was possible to demonstrate in the stable cell lines of the medullary raphe neurons and in the raphe midline area of the midbrain a relevant role of the FGFR1-5HT1A heteroreceptor complexes in plasticity.

FGF-2 and 5-HT1A agonist cotreatment produced a marked enhancement of the number of processes formed by each medullary raphe cell and of the amounts of 5-HT immunoreactivity per cell. These plastic changes were specific since they were blocked by FGFR1 inhibitors and 5-HT1A antagonists and by siRNAs for FGFR1 and for 5-HT1A. The dependency on the heteroreceptor complex was indicated by the ability of the TMV but not the TMII peptide to block these plasticity events. In agreement, this cotreatment using the same cell model led to a marked synergistic increase in the FGFR1 and ERK1/2 phosphorylation levels vs single treatment with the agonists. Like the structural plasticity events these signaling events were specific since they were blocked by FGFR1 inhibitors and 5-HT1A antagonists and by siRNAs for FGFR1 and for 5-HT1A. The dependency on the heteroreceptor complex was again indicated by the ability of the TMV but not the TMII peptide of the 5-HT1A receptor to block these plasticity events. This increase in ERK signaling, likely involved in mediating the increase in structural plasticity observed, may be produced by the observed 5-HT1A agonist induced transactivation of the FGFR1 as seen from its increased phosphorylation levels. The molecular mechanism may involve an enhancing allosteric receptor–receptor interaction in the heteroreceptor complex [9].

The studies using i.c.v. injections of FGF-2 and/or the 5-HT1A agonist 8-OH-DPAT in rats gave in vivo evidence that the cotreatment with these agonists can synergistically increase ERK1/2 and FGFR1 phosphorylation levels in the caudal midline region containing the dorsal and median raphe nuclei rich in 5-HT nerve cell bodies [10]. Thus, both *in vitro* and in vivo findings support the view that the signaling of the FGFR1-5-HT1A heteroreceptor complexes in the dorsal and median raphe 5-HT nerve cells [5] may have a role in enhancing neuroplasticity in these neurons. It is of substantial interest that i.c.v. TM V of the 5-HT1A receptor, which belongs to the receptor interface, can markedly counteract the synergistic increase in FGFR1 and ERK1/2 phosphorylation after combined i.c.v. treatment with FGF-2 and 8-OH-DPAT. These results in fact give evidence that the FGFR1-5-HT1A heteroreceptor complexes are involved in mediating these actions in the brain. This is strongly supported by the observation that i.c.v. TM II, which does not belong to the receptor interface, in the same dose failed to counteract these effects.

It should be noticed that BDNF, known to have antidepressant-like effects [11], in primary raphe cultures produces increases in neuritic lengths of 5-HT nerve cells via TrkB receptors which is higher in the absence of 5-HT1A receptors [12]. These results indicate that there exist in embryonic development indirect or direct TrkB-5-HT1A receptor interactions in 5-HT nerve cells which are antagonistic in nature. Should TrkB-5-HT1A heteroreceptor complexes exist in the raphe 5-HT nerve cells, the receptor–receptor interactions within them would therefore likely be antagonistic in contrast to the synergistic receptor–receptor interactions found in the current study in the discovered FGFR1-5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT nerve cells [5]. In addition, the enhancement of the neuritic lengths of 5-HT nerve cells observed in the 5-HT1A null mutant mice may be possible also through a compensatory mechanism involving the formation of FGFR1-5-HT1B heteroreceptor complexes [5].

It may be speculated that certain types of depression may develop as a result of a disruption of the FGFR1-5-HT1A heteroreceptor complex in midbrain 5-HT raphe cells and their hippocampal target neurons [1]. This reduces the neuroplasticity and

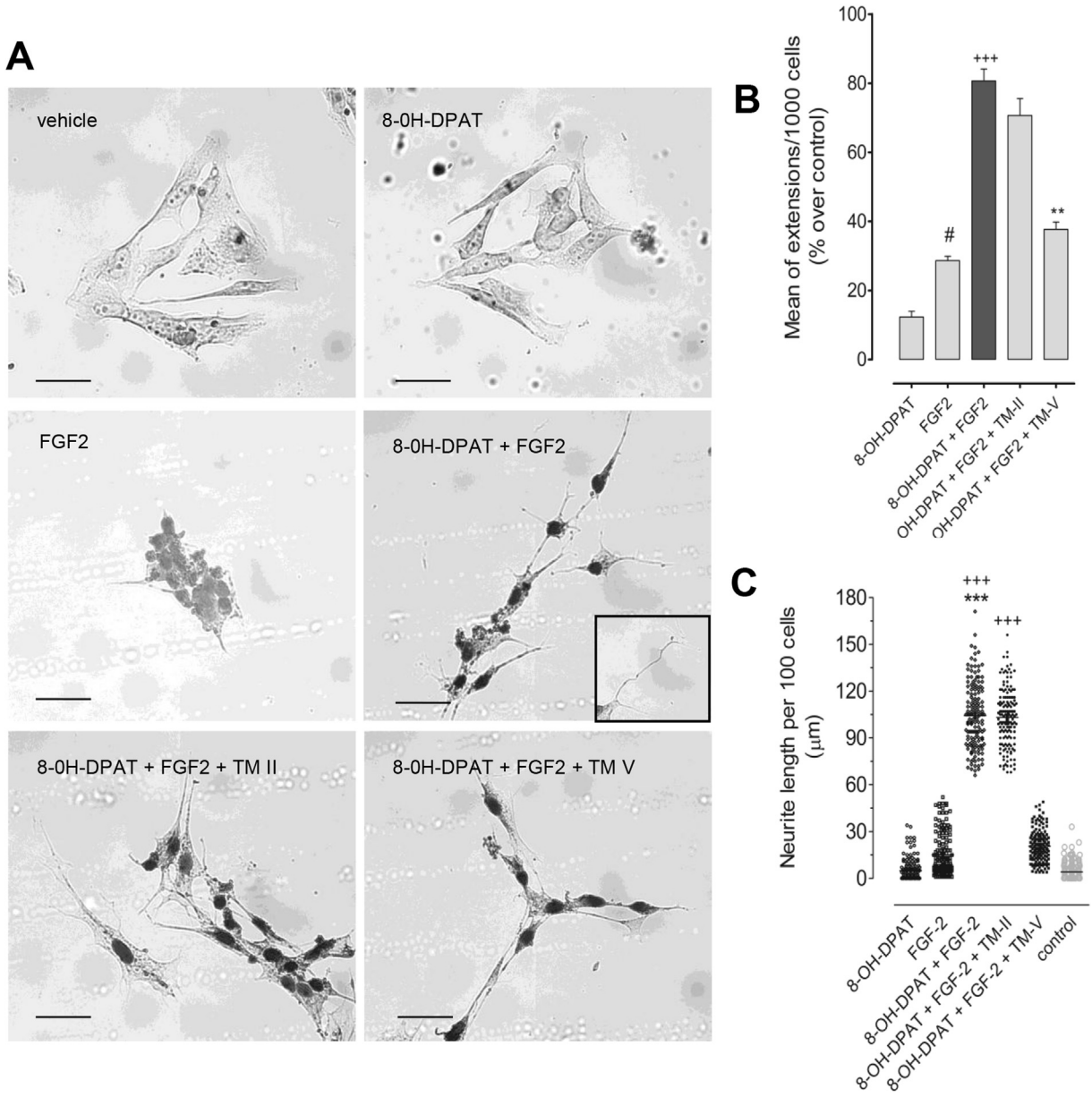

Fig. 3. FGFR1-5-HT1A receptor–receptor interactions lead to structural plasticity in RN33B cells. **(A)** 5-HT1A agonist markedly enhanced FGF-2-mediated RN33B cell differentiation (neurite density). Undifferentiated RN33B cells were treated without (vehicle) or with FGF-2 (50 ng/ml) and/or 8-OH-DPAT (100 nM), in the presence of TM-II and TM-V (0.4 μM) as indicated. Each 24 h, the medium was replaced and fresh ligands were added. After a total incubation time of 3d, the cells were fixed and photographed. **(B)** Extensions were analyzed with light microscopy. Average number of extensions per 1000 cells and mean length per 100 cells are given. Scale bar = 25 μm for all panels. 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 ($^{+++}P < 0.001$); the group treated with TM peptide V (0.4 μM) is highly significantly reduced versus the combined treated group ($^{**}P < 0.01$); FGF-2 is significantly different from 8-OH-DPAT alone ($\#P < 0.05$). **(C)** Extension lengths/100 cells are presented for the various treatment groups as a scattered plot. The quantification includes the calculation of mean ± S.E.M., n = 3. The combined group is significantly different from 8-OH-DPAT and FGF-2 alone ($^{***}P < 0.001$); combined group as well as treated TM peptide II group are highly significantly increased versus the control group and the group treated with TM peptide V group ($^{+++}P < 0.001$). 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.

trophism of large numbers of midbrain raphe 5-HT neurons. This can leads to dysregulation of the 5-HT1A autoreceptor with abnormal reductions of the firing rate in these neurons due to their increased coupling to GIRK channels. Such events would have consequences for large parts of the tel-and diencephalon which is modulated by ascending midbrain raphe 5-HT neurons via their global 5-HT nerve terminal networks in these parts of the brain operating via extrasynaptic volume transmission [15–17]. Future work will show if this hypothesis has a value in understanding mechanisms of major depression. The hippocampal and midbrain raphe 5-HT1A-FGFR1 heteroreceptor complexes are proposed to be a novel target for anti-depressant drug development. The activation of this target can lead to drugs with more rapid and robust antidepressant actions linked to trophic actions inducing recovery from atrophy in the hippocampus and in ascending midbrain raphe 5-HT neurons, associated with increase outgrowth of 5-HT nerve terminal networks.

Taken together, the 5-HT1A autoreceptors by being part of a FGFR1-5-HT1A heteroreceptor complex in midbrain raphe 5-HT nerve cells appears to have an additional functional role in

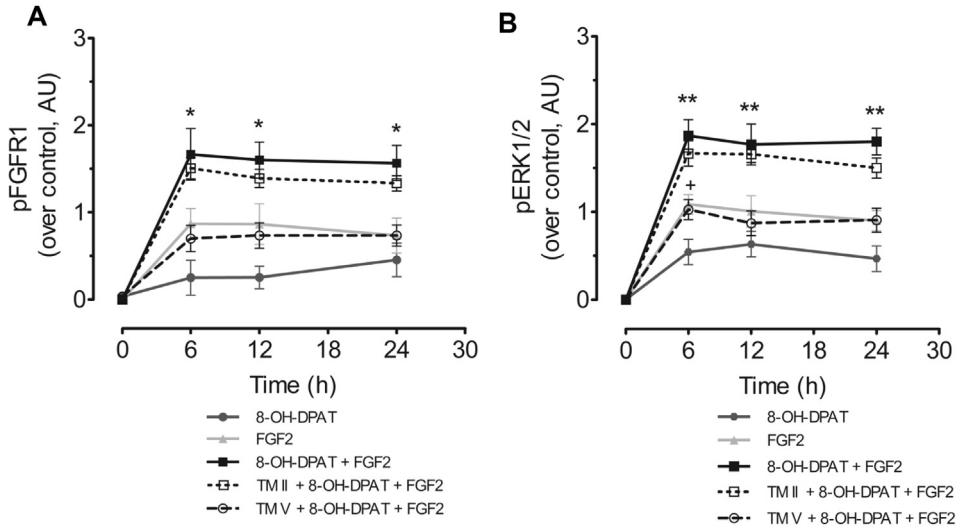

Fig. 4. (A–B) FGFR1 transactivation and ERK1/2 phosphorylation after i.c.v. treatment with FGF-2 and 8-OH-DPAT in the caudal midline raphe area of the midbrain. Synergistic activation of ERK1/2 phosphorylation and FGFR1 phosphorylation was observed in the caudal midline area of the rat midbrain containing the dorsal and median raphe nuclei after i.c.v. co-injection of FGF-2 (50 ng) and/or 8-OH-DPAT (200 nmoles). Data are mean ± S.E.M. Three rats per group (total of 12 groups). Combined treated group (8-OH-DPAT + FGF-2) and the TM peptide II treated group (TM II, 0.4 μM + 8-OH-DPAT + FGF-2) are significantly different from FGF-2 and 8-OH-DPAT alone group and the TM peptide V treated group (TM V, 0.4 μM + 8-OH-DPAT + FGF-2) at the time points studied (FGFR1 transactivation *: $P < 0.05$, ERK1/2 phosphorylation **: $P < 0.01$); activation of ERK1/2 at six hours in the FGF-2 alone group and the TM peptide V treated group were significantly different from that in the 8-OH-DPAT alone group (+: $P < 0.05$). Statistical analysis was performed by Two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The P value 0.05 and lower was considered significant.

midbrain 5-HT neuron systems besides playing a key role in reducing the activity of these neurons as an autoreceptor [13,14]. Thus, by transactivation of FGFR1 in this heteroreceptor complex and increasing its ERK1/2 signaling the 5-HT1A autoreceptor may improve the neuroplasticity of the many midbrain 5-HT nerve cells in which it exists potentially leading to antidepressant effects.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.133.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.133.

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