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5-Hydroxytryptamine 2B receptor regulates cell-cycle progression: Cross-talk with tyrosine kinase pathways.

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Running title: 5-HT_{2B} receptor activates c-Src

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ABSTRACT

In this paper, we present evidence that activation of 5-Hydroxytryptamine 2B (5-HT_{2B}) receptors by serotonin (5-HT) leads to cell cycle progression through retinoblastoma protein hyperphosphorylation, and through activation of both cyclin D1/cdk 4 and cyclin E/cdk 2 kinases by a mechanism that depends on induction of cyclin D1 and cyclin E protein levels. The induction of cyclin D1 expression, but not that of cyclin E is under mitogen-activated protein kinase (MAPK) control, indicating an independent regulation of these two cyclins in the 5-HT_{2B} receptor mitogenesis. Moreover, by using the specific platelet-derived growth factor receptor (PDGFR) inhibitor AG1296 or overexpressing a kinase mutant PDGFR, we showed that PDGFR kinase activity is essential for 5-HT_{2B}-triggered MAPK/cyclin D1 but not cyclin E signalling pathways. 5-HT_{2B} receptor activation also increases activity of the Src family kinase, c-Src, Fyn and c-Yes. Strikingly, c-Src, but not Fyn or c-Yes, is the crucial molecule between Gq-coupled 5-HT_{2B} receptor and the cell cycle regulators. Inhibition of c-Src activity by 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) or depletion of c-Src is sufficient to completely abolish the induction of 5-HT-induced (i) PDGFR tyrosine kinase phosphorylation and MAPK activation, (ii) cyclin D1 and cyclin E expression levels, and (iii) thymidine incorporation. This paper elucidates a model of 5-HT_{2B} receptor mitogenesis in which c-Src acts alone to control cyclin E induction and in concert with the receptor tyrosine kinase PDGFR to induce cyclin D1 expression via the MAPK/ERK pathway.

Abbreviations: Angiotensin (Ang); cyclin-dependent kinase (cdk); epidermal growth factor receptor (EGFR); G-protein-coupled receptors (GPCR); lysophosphatidic acid, (LPA); mitogen activated protein kinase, (MAPK); platelet derived growth factor receptor, (PDGFR); receptor tyrosine kinase (RTK); retinoblastoma protein (pRb); Serotonin, 5-hydroxytryptamine (5-HT); 5-hydroxytryptamine 2B (5-HT_{2B}).

Serotonin (5-hydroxytryptamine, 5-HT) is involved in regulating cellular functions of central and peripheral nervous systems, endocrine and exocrine organs, as well as vascular and hematopoietic systems (1). These actions are mediated by numerous cognate receptors. For instance, 5-HT acts as a growth factor in a variety of cells, a function which is mediated by 5-HT_{1A-B} and 5-HT_{2A-B-C} receptor subtypes (2-5). The 5-HT_{2B} receptor belongs to the G-protein-coupled receptors (GPCR) family. Binding of 5-HT to 5-HT_{2B} receptor activates G_q protein, thereby stimulating phospholipase C, which initiates a rapid release of inositol trisphosphate, and results in a rise in intracellular calcium levels (6). The signal transduction pathways activated downstream of the 5-HT_{2B} receptor include the Ras and mitogen activated protein kinase, MAPK (MAPK/ERK subfamily) pathways (3). Despite numerous studies of 5-HT signal transduction pathways, the critical steps from signal integration to its proliferative action have not been fully elucidated.

The regulation of cell proliferation is a complex process which is primarily regulated by external mitogenic factors. Once cells are induced to proliferate, passage through the mitotic cell cycle is directed by activation of a series of cyclins and their catalytic subunits, cyclin-dependent kinases (cdks) (7). In most cases, mitogenic factors act by stimulating both the proliferation of quiescent cells arrested in G₀ and the progression of cell cycle through the restriction point in late G₁. Progression through G₁ and the G₁/S transition in mammalian cells is regulated by cdk 4 and cdk 6 which form complexes with cyclin D1, and by cdk 2 which forms a complex with cyclin E (7). The G₁-cyclin/cdk complexes drive the phosphorylation of retinoblastoma protein (pRb) that represents the limiting event in G₁ progression (8). However, the mechanism whereby 5-HT triggers G₁ progression, to our knowledge, has not been examined.

The link between upstream signalling circuitry and the cell cycle machinery is of wide interest. The best identified candidates for this link are Ras and MAPK (8, 9). Cyclin D1 and cyclin E expression are induced by several growth factors including estrogen, and heregulin, possibly through Ras/MAPK pathways (10, 11). The functional link between receptor tyrosine kinase (RTK) pathways and cyclin-dependent kinases appears well established; however, little is known about the detailed G₁ effectors of mitogenic signalling cascades triggered by GPCRs. Recently, GPCRs have been shown to stimulate rapid tyrosine phosphorylation of RTKs, a phenomenon known as transactivation (12). Cytoplasmic tyrosine kinases of the Src family have independently been implicated in activation of MAPK pathway by GPCR (13-15). However, the identification of downstream targets and the role in biological responses of the cross-talk between GPCRs and tyrosine kinase activation have not been elucidated especially in the cell cycle context.

We previously reported that expression of a single 5-HT receptor, 5-HT_{2B}, in the non-transformed mouse fibroblast LMTK- cells is mitogenic and transforming since these cells form foci and induce tumors in nude mice. Also, the same receptor is overexpressed with a similar coupling to Ras in human carcinoid tumor cells (3). Since the uncontrolled cell proliferation is the hallmark of cancer cells, the studying of the complete signal transduction system from the membrane to the cell cycle machinery is essential to understand 5-HT-dependent transformation of this cell line.

In this study, the mechanism by which 5-HT binding to the 5-HT_{2B} receptor links mitogenic signals from the membrane to activation of the cell cycle machinery was examined in a 5-HT_{2B} receptor-transformed cell line. We present evidence for the differential involvement of Src and Platelet derived growth factor receptor (PDGFR) in 5-HT_{2B}-mediated cell cycle progression.

MATERIALS AND METHODS

Antibodies. Antibodies, rabbit polyclonal anti-cdk 2 (M2), anti-cdk 4(c-22), anti-cyclin E (M-20), anti-p27kip (M-197), anti -ERK1/2, anti-c-Yes (3) and anti-b-PDGFR(954), mouse monoclonal anti-cyclin D1(72-13G or R-124), anti-Fyn (15) antibody, and GST-Rb were from Santa Cruz. The anti active MAPK was from Promega and the monoclonal anti-pRb antibody from Pharmingen. Mouse monoclonal anti-phosphotyrosine PY20 is from UBI. Respective secondary antibodies were obtained from Pierce.

Cell Culture and transfection. Mouse fibroblast LMTK- cells were stably transfected with an expression vector (pSG5), carrying the mouse 5-HT_{2B} receptor cDNA using Lipofectamine (GIBCO) as previously described (16) to generate LM6 cells. The plasmid (pcDNA3) carrying a mutant PDGFR-b (K634 A) at kinase activity site with the ATP-binding lysine mutated to alanine (17) provided by Drs. R_nstrand and Heldin (Sweden) was introduced into LM6 cells using high efficiency transferrin receptor-mediated-Lipofectamine transfection. Transferrin in combination with Lipofectamine results in almost 100 % transfection of cells (18). LM6 cells grown in 100 mm dishes were treated with 4 ml of serum- and antibiotic- free medium, including 50 mM of Lipofectamine, 10 mg of total plasmid DNA and 16 mM of transferrin overnight, then medium including 10% serum was added for 24h. 48 h after transfection, cells were serum-starved for 36h then treated and lysed. With this protocol, transfection efficiency in LM6 cells reached 85-90% as measured by cotransfection of a b-Galactosidase-containing construct.

To reduce endogenous protein expression, LM6 cells were transfected with phosphorothioate-protected oligodeoxyribonucleotides using also transferrin receptor-mediated-Lipofectamine transfection (19). After transfection in similar conditions, LM6 cells were serum-starved for 36h then treated and lysed. Mouse p42 and p44 MAPK mRNA, antisense (5'-GCCGCCGCCGCCGCAT-3') or sense (5'-ATGGCGGCGGCGGCGGC-3') (20), the c-Src , sense (5'-taccgcgtgttcttcgtt-3') and antisense (5'-ttgctcttcttctgcccatt-3') (21), the Fyn, sense (5'-GATAAAGAAGCAGCGAA-3') and antisense (5'-TTCGCTGCTTCTTTATC-3') (22) were used. The efficiency of transfection was verified by western blot analysis. For example, at least 70 % of cells became transfected for MAPK antisense oligonucleotides within 5 hours and reached a maximum (approximately 95 %) 16h after adding medium including serum.

Cell cycle analysis. Progression through the cell cycle was monitored by flow cytometric analysis of DNA content of cell populations stained with propidium iodide. Cell cycle analysis was carried out with a fluorescence-activated cell sorter (Becton Dickinson FACScan flow cytometer). A minimum of 10 000 cells were analysed in each instance. The percentage of cells within G₁, S and G₂ and M phases was determined using CellQuest software.

Thymidine incorporation. Cells were seeded on 24 well plates at a density of 10³ cells/well in a Dulbecco medium containing 10% FCS, grown overnight and serum starved for 36-48 hours. Quiescent cells were treated with 5-HT at different concentrations and time points, and 0.5 mCi of (3H)-thymidine was added to the culture during the last 4 hours of incubation. The free radioactivity was washed in 5% trichloroacetic acid, the incorporated radioactive thymidine was quantified by scintillation counting.

Cell lysis, immunoprecipitation and immunoblotting. After treatment of quiescent cells with 5-HT at different time point, cells were lysed in an immunoprecipitation buffer (50 mM Tris-HCl,

pH 7.5, 120 mM NaCl, 1% Nonidet P 40, 2 mM sodium orthovanadate, 10 mM NaF, containing the protease inhibitors aprotinin, leupeptin and phenylmethylsulphonylfluoride). The lysates were precleared by centrifugation at 13 000 g for 10 minutes. Electrophoresis was performed in SDS-polyacrylamide gels, using 100 mg of total cell protein for each sample line. After electro-transfer, the membrane was blocked in PBS containing 0.2 % Tween 20 (T-PBS) and 5 % non fat dry milk for 1 hour at room temperature, incubated with an appropriate antibody in T-PBS overnight at 4°C and with the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 hour. Antibody-antigen complexes were detected with ECL kit per manufacturers instruction. For immunoprecipitations, lysates containing equal amounts of protein (500 µg) were incubated with the appropriate antibody (1 mg /sample) overnight at 4°C, 25 mg /ml of protein A/G Sepharose beads added for 1 hour and the immune complexes washed two times with immunoprecipitation buffer. These immunocomplexes were separated by electrophoresis in SDS 6-12 % polyacrylamide gels and transferred to nitro-cellulose as described above. Loading homogeneity was verified by stripping and reprobing the blots according to ECL kit recommendations. Densitometric analysis was performed with densitometry image analyser (Bio-rad, GS-700).

Cyclin/cdk associated kinase assay. For cyclin E/cdk 2 kinase activity, the lysate (500 µg) was immunoprecipitated with cyclin E antibody, and histone H1 (2.5 µg) was used as a substrate. For cyclin D1/cdk 4 kinase assay, the lysate (500 µg) was immunoprecipitated with cyclin D1 antibody, and glutathione S-transferase-retinoblastoma protein (GST-Rb, 10 µg) was used as a substrate according to the information found in ref. (23).

Kinase assay for Src family kinases. The enzyme assay designed to measure the phosphotransferase activity of Src family kinases in immunoprecipitates was used. The assay kit is based on phosphorylation of a specific substrate peptide derived from p34-cdc2 (24) by Src kinase. The enzymatic activity was exhibited as fold increase (basal activity is 32.73 pmol/min/cell).

Data analysis and statistics. Statistical analysis were performed according to the Kruskal and Wallis test. The chosen significance criteria was $p < 0.05$. All values given are representative of at least two duplicated independent experiments.

RESULTS

5-HT_{2B} receptor activation stimulates S phase entry, pRb phosphorylation and G1 cyclin kinase activity. We determined the kinetics of thymidine incorporation of quiescent fibroblasts expressing 5-HT_{2B} receptor (LM6). A peak of DNA synthesis was observed after 16 hours of 5-HT treatment, at which time the rate of thymidine incorporation was increased 5 fold over uninduced cells (figure 1A). Activation of 5-HT_{2B} receptor stimulated thymidine incorporation in a dose-dependent manner reaching a maximum at 1 mM of 5-HT and could be blocked by ritanserin (1 mM), a selective antagonist of 5-HT_{2B} receptors (not shown). Flow cytometric analysis (FACS) of serum starved LM6 cells, stimulated with 5-HT for 0-16 hours showed that over 60 % of cells had begun replicating their DNA by the time the peak of thymidine incorporation occurred (figure 1B). The proportion of cells in S phase increased more than 30% after 16 hours, concomitant with a decrease in the number of cells in G1 phase (from 80% to 50%, figure 1C).

In growth-arrested LM6 cells, 70 % of pRb was hypophosphorylated (figure 2A top). 5-HT_{2B} receptor stimulation resulted in an increase of hyperphosphorylated pRb within 4 hours and in the almost complete disappearance of the hypophosphorylated form of pRb by 8 hours. A

maximum was reached at 12-16 hours when over 80 % of pRb was hyperphosphorylated. The kinetics of pRb hyperphosphorylation preceded the time of entry into S phase. Cyclin D1/cdk 4 activity increased significantly after 4 hours of 5-HT treatment (1 mM), reaching a maximum at 8 hours (figure 2A middle). After 5-HT2B receptor stimulation, cyclin E/cdk 2 activity increased slowly and steadily between 4 to 8 hours, peaking at 12 hours (figure 2A bottom). The time point when cyclin E/cdk 2 activity peaks corresponds to the G1/S phase transition. Following 5-HT2B receptor stimulation, the initial hyperphosphorylation of pRb is coincident with the peak in cyclin D1/cdk 4 activation at 8 hours. Subsequent progressive hyperphosphorylation of pRb could be caused by cyclin E cdk2 activation, which reached a maximum at 12 h. This finding is consistent with progressive hyperphosphorylation of pRb by cyclin D1- and cyclin E-dependent kinase complexes.

5-HT2B receptor stimulation increases cyclin D1 and cyclin E expression levels. Expression of cyclin D1 protein increased within 2h following 5-HT2B receptor stimulation and continued to increase steadily, achieving maximum levels by 6-8 hours (figure 2B, left). Cyclin E expression levels also increased, but more slowly, beginning 2h after 5-HT2B receptor stimulation and peaking at 12 hours (figure 2B right). The profiles of cyclin D1 and cyclin E expression levels induced by 5-HT correlates with kinetics of cyclin D1- and cyclin E-associated kinase activity (figure 2A). The levels of other cyclins such as cyclin A (data not shown), or cdk 2, cdk 4 and p27kip protein levels remained essentially unchanged during this period (figure 2C).

MAPK regulates Cyclin D1 but not Cyclin E levels in the 5-HT2B receptor mitogenic signalling and is regulated by tyrosine kinases. In MAPK antisense oligonucleotides transfected cells, (Fig. 3A) 5-HT (1 μ M) was able to increase cyclin E expression to the same extent as seen in the sense oligonucleotide transfected cells whereas cyclin D1 expression was completely abolished (figure 3B). PD 098059, a specific inhibitor of MEK-1 (25), at a concentration of 15 mM that maximally inhibits MAPK activation (figure 3C), significantly inhibited 5-HT-induced cyclin D1 but not cyclin E levels. Genistein, a general tyrosine kinase inhibitor, (at 15 mM concentration) inhibited 5-HT-induced MAPK activity as assessed by the relative ERK1/ERK2 activity (figure 3C). In the same assay, both the PDGFR specific inhibitor, tyrphostin AG 1296 (at 0.1 mM concentration) (26) and the Src family kinases inhibitor, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine (PP1) (at 1 mM) (27) reduced 5-HT-induced MAPK activity (figure 3C). Since various inhibitors of tyrosine kinases inhibited 5-HT-induced MAPK activation (figure 3C), we investigated their specific action on 5-HT-induced DNA synthesis by measuring thymidine incorporation. In addition to genistein (15 mM), AG 1296 (at 0.1 mM) significantly reduced 5-HT-induced DNA synthesis by 85% and 65% respectively, indicating that activation of PDGFR could regulate cell cycle machinery (data not shown).

5-HT2B receptor activation triggers tyrosine phosphorylation of PDGFR. Time course experiments revealed that in the absence of PDGF, 5-HT (1 μ M) induces PDGFR- β tyrosine phosphorylation reaching to maximum within 10 minutes in the absence of variation in the level of PDGFR protein (figure 4A). Ligand-independent phosphorylation of PDGFR by 5-HT could be completely blocked by AG 1296 at 0.1 μ M concentration (figure 4B). Overexpression of a kinase mutant PDGFR-b (K634A) diminished PDGFR phosphorylation by 5-HT (data not shown) indicating 5-HT2B receptor stimulation transactivates PDGFR. c-Src depletion (figure 4C) or c-Src inhibition by PP1 (at 1 μ M) (figure 4D) abolished 5-HT-induced PDGFR- β phosphorylation

without changing PDGFR protein levels.

5-HT_{2B} receptor stimulation activates Src family kinase activity and c-Src activation is required for thymidine incorporation. Stimulation of LM6 cells by 5-HT (1 μ M) raised the phosphorylation level of c-Src within 5 min (figure 5A) whereas this phosphorylation was completely blocked by PP1 (1 μ M), which also blocked PDGFR phosphorylation induced by 5-HT (figure 4D). No change in the level of Src protein was observed during this assay (Fig. 5A). In a Src kinase assay (24), 5-HT provoked a 1.5-fold increase in c-Src kinase activity at 1 min, reaching to the maximum (8-fold increase) at 3 min, which was sustained after 10 min of stimulation (Fig. 5B). This increase could be blocked by PP1 (1 M) but not AG 1296 (1 M) (data not shown). Other Src family kinases (Fyn and c-Yes) expressed in fibroblast cells could substitute for c-Src. Therefore, we also assayed Fyn and c-Yes activity in response to 5-HT. Fyn activity rose when 5-HT_{2B} activation reached the maximum at 10 min (Fig. 5B). c-Yes activity was elevated later than c-Src activity was. c-Src-antisense transfection (but not sense) inhibited MAPK activity and thymidine incorporation, whereas Fyn antisense or sense transfection did not alter either activity (Fig. 5 C and D).

c-Src is required for both cyclin D1 and Cyclin E induction in 5-HT_{2B} receptor mitogenic signalling. AG 1296 (0.1 mM) at the concentration that totally inhibit PDGFR-b phosphorylation, significantly reduced cyclin D1 expression levels induced by 5-HT_{2B} receptor stimulation, whereas induction in cyclin E expression by 5-HT remained unchanged (figure 6A). Similar data were obtained when the cell were pretreated with higher concentration of AG1296 (1mM). Moreover, when the kinase mutant PDGFR (K634A) was overexpressed, cyclin D1 but not cyclin E expression levels upon 5-HT_{2B} receptor stimulation was significantly reduced (figure 6B). These data indicate that the signalling pathway downstream of PDGFR does not regulate cyclin E expression, however, PDGFR plays a key role in regulating 5-HT-induced cyclin D1 expression. Furthermore, PP1 at the concentration that totally inhibits c-Src kinase activity (1 mM), completely abolished both cyclin D1 and cyclin E levels induced by 5-HT_{2B} receptor stimulation (figure 6A). Similar results in response to 5-HT were obtained in c-Src depleted cell line. However, in the Fyn depleted cell line neither cyclin E nor cyclin D1 expression level were changed in response to 5-HT (figure 6B). Supporting evidence of these findings is the total inhibition by AG 1296 of the initial hyperphosphorylation of pRb within the first 8h following 5-HT_{2B} receptor stimulation, a period in which pRb is mainly regulated by activation of cyclin D1/cdk 4 kinase; however, 12h after stimulation, progressive hyperphosphorylation of pRb through cyclin E/cdk 2 kinase activity was completely blocked by PP1 but not by AG 1296 (figure 6C).

DISCUSSION

In absence of growth factors, 5-HT is particularly potent at inducing cell cycle progression of L-cells expressing the 5-HT_{2B} receptor (LM6) (figure 1). Stimulation of 5-HT_{2B} receptor induces pRb hyperphosphorylation, by activating both cyclin D1/cdk 4 and cyclin E/cdk 2 complexes through its ability to increase the cyclin D1 and cyclin E protein levels (figure 2). The induction of cyclin D1 and cyclin E proteins which is important for progression of G1 phase and for transition to S phase, is, therefore, a critical target for 5-HT_{2B} receptor-induced proliferative signalling in G1 phase. Recently, the GPCR, Angiotensin II (AngII) receptor activation has been shown to stimulate G1 phase progression through the induction of cyclin D1 (28). We elucidated that the regulation of G1 cyclin/cdk complex activity by 5-HT is independent of the cdk inhibitor, p27kip,

because p27kip did not shift from its association with cyclin D1/cdk4 to cyclin E/cdk2 (data not shown). Activation of the RTK ErbB-2 by heregulin in the T-47D human breast cancer cells (10) or of the estrogen receptor in MCF-7 human breast cancer cells (23) triggers cell cycle progression without significant decrease in p27kip expression levels. To our knowledge, this is the first demonstration that 5-HT_{2B} receptor activation regulates cell cycle progression by inducing cyclin E and cyclin D1 expression levels.

Preincubation of 5-HT_{2B} expressing cells with PD 098059 at a concentration that completely inhibits MAPK activation, also blocks 5-HT-induced thymidine incorporation. In MAPK-depleted cells, 5-HT is still able to induce cyclin E expression, whereas cyclin D1 induction is completely abolished, indicating that the MAPK pathway is necessary for 5-HT-dependent regulation of cyclin D1 but not of cyclin E levels (figure 3). Interestingly, MAPKs are required to pass the G1 restriction point in fibroblasts and inhibition of MAPK activity abolishes cyclin D1 promoter activity (29). Inhibition of the MAPK by PD 098059 blocks heregulin-induced cell cycle progression that is mainly due to cyclin D1 induction in T-47D human breast cancer cells (10). Therefore, our data indicate that, in 5-HT_{2B} receptor mitogenic signalling, only cyclin D1 expression is regulated via MAPK, however, another pathway independent of MAPK activation should regulate cyclin E expression.

Based on the initial observation that treatment of the 5-HT_{2B} expressing cells with a selective PDGFR kinase inhibitor AG 1296 or a Src family kinase inhibitor PP1 strongly reduced 5-HT-induced MAPK activation, pRb hyperphosphorylation as well as thymidine incorporation, tyrosine kinases seemed to be involved in the 5-HT_{2B} mitogenic signalling. Tyrosine kinases were proposed to mediate MAPK activation by 5-HT_{1A-2A} (5, 30) and Ang II receptors. Furthermore, the fact that activation of 5-HT_{2B} receptor stimulates PDGFR- β phosphorylation in the absence of PDGF demonstrates the existence of cross-talk between Gq-coupled receptor and RTKs (figure 4). The requirement for the PDGFR kinase activity in 5-HT_{2B} receptor mitogenic pathway is demonstrated either by using the PDGFR kinase inhibitor AG 1296 or by overexpressing a kinase mutant PDGFR in LM6 cells in which PDGFR phosphorylation upon stimulation was totally abolished. Gi/Gq-coupled receptors are known to activate the ERK pathway via both tyrosine kinase-dependent and -independent pathways. For example, receptors for endothelin, Ang II, lysophosphatidic acid (LPA), and muscarinic acetylcholine receptor have been shown to stimulate ligand-independent tyrosine phosphorylation of PDGFR (31), insulin-like growth factor-1 receptor β subunit (32), EGFR, and ErbB-2 (12). LPA and thrombin receptors has been shown to mediate ERK 1/2 activation and transactivation of the EGF receptor largely via pertussis toxin-sensitive G proteins (33). Since we have shown that cholera or pertussis toxin and antibodies against Gi or Gs were ineffective whereas anti G α_q antibody almost completely blocks GTPase activation triggered by 5-HT in the same cell line (6), we excluded the possible involvement of Gi-protein in 5-HT_{2B} mitogenic signalling. In addition, activation of 5-HT_{2B} receptor appears to preferentially transactivate PDGFR, since 5-HT does not phosphorylate ErbB-2 receptor also present on LM6 cells where endogenous EGFR is not expressed (34). Therefore, ligand-independent activation of RTKs by GPCRs occurs in a cell specific manner. Preferential coupling to or, specific expression of the RTKs could explain this specificity. Moreover, treatment of the cells with PDGFR kinase inhibitor AG 1296 or overexpressing a kinase mutant PDGFR totally abolished MAPK activity and Cyclin D1 expression in response to 5-HT (figure 4, 6). These data suggest the requirement of the PDGFR kinase activity for transduction of the 5-HT_{2B} receptor-induced MAPK activity and cyclin D1 expression. In the 5-HT_{2B} receptor mitogenic signalling, the PDGFR, thus, serves as a signal transducer of 5-HT-mediated regulation of MAPK activity as well as cyclin D1 but not cyclin E expression. This study demonstrates that PDGFR kinase activity is essential for 5-HT_{2B}-triggered

MAPK cyclin D1 activation; however, this transactivation is not sufficient for cell-cycle progression.

Src family kinases have been suggested to mediate LPA-or Gbg-induced tyrosine phosphorylation of SH2-containing protein (Shc) and subsequent activation of MAPK, given that inhibition of the Src kinases markedly attenuated these responses (13, 15). Although, if c-Src is "upstream or downstream" of RTKs is still in debate, there is supporting evidence that c-Src activation precedes RTKs transactivation by GPCR. Previously, overexpression of the Src kinase inhibitor kinase Csk has been shown to attenuate LPA and alpha2-adrenergic receptor-mediated EGFR phosphorylation in COS-7 cells (15). Recently Src family kinases have been shown to be required for integrin but not PDGFR signal transduction in fibroblast cells obtained from knockout embryos where Src family kinases were non-functional (35); this work clearly indicated that Src family kinases are not downstream of PDGFR signalling, because PDGF could fully activate mitogenic pathway in absence of c-Src, Fyn and c-Yes. Our data strongly suggest that c-Src plays an "upstream" role in the transactivation of PDGFR in the 5-HT2B mitogenic signalling, based on the observation that PP1 and more convincingly that c-Src depletion abolished 5-HT-induced PDGFR phosphorylation (figure 4). This finding indicates that in 5-HT2B mitogenic pathway, c-Src targets phosphorylation sites of the PDGFR that modulate PDGFR activation. Moreover, inactivating PDGFR by a specific inhibitor, AG 1296, or overexpressing the kinase mutant PDGFR did not affect Src kinase activation by 5-HT, confirming that PDGFR is not upstream of Src in 5-HT2B mitogenic signalling. The kinetic of c-Src kinase activation by 5-HT also shows Src kinase activation earlier than PDGFR phosphorylation. The observed 5-HT-induced Src kinase activity suggests that c-Src acts as a kinase rather than as a scaffolding protein in 5-HT2B receptor mitogenic pathway, and the autophosphorylation site of c-Src must be activated (36). Each of the Src family kinases (c-Src, Fyn and c-Yes) that we examined were activated by 5-HT. However, c-Src activation in response to 5-HT, is stronger and appears earlier than that of either c-Yes or Fyn (figure 5). Both the Fyn and c-Yes kinase activity following 5-HT2B stimulation do not correlate with PDGFR transactivation. Depletion of c-Src but not of Fyn, completely abolished 5-HT2B receptor-induced thymidine incorporation, MAPK activation and cyclin D1 and E expression. PP1 totally inhibited 5-HT-induced initial and progressive hyperphosphorylation of pRb (Fig. 6), which is regulated by cyclin D1- and cyclin E-dependent kinase activity, respectively. These data strongly support the notion that c-Src is the key factor in 5-HT2B receptor-induced cell-cycle progression. Fyn and c-Yes do not substitute for c-Src, but might be involved in other biological effects of the 5-HT2B receptor. Anti-pp60-c-Src but not c-Yes or Fyn antibodies blocked the Gq-coupled angiotensin II receptor-stimulated tyrosine phosphorylation (36), lending further support to a specialized role for c-Src in the Gq-dependent mitogenic process.

Our data support a model for the 5-HT mitogenic signalling in which c-Src is a crucial molecule that links the Gq-coupled 5-HT2B receptor and cell cycle regulators: c-Src alone controls cyclin E induction and transactivates receptor tyrosine kinase PDGFR to induce cyclin D1 expression via MAPK/ERK pathway (figure 7).

References

1. Wilkinson, L. O. & Dourish, C. T. (1991) in Serotonin receptor subtypes: Basic and clinical aspects, ed. Peroutka, S. J. (Wiley-Liss, New-York), Vol. 15, pp. 147-210.
2. Julius, D. (1991) *Annu. Rev. Neurosci.* 14, 335-360.
3. Launay, J.-M., Birraux, G., Bondoux, D., Callebert, J., Choi, D.-S., Loric, S. & Maroteaux, L. (1996) *J. Biol. Chem.* 271, 3141-3147.
4. Seuwen, K., Magnaldo, I. & Pouyssegur, J. (1988) *Nature* 335, 254-6.
5. Garnovskaya, M. N., van Biesen, T., Hawe, B., Casanas Ramos, S., Lefkowitz, R. J. & Raymond, J. R. (1996) *Biochemistry* 35, 13716-22.
6. Loric, S., Maroteaux, L., Kellermann, O. & Launay, J.-M. (1995) *Mol. Pharmacol.* 47, 458-466.
7. Jacks, T. & Weinberg, R. A. (1998) *Science* 280, 1035-6.
8. Peeper, D. S., Upton, T. M., Ladha, M. H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J. A. & Ewen, M. E. (1997) *Nature* 386, 177-81.
9. Le Gall, M., Grall, D., Chambard, J. C., Pouyssegur, J. & Van Obberghen-Schilling, E. (1998) *Oncogene* 17, 1271-7.
10. Fiddes, R. J., Janes, P. W., Sivertsen, S. P., Sutherland, R. L., Musgrove, E. A. & Daly, R. J. (1998) *Oncogene* 16, 2803-13.
11. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W. & Sutherland, R. L. (1997) *J. Biol. Chem.* 272, 10882-94.
12. Daub, H., Weiss, F. U., Wallasch, C. & Ullrich, A. (1996) *Nature* 379, 557-60.
13. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. & Schlessinger, J. (1996) *Nature* 383, 547-50.
14. Thomas, S. M. & Brugge, J. S. (1997) *Annu Rev Cell Dev Biol* 13, 513-609.
15. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J. & Lefkowitz, R. J. (1996) *J. Biol. Chem.* 271, 19443-50.
16. Nebigil, C. G. (1997) *Biochemistry* 36, 15949-58.
17. Emaduddin, M., Ekman, S., Ronnstrand, L. & Heldin, C. H. (1999) *Biochem. J.* 341, 523-8.
18. Cheng, P. W. (1996) *Hum. Gene Ther.* 7, 275-82.
19. Shichiri, M., Sedivy, J. M., Marumo, F. & Hirata, Y. (1998) *Mol. Endocrinol.* 12, 172-80.
20. Sale, E. M., Atkinson, P. G. & Sale, G. J. (1995) *EMBO J.* 14, 674-84.
21. Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. B. & Baron, R. (1996) *Nature* 383, 528-31.
22. Li, Y. & Chen, B. (1995) *J Leukoc Biol* 57, 484-90.
23. Planas-Silva, M. D. & Weinberg, R. A. (1997) *Mol. Cell. Biol.* 17, 4059-69.
24. Cheng, H. C., Nishio, H., Hatase, O., Ralph, S. & Wang, J. H. (1992) *J. Biol. Chem.* 267, 9248-56.
25. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. & Saltiel, A. R. (1995) *J. Biol. Chem.* 270, 27489-94.
26. Gazit, A., App, H., McMahon, G., Chen, J., Levitzki, A. & Bohmer, F. D. (1996) *J. Med. Chem.* 39, 2170-7.
27. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A. & Connelly, P. A. (1996) *J. Biol. Chem.* 271, 695-701.
28. Watanabe, G., Lee, R. J., Albanese, C., Rainey, W. E., Battle, D. & Pestell, R. G. (1996) *J. Biol. Chem.* 271, 22570-7.
29. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. & Pouyssegur, J. (1996) *J. Biol. Chem.*

271, 20608-16.

30. Florian, J. A. & Watts, S. W. (1998) *J. Pharmacol. Exp. Ther.* 284, 346-55.
31. Linseman, D. A., Benjamin, C. W. & Jones, D. A. (1995) *J. Biol. Chem.* 270, 12563-8.
32. Rao, G. N., Delafontaine, P. & Runge, M. S. (1995) *J. Biol. Chem.* 270, 27871-5.
33. Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J. & Luttrell, L. M. (1999) *J. Biol. Chem.* 274, 13978-84.
34. Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G. & Gudermann, T. (1998) *Proc. Natl. Acad. Sci. USA* 95, 8985-90.
35. Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A. & Soriano, P. (1999) *EMBO J.* 18, 2459-71.
36. Schieffer, B., Paxton, W. G., Chai, Q., Marrero, M. B. & Bernstein, K. E. (1996) *J. Biol. Chem.* 271, 10329-33.

Figure legends

Figure 1

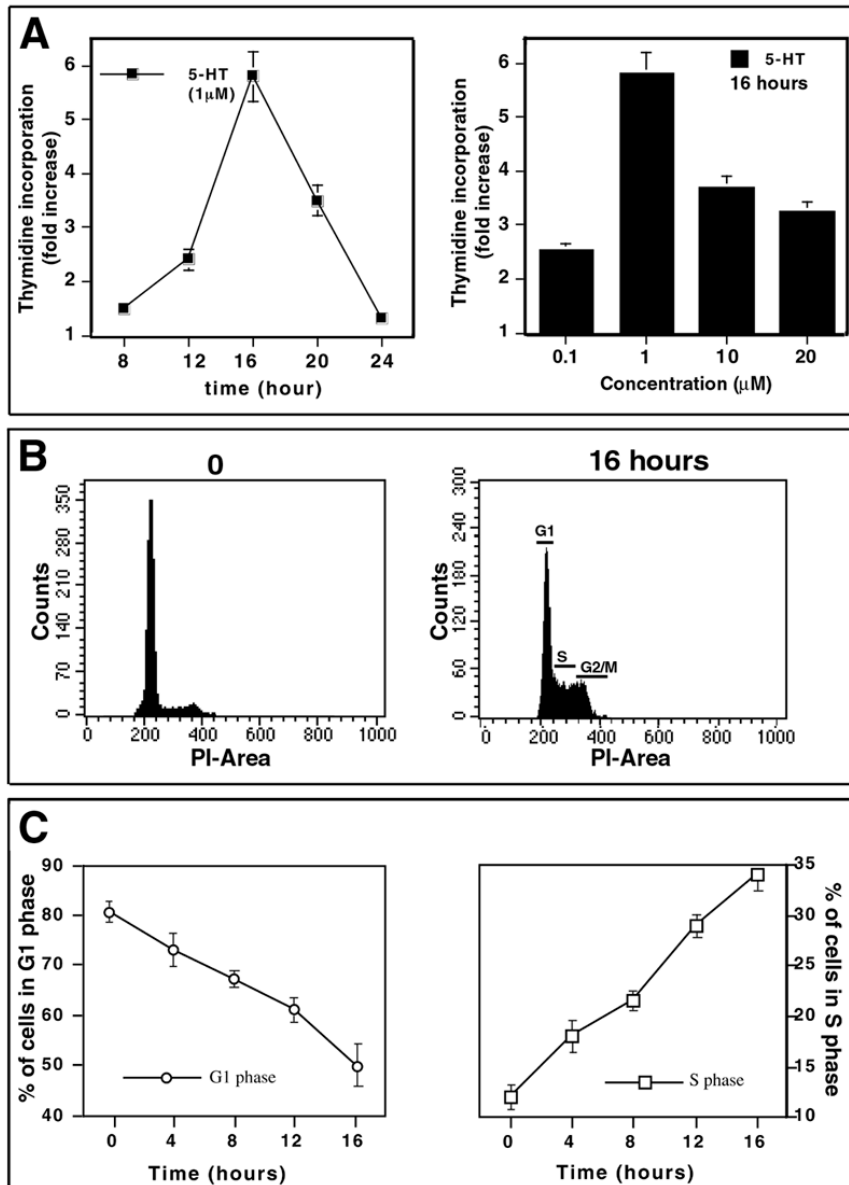


Fig. 1. 5-HT_{2B} receptor activation stimulates S phase entry. (A) 5-HT increases thymidine incorporation. Quiescent LM6 cells were treated with 5-HT (1 mM) for the indicated time (left), or with different concentrations of 5-HT for 16 hours (right). (B) 5-HT increases the percentage of LM6 cells in S phase. Representative cell cycle profiles are presented at zero time (left) and 16 hours (right) after 5-HT treatment. (C) Progression of LM6 cells from G1 (left) to S (right) phase after 5-HT (1 mM) treatment. Thymidine incorporation and the percentage of cells in G1, S and G2/M phase were determined as described in Materials and Methods. Values represent the mean \pm SEM for duplicate samples obtained from at least three independent experiments.

Figure 2

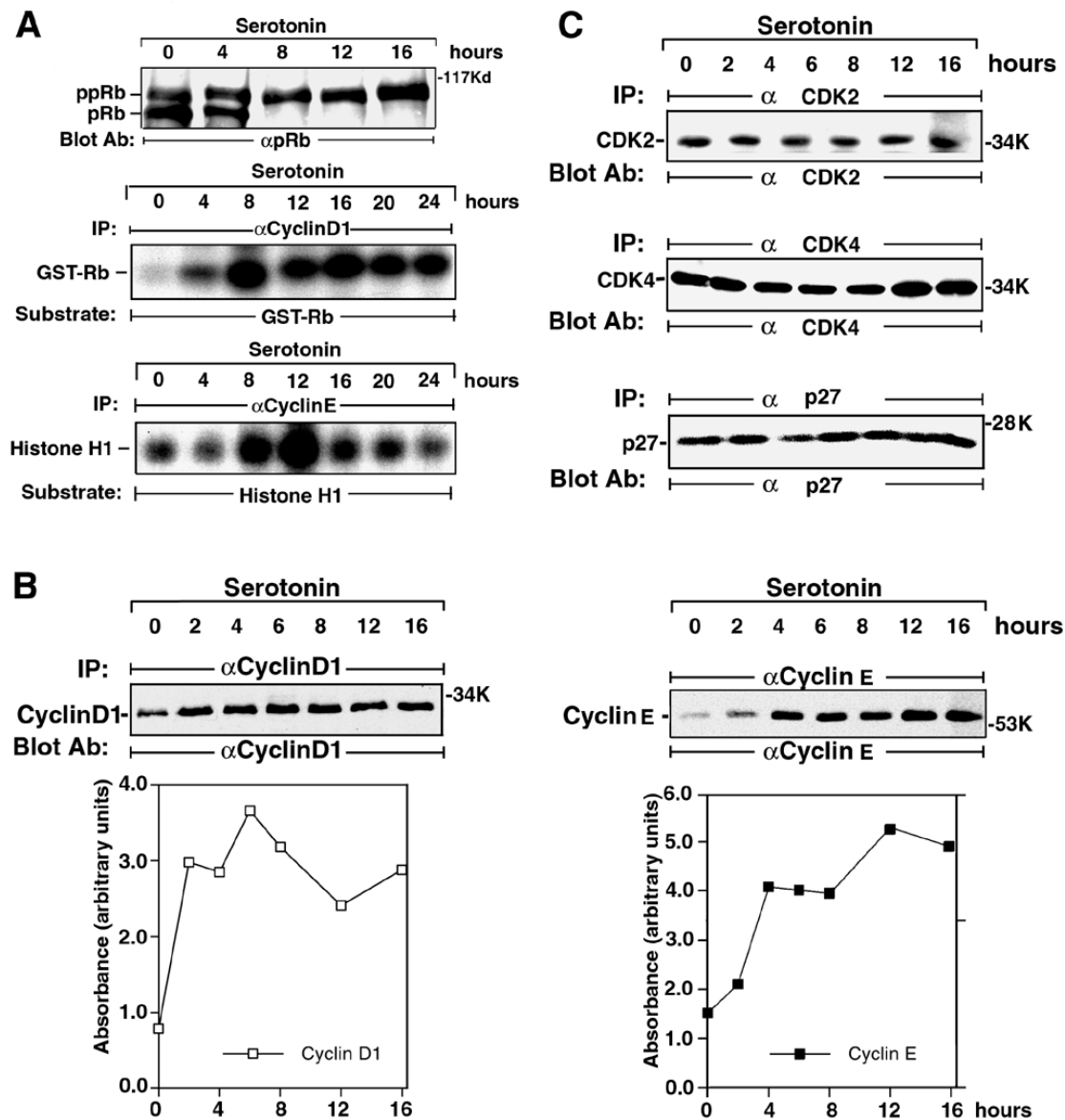


Fig. 2. 5-HT_{2B} receptor activation stimulates pRb phosphorylation and G1 cyclin kinase activity. (A) Stimulation of pRb hyperphosphorylation and G1 cyclin levels by 5-HT. Membranes were probed with pRb antibody which recognise both hypo and hyper phosphorylated state of protein. The positions of hypophosphorylated pRb, and hyperphosphorylated ppRb are indicated (top). For cyclin D1/cdk 4 kinase assay (middle) or for cyclin E/cdk 2 kinase assay (bottom), the 5-HT-dependent phosphorylation of both complexes was assayed by autoradiogram as described in the Materials and Methods section. (B) 5-HT increases protein levels of G1 cyclins (D1 and E). (C) Protein levels of cdk 4, cdk 2 or cdk inhibitor, p27kip are not affected by 5-HT. Cyclin D1, cyclin E, (B) cdk 4, cdk 2 or p27kip (C) were detected with ECL and quantified by densitometry. These data are representative of at least three individual experiments. The position of cyclin D1, cyclin E, cdk 4, cdk 2 and p27kip are indicated.

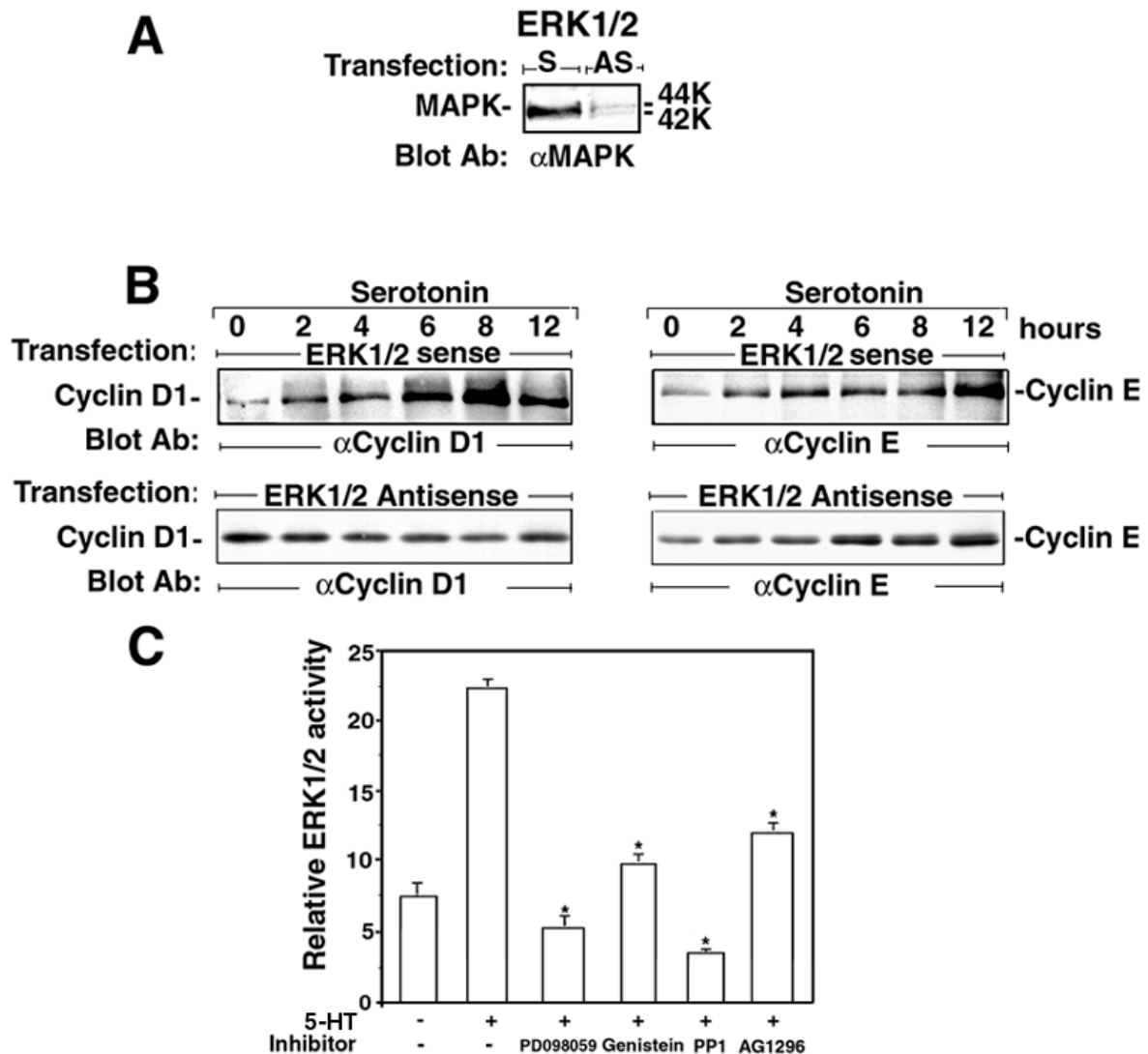
Figure 3

Fig. 3. MAPK regulates Cyclin D1 but not Cyclin E levels in the 5-HT_{2B} receptor mitogenic signalling and is regulated by tyrosine kinases. (A) Depletion of MAPK by antisense transfection. LM6 cells were transiently transfected with antisense (AS) or sense (S) oligodeoxyribonucleotides of mouse MAPK (ERK1/2) mRNA and probed with MAPK antibody. (B) 5-HT-induced cyclin D1 expression is abolished in MAPK depleted cells but not that of cyclin E. Following visualisation with anti-cyclin D1 antibody, the membrane were washed and re-probed with cyclin E antibody. The position of cyclin E, cyclin D1 are indicated. (C) MAPK activation is regulated by tyrosine kinases. After pretreatment with inhibitors for MAPK kinase (PD 098059) (15 mM), tyrosine kinase (genistein) (15 mM), Src (PP1) (1 mM) and PDGFR (AG 1296) (0.1 mM), the cells were incubated with 5-HT (1 mM) for 10 min, then revealed with anti-active MAPK antibody. After revealing immunocomplexes by ECL, the relative active ERK1 and ERK2 proteins levels were assessed using a luminometer. The star indicates significant inhibition of the parameters compared to 5-HT stimulation alone ($p < 0.05$, $n > 3$). All values represent the mean \pm SEM for duplicate samples and are representative of at least four individual experiments.

Figure 4

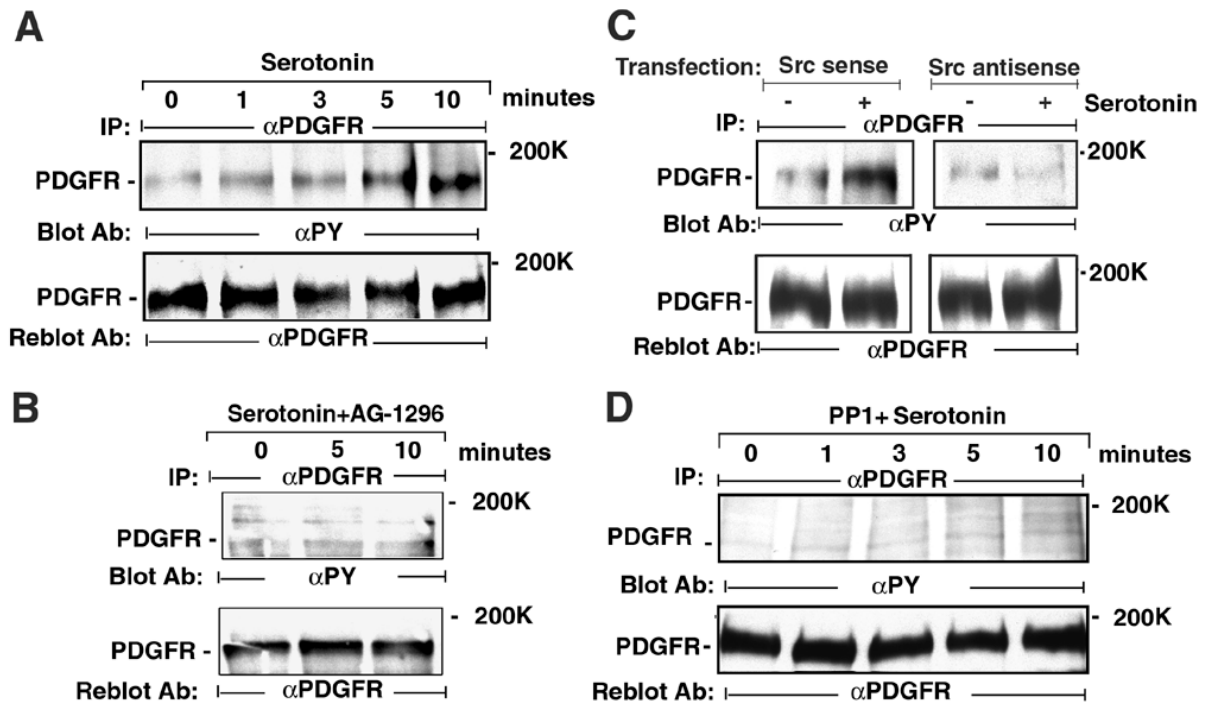


Fig. 4. 5-HT_{2B} receptor activation triggers tyrosine phosphorylation of PDGFR. (A) 5-HT stimulates PDGFR phosphorylation. Serum starved cells were incubated with 5-HT (1 mM) for the indicated times. (B) AG 1296 inhibits 5-HT-induced PDGFR phosphorylation. Serum starved cells were pretreated with AG 1296 (0,1 mM) for 15 minutes prior to stimulation with 5-HT (1 mM) for the indicated times. (C) c-Src depletion inhibits 5-HT-induced PDGFR phosphorylation. After transfection of LM6 cells with c-Src sense or antisense oligonucleotides, serum starved cells were stimulated with vehicle (-) or 5-HT (1 mM) (+) for 5 min. (D) PP1 inhibits 5-HT-induced PDGFR phosphorylation. Serum starved LM6 cells were pretreated with PP1 (1 mM) for 15 minutes prior to stimulation with 5-HT (1 mM) for the indicated times. After cell lysis, 250 mg of samples were immunoprecipitated with anti-b-PDGFR antibody. The induction of PDGFR tyrosine phosphorylation was analysed following immunoblotting with anti-phosphotyrosine antibody (upper), or after stripping, the same blot was reprobbed with anti-PDGFR antibody (lower). The position of PDGFR-b is indicated. These data are representative of at least two individual experiments.

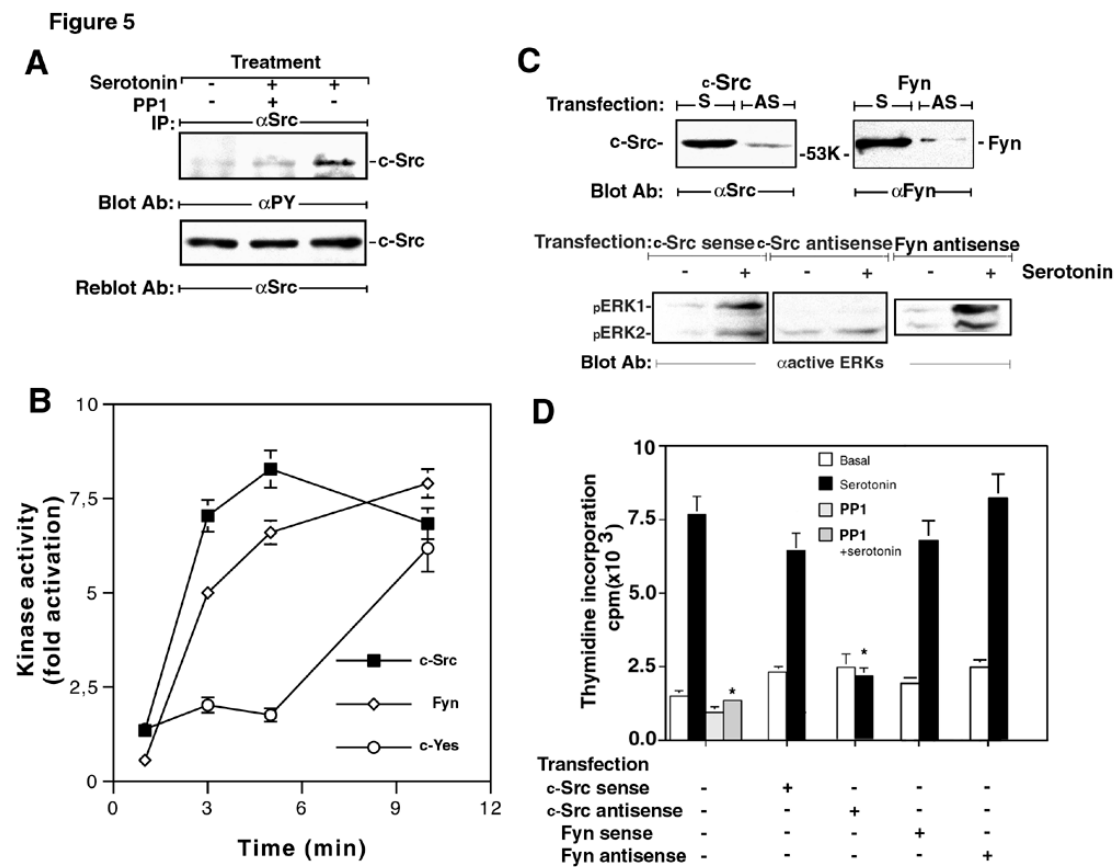


Fig. 5. 5-HT induces Src family kinase activation and activation of c-Src is required for MAPK and thymidine incorporation. (A) 5-HT induces phosphorylation of c-Src. Serum starved LM6 cells were pre-treated with either vehicle or PP1 (1 mM) for 15 minutes prior to stimulation with 5-HT (1 mM) for 5 minutes. After cell lysis, 250 mg of samples were immunoprecipitated with anti-Src antibody. The induction of c-Src tyrosine phosphorylation was analysed following immunoblotting with anti-phosphotyrosine antibody (upper) or after stripping reprobed with anti-Src antibody (lower). The position of c-Src is indicated. These data are representative of at least two individual experiments. (B) 5-HT induces Src family kinase activity. Quiescent cells were treated with 5-HT (1 mM) for the indicated times, washed, lysed and proteins were extracted. After immunoprecipitation with either c-Src, Fyn or c-Yes specific antibody, in vitro kinase activity was assessed using as exogenous substrate a peptide derived from p34-cdc2 as described under material and methods. (C) Depletion of Src but not Fyn by antisense oligonucleotide transfection reduces MAPK activity induced by 5-HT. LM6 cells were transiently transfected with antisense (AS) or sense (S) oligodeoxyribonucleotides of mouse c-Src or Fyn mRNA. Quiescent cell extract treated with vehicle (upper, -) or 5-HT (+) for 5 min, then lysed, and protein extracts were probed with corresponding antibodies (c-Src or Fyn, upper) or anti-active MAPK antibody (lower). The position of c-Src, Fyn and active ERK1 or ERK2 are indicated. (D) c-Src but not Fyn depletion abolishes 5-HT-induced thymidine incorporation. After the transfection of either c-Src or Fyn sense or antisense oligonucleotides, quiescent cells were treated with 5-HT (1 mM) for 16 h, thymidine incorporation was detected in these transfected cell lines or in PP1 (1 mM) pretreated (for 15 min) LM6 cells. All values represent the mean \pm SEM for duplicate samples obtained from at least two independent experiments.

Figure 6

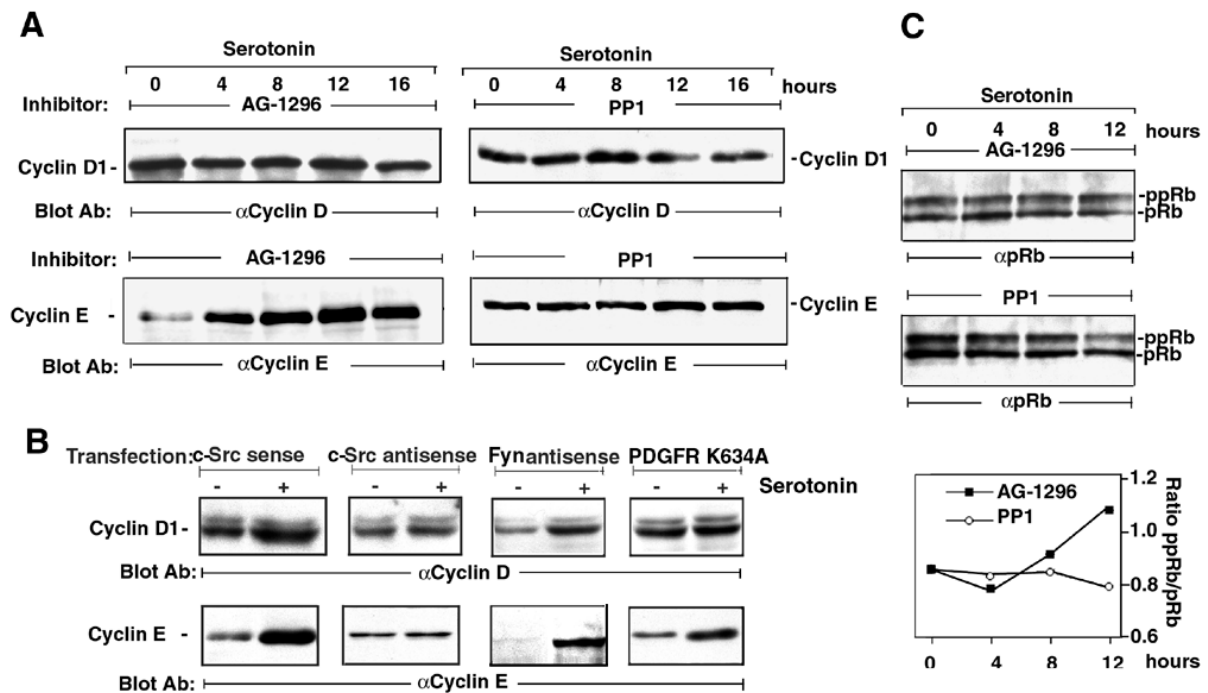


Fig. 6. c-Src is required for both cyclin D1 and Cyclin E induction in 5-HT_{2B} receptor mitogenic signalling. (A) Effect of tyrosine kinase inhibition on 5-HT-induced cyclin levels in LM6 cells. After pretreatment with AG 1296 (0,1 mM) or PP1 (1 mM) for 15 min, the cells were incubated with 5-HT at different time point, then revealed with cyclin D1 (R-124) antibody or with cyclin E antibody. The position of cyclin E, cyclin D1 are indicated. (B) Effect of depletion of c-Src and Fyn or overexpression of a kinase mutant PDGFR in LM6 cells on 5-HT-induced cyclin D1 and cyclin E levels. LM6 cells were transiently transfected with antisense or sense oligodeoxyribonucleotides of mouse c-Src or Fyn mRNA or plasmid carrying kinase domain mutated PDGFR (K634A). Quiescent cells were treated with vehicle (upper), (-, lower) or 5-HT (+, lower) for 12 h, then protein extracts were revealed with cyclin D1 antibody (72-12C). Following visualisation of immunocomplexes, the membrane were washed and reprobred with cyclin E antibody. The position of cyclin E, cyclin D1 are indicated. (C) The effect of AG 1296 or PP1 on 5-HT-induced pRb hyperphosphorylation. Serum starved LM6 cells were pre-treated either with vehicle, PP1 (1 mM) or AG 1296 (0.1 mM) for 10 minutes prior to stimulation with 5-HT (1 mM) for the indicated times. Total cell lysates were analysed using pRb antibody. The position of pRb and ppRb are indicated. Antibody-antigen complexes were detected with ECL and quantified by densitometric analysis. These data are representative of at least two individual experiments.

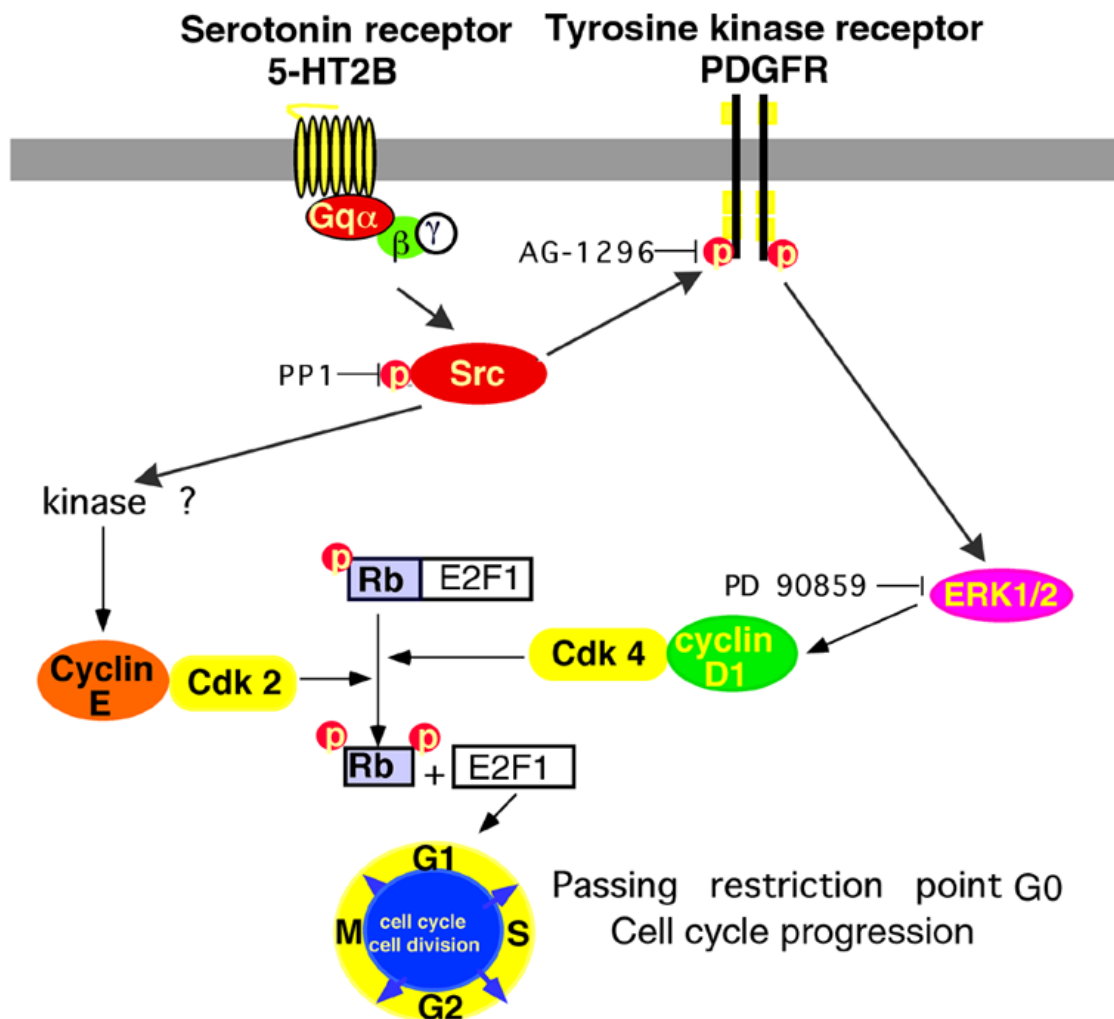


Fig. 7. c-Src and PDGFR are functionally connected to each other and provides links to cell cycle regulators in Gq-coupled 5-HT2B receptor mitogenic signalling. c-Src either acts alone to control cyclin E induction or in concert with a receptor tyrosine kinase PDGFR to induce cyclin D1 expression via ERK MAPK pathway. This action raises the level of pRb phosphorylation and triggers cell cycle progression.

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