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p90 Ribosomal S6 Kinase 2, a Novel GPCR Kinase, Is Required for Growth Factor-Mediated Attenuation of GPCR Signaling†

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ABSTRACT: The 5-hydroxytryptamine 2A (5-HT_{2A}) receptor is a member of the G protein-coupled receptor superfamily (GPCR) and plays a key role in transducing a variety of cellular signals elicited by serotonin (5-HT; 5-hydroxytryptamine) in both peripheral and central tissues. Recently, we discovered that the ERK/MAPK effector p90 ribosomal S6 kinase 2 (RSK2) phosphorylates the 5-HT_{2A} receptor and attenuates 5-HT_{2A} receptor signaling. This raised the intriguing possibility of a regulatory paradigm whereby receptor tyrosine kinases (RTKs) attenuate GPCR signaling (i.e., "inhibitory cross-talk") by activating RSK2 [Strachan et al. (2009) *J. Biol. Chem. 284*, 5557−5573]. We report here that activation of multiple endogenous RTKs such as the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR), and ErbB4 significantly attenuates 5-HT_{2A} receptor signaling in a variety of cell types including mouse embryonic fibroblasts (MEFs), mouse vascular smooth muscle cells (mVSMCs), and primary cortical neurons. Importantly, genetic deletion of RSK2 completely prevented signal attenuation, thereby suggesting that RSK2 is a critical mediator of inhibitory cross-talk between RTKs and 5-HT_{2A} receptors. We also discovered that P2Y purinergic receptor signaling was similarly attenuated following EGFR activation. By directly testing multiple endogenous growth factors/RTK pathways and multiple Gq-coupled GPCRs, we have now established a cellular mechanism whereby RTK signaling cascades act via RSK2 to attenuate GPCR signaling. Given the pervasiveness of growth factor signaling, this novel regulatory mechanism has the potential to explain how 5-HT_{2A} receptors are regulated *in vivo*, with potential implications for human diseases in which 5-HT_{2A} or RTK activity is altered (e.g., neuropsychiatric and neurodevelopmental disorders).

The GPCR[1] superfamily mediates essential functions in organisms as diverse as unicellular choanoflagellates and humans (*1*, *2*). In humans, GPCRs comprise approximately 2% of the genome to transduce signals elicited by both endogenous and exogenous ligands (*3*−*5*). Not surprisingly, GPCR dysregulation is associated with many human diseases (*6*), thus explaining why GPCRs are successful therapeutic targets and remain the focus of intense drug discovery efforts (*7*).

The Gq-coupled 5-HT_{2A} receptor, in particular, is one of 14 GPCRs that mediates the pleiotropic actions of 5-HT in both peripheral and central tissues (*8*, *9*). The 5-HT_{2A} receptor is an important therapeutic target for a large number of psychiatric and medical diseases (*9*) and is also the site of action of most, but not all, hallucinogens which function as 5-HT_{2A} receptor agonists (*10*, *11*) (Keiser et al. (2009) in press). Additionally, atypical antipsychotics (e.g., clozapine) are thought to mediate their therapeutic actions, at least in part, by antagonizing 5-HT_{2A} receptors (*12*).

We recently discovered a novel regulatory mechanism whereby RSK2 interacts with 5-HT_{2A} serotonin receptors and attenuates receptor signaling via direct receptor phosphorylation (*13*, *14*). RSK2 is a multifunctional ERK/MAPK effector activated downstream of growth factor signal cascades involving RTKs (*15*). This raised the intriguing possibility of a new regulatory mechanism whereby RTKs attenuate GPCR signaling (referred to here as "inhibitory cross-talk") by activating RSK2. These studies led to the initial discovery that activation of the EGFR attenuates 5-HT_{2A} receptor signaling, presumably via RSK2 activation (*14*). These preliminary data were intriguing for several reasons including (1) they suggested for the first time that the 5-HT_{2A} receptor is part of an emerging regulatory paradigm whereby activated RTKs attenuate GPCR signaling (*16*−*22*), and (2) they were the first to identify RSK2 as a novel mediator of inhibitory cross-talk between growth factor-activated RTKs and a GPCR.

In this paper, we show that activation of various endogenous RTKs (i.e., EGFR, PDGFR, and ErbB4) significantly attenuates

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[1]Abbreviations: 5-HT, serotonin; 5-HT_{2A}, serotonin 2A receptor; GPCR, G protein-coupled receptor; RSK2, p90 ribosomal S6 kinase 2; ERK/MAPK, extracellular signal regulated kinase/mitogen-activated protein kinase; CRC, concentration−response curve; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IGF-1, insulin-like growth factor 1; IGF-1 R, insulin-like growth factor 1 receptor; TGF-α, transforming growth factor α; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; NRG-1, neuregulin 1; GFP, green fluorescent protein; 5-methoxy-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's buffered salt solution; MEF, mouse embryonic fibroblast; mVSMC, mouse vascular smooth muscle cell; RFU, relative fluorescent unit.

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5-HT_{2A} receptor signaling in multiple cell types (i.e., in MEFs, mVSMCs, and primary cortical neurons). In contrast, insulin-like growth factor 1 (IGF-1), which only weakly activates RSK2, fails to attenuate 5-HT_{2A} receptor signaling. Together with evidence that genetic deletion of RSK2 is sufficient to prevent RTK-mediated signal attenuation in all tested cellular backgrounds, these findings support a novel role for RSK2 in inhibitory cross-talk between RTKs and the 5-HT_{2A} receptor. Significantly, we also discovered that P2Y purinergic receptor signaling, which is regulated by RSK2, was similarly attenuated following EGF receptor activation in wild-type (RSK2+/+) MEFs. By testing several endogenous growth factors/RTK pathways and multiple Gq-coupled GPCRs, we have now established a cellular mechanism whereby RTK signaling cascades attenuate GPCR signaling through RSK2. These findings provide an initial framework for a conserved regulatory mechanism whereby RTKs act via RSK2 to attenuate GPCR signaling and, given the complexity of cellular signaling, have the potential to explain how these receptors are regulated *in vivo*.

Moreover, because null mutations of RSK2 lead to Coffin−Lowry syndrome which exhibits behaviors characteristic of 5-HT_{2A} dysregulation including a schizophrenia-like psychosis and cognitive impairment (*23*), these findings may explain, in part, some of the clinical manifestations of this neurodevelopmental disease.

EXPERIMENTAL PROCEDURES

Materials. Cell culture reagents including fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin−EDTA, F-12 nutrient mixture, OptiMEM, neurobasal medium, B27 supplement, Hank's buffered salt solution (HBSS), sodium pyruvate, penicillin, and streptomycin were supplied by Gibco (Invitrogen, Carlsbad, CA). Serotonin, 5-methoxy-*N*,*N*-dimethyltryptamine (5-methoxy-DMT), human EGF, human PDGF-AB and PDGF-BB, human TGF-α, IGF-1, papain, probenecid, bovine serum albumin (BSA), low molecular weight poly(L-lysine), sodium tetraborate, L-glutamine, and all other standard reagents were supplied by Sigma-Aldrich Corp. (St. Louis, MO). Boric acid was supplied by EMD Chemicals (Gibbstown, NJ). MDL-100907 and lisuride were acquired as previously detailed (*12*). Collagenase II was obtained from Worthington Biochemical Corp. (Lakewood, NJ), and elastase (grade II) was supplied by Roche Applied Science (Indianapolis, IN). Restriction endonucleases were supplied by New England Biolabs (Ipswich, MA). [^{3}H]Ketanserin was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Protein A/G agarose was supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Serum Dialysis. We extensively dialyzed FBS to remove the 5-HT present in serum. Briefly, 500 mL of FBS was placed into dialysis tubing (Spectra/Por 3500 MWCO; Spectrum Laboratories, Rancho Dominguez, CA) and equilibrated with 4 L of cold dialysis buffer (120 mM NaCl, 10 mM Tris-HCl, pH 7.5 at room temperature) for 24 h at 4 °C with stirring. The buffer was changed five times totaling 120 h of dialysis. The dialyzed FBS was then sterile-filtered (0.22 μm, Millipore), and aliquots were stored at −20 °C until further use. HPLC−electrochemical detection analysis of the dialyzed serum determined that, when used at a concentration of 5%, our dialyzed culture medium contained 33 times less 5-HT than commercially dialyzed FBS (i.e., 0.039 nM vs 1.3 nM 5-HT).

Cell Culture and Transfection. The RSK2+/+ and RSK2 knockout (RSK2−/−) MEFs stably expressing similar levels of 5-HT_{2A} receptors were generated previously by Sheffler et al. (*13*) using MEFs originally isolated from RSK2+/+ and RSK2−/− mice (*24*). Mouse VSMCs and cortical neurons were isolated as detailed below. HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured at 37 °C in a humidified environment in the presence of 5% CO_2. Specifically, HEK293T and mVSMC cell lines were maintained in standard medium (DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin). Polyclonal populations of MEFs stably expressing FLAG-tagged rat 5-HT_{2A} receptors were cultured in standard medium supplemented with 4 μg/mL puromycin to maintain selection pressure. Primary cortical neurons were maintained in complete neurobasal medium (neurobasal medium, 1× B27 supplement, 0.5 mM L-glutamine, 25 μM glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin). Fugene6 (Roche) was used exactly as described by the manufacturer to transiently transfect subconfluent HEK293T cells.

cDNA Constructs. For generation of stable cell lines, the rat 5-HT_{2A} receptor containing a cleavable N-terminal *Haemophilus influenzae* hemagglutinin membrane insertion signal sequence (*25*) and N-terminal FLAG (DYKDDDDK) affinity tag (FLAG-5-HT_{2A}) (*26*) was subcloned into the pBABE retroviral vector containing a puromycin resistance gene (FLAG-5-HT_{2A}-pBABEpuro) (*27*). Briefly, 5′ *Eco*RI and 3′ *Sal*I restriction sites were introduced into the FLAG-5-HT_{2A} sequence via the following PCR primers: 5′AAA**GAATTC**GCCACCATGAAGACGATCAT3′ (*Eco*RI highlighted in bold) and 5′AAA**GTCGAC**TCACACACAGCTAACCTTTTC3′ (*Sal*I highlighted in bold). The FLAG-5-HT_{2A}-pBABEpuro construct was sequence-verified (Case Western Reserve University Genomics Core Facility, Cleveland, OH) and determined via competition radioligand binding assays to bind 5-HT with characteristic affinity (http://pdsp.med.unc.edu/pdsp.php) (*28*).

For infection of primary cortical neurons, the rat 5-HT_{2A} receptor containing the green fluorescent protein (GFP) inserted between amino acids 452 and 453 within the C-terminus (5-HT_{2A}-GFP-CT) (*29*) was subcloned into the FUGW lentiviral construct (5-HT_{2A}-GFP-CT-FUGW) (*30*). Briefly, 5′ *Xba*I and 3′ *Eco*RI restriction sites were introduced into the 5-HT_{2A}-GFP-CT sequence via the following primers: 5′AAAA**TCTAGA**GCCACCATGGAAATTCTTTGTGAAG3′ (*XbaI* highlighted in bold) and 5′TTTT**GAATTC**TCACACACAGCTAACCTTTTCATTC3′ (*Eco*RI highlighted in bold). The resulting 5-HT_{2A}-GFP-CT-FUGW construct was sequence-verified by automated sequencing (UNC-Chapel Hill DNA Sequencing Facility, Chapel Hill, NC).

Microarray Analysis and Pathway Generation. Microarray studies were performed previously by Sheffler et al. (*13*) to compare gene expression profiles in RSK2+/+ and RSK2−/− MEFs. For pathway analysis of RSK2−/− and RSK2+/+ fibroblast gene expression patterns, GenMAPP and MAPPFinder software packages were used as previously detailed (*13*, *31*, *32*).

Isolation of Mouse Aortic Vascular Smooth Muscle Cells. Mouse aortic VSMCs were isolated from 12-week-old mice (three mice per genotype), as previously detailed (*33*). Briefly, mice were sacrificed by cervical dislocation and immediately perfused with 25 mL of 1× HBSS (without Ca^{2+} and Mg^{2+}). Under sterile conditions, the abdominal/thoracic aorta extending from the ilial bifurcation to aortic arch was carefully microdissected and rinsed with HBSS. The pooled aorta were

then incubated with collagenase buffer (175 units/mL in HBSS, filtered through 0.2 μm polyethersulfone membrane) for 15 min at 37 °C in the presence of 5% CO_2. After the adventitial layer was removed, the aorta were incubated with DMEM supplemented with 10% FBS overnight at 37 °C in the presence of 5% CO_2. The next day the aorta were cut into 2 mm segments and incubated with digestion buffer (175 units/mL collagenase and 0.125 mg/mL elastase in HBSS, filtered through 0.2 μm polyethersulfone membrane) for 1 h at 37 °C in the presence of 5% CO_2. Following digestion, the tissue was dissociated with a glass Pasteur pipet, DMEM supplemented with 10% FBS was added, and the cells were collected via centrifugation (200g for 8 min). The cells were resuspended in DMEM supplemented with 20% FBS and transferred to a T-25 cm flask, and the cells were incubated overnight at 37 °C in the presence of 5% CO_2. The next day the cells were carefully washed with DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured normally with medium changes every 3 days. Cells were determined to be SMCs via immunofluorescence assays using the rabbit polyclonal smooth muscle α-actin antibody exactly as described by the manufacturer (1:1000; Abcam Inc., Cambridge, MA). For all experiments, mVSMCs were used between passages three and seven.

Isolation of Primary Cortical Neurons. Cortical neurons were prepared as previously detailed (*34*−*36*). Briefly, pups (postnatal day <2) were genotyped and euthanized by decapitation. The entire frontal cortex of RSK2+/+ and RSK2−/− animals was microdissected from whole brain, followed by digestion in neurobasal medium containing 0.1% papain and 0.2% BSA at 37 °C for 20 min. The medium was then replaced with complete neurobasal medium, and the digested tissue was mechanically dissociated via trituration with a glass Pasteur pipet. The supernatant was transferred to a new sterile 1.5 mL tube, and cells were collected via centrifugation (200g for 5 min). The cell pellet was then resuspended in conditioned complete neurobasal medium. Cells were counted and seeded at a density of 50000 cells/well onto poly(L-lysine)-coated 96-well plates (0.1 mg/mL low molecular mass poly(L-lysine), 0.625% boric acid, 0.955% sodium tetraborate) and cultured normally.

Lentivirus Production and Infection. Lentiviral infection of primary cortical neurons was performed essentially as previously described (*35*). In brief, a predetermined mixture of 5-HT_{2A}-GFP-CT-FUGW and the viral packaging constructs VSVG and Delta 8.9 (ratio = 3.3 FUGW:2.5 Delta 8.9:1 VSVG) were cotransfected into HEK293T cells using Fugene6. Forty-eight hours after transfection the medium containing virus was removed and pooled, and a virus pellet was obtained via centrifugation (26000*g* for 5 h). The virus pellet was resuspended in PBS, concentrated approximately 40-fold using Amicon UltraCel 100K filters (Millipore), and then tested for infection and expression of 5-HT_{2A}-GFP in HEK293T cells. A predetermined amount of concentrated lentivirus was then applied to primary cortical neurons cultured for 7−10 days *in vitro*.

Immunoprecipitation and Western Blotting. Immunoprecipitation of RSK2 and detection of Ser(P)-386 following growth factor treatment were performed as previously described (*37*). Briefly, RSK2+/+ and RSK2−/− cells were treated with EGF (100 ng/mL) or IGF-1 (10nM) for various times and solubilized with cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% tergitol, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, EDTA-free protease inhibitors, 50 mM NaF, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, and 0.1 mM sodium orthovanadate, pH 8.0) for 20 min at 4 °C. Supernatants were collected via centrifugation (15000*g*, 30 min) and equal amounts of protein A/G-cleared lysate were incubated with mouse monoclonal anti-RSK2 (2 μg) for 2 h at 4 °C, followed by incubation with protein A/G agarose for 2 h at 4 °C. Immunopurified complexes were extensively washed with lysis buffer, eluted with 2× SDS sample buffer (125 mM Tris-HCl, 4% sodium dodecyl sulfate, 20% glycerol, 200 mM dithiothreitol, 0.2% Bromphenol Blue, pH 6.8), and stored at −80 °C until further use.

Proteins were immunoblotted using standard procedures (*28*). Specifically, proteins were resolved on 10% SDS−PAGE gels, electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and blocked with standard blocking buffer (Tris-buffered saline, 0.1% Tween-20, and 5% nonfat dehydrated milk) for 1 h at room temperature. Membranes were then incubated with primary antibodies diluted in standard blocking buffer or phospho-specific blocking buffer (5% BSA and TBST). Specifically, RSK2 was detected using the goat polyclonal RSK2 antibody (1:1000; Santa Cruz Biotechnology, Inc.) and the rabbit polyclonal Ser(P)-386 antibody (1:1000; Cell Signaling Technology, Inc., Danvers, MA). The EGFR was detected using the rabbit polyclonal EGFR antibody (1:500; Santa Cruz Biotechnology, Inc.) and the mouse monoclonal Tyr(P)-1068 antibody (1:500; Cell Signaling Technology, Inc.). Phosphorylated IGF-1 R was detected using the rabbit polyclonal Tyr(P)-1158/1162/1163 antibody (1:1000; Upstate Millipore). β-actin was detected in cell lysates using the mouse monoclonal β-actin antibody (1:30000, Sigma). Membranes were washed extensively with Tris-buffered saline + 0.1% Tween-20 (TBST) and subsequently incubated for 1 h at room temperature with secondary horseradish peroxidase-conjugated antibodies raised against mouse, rabbit, and goat IgG (1:1000; Vector Laboratories, Burlingame, CA). Membranes were washed extensively, and proteins were detected using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Inc., Rockford, IL). Immunoreactive bands were imaged and quantified using Kodak Imaging software (Eastman Kodak, New Haven, CT). Sum pixel intensity values were analyzed via the one-tailed, paired *t* test (significance defined as $p < 0.05$) (Graphpad Software, Inc., La Jolla, CA).

Fluorometric Imaging Plate Reader (FLIPR^{tetra}) Analysis of Intracellular Ca^{2+} Release. Intracellular Ca^{2+} release was measured in MEFs and mVSMCs via FLIPR^{tetra} assays using a Ca^{2+} assay kit (Molecular Devices, Sunnyvale, CA) as previously detailed (*13*, *14*). Briefly, MEFs were plated at a density of 25000 cells/well into black-wall, clear-bottom 96-well tissue culture plates (Greiner Bio-One, Monroe, NC), whereas mVSMCs were plated at a density of 10000 cells/well into black-wall, clear-bottom 384-well tissue culture plates (Greiner Bio-One). The cells were cultured in dialyzed medium (DMEM, 5% FBS dialyzed to <0.05 nM 5-HT, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin) and serum-free medium (DMEM, 0.1% BSA, 100 units/mL penicillin, and 100 μg/mL streptomycin) for 24−40 h before the assay for normal and growth factor desensitization experiments, respectively. For both experiments, the cells were incubated with Ca^{2+} assay buffer (20 mM HEPES, 1× HBSS, 2.5 mM probenecid, and Ca^{2+} assay reagent, pH 7.4) for 60 min at 37 °C prior to initiating the FLIPR program. However, for growth factor desensitization experiments the cells were incubated with growth factors diluted in Ca^{2+} assay buffer (for 30 and 60 min time points). After dye loading, the FLIPR^{tetra} was programmed to add agonist approximately 10 s after establishing baseline relative

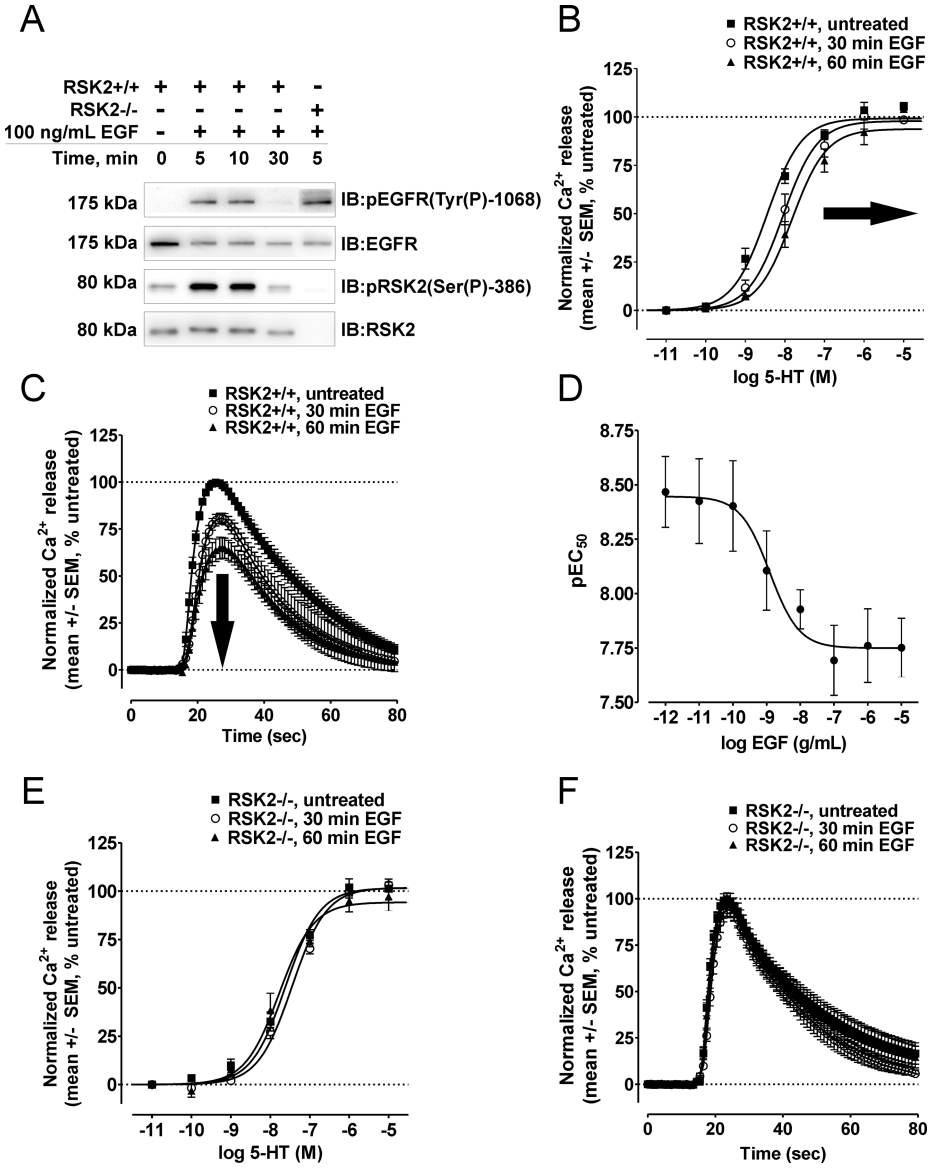

FIGURE 1: RSK2 is required for inhibitory cross-talk between the EGFR and the 5-HT_{2A} receptor. The EGFR and RSK2 were activated with 100 ng/mL EGF (A), and then 5-HT_{2A}-mediated Ca^{2+} responses were measured via $FLIPR^{tetra}$ assays in RSK2+/+ (B−D) and RSK2−/− (E, F) MEFs. (A) Immunoblots showing that EGF treatment (100 ng/mL) activated the EGFR (Tyr(P)-1068, top panels) and RSK2 (Ser(P)-386, bottom panels) in RSK2+/+ MEFs. The EGFR was similarly activated in RSK2−/− MEFs; however, RSK2 was not detected in RSK2−/− MEFs (bottom panel). Shown are representative immunoblots of three independent experiments. (B) In RSK2+/+ MEFs, CRCs for 5-HT were significantly shifted rightward (i.e., decreased 5-HT potency, bold arrow) following 30 min (○) and 60 min (▲) EGF treatments relative to untreated cells (■). Shown are the results (mean ± SEM) of three to ten independent experiments performed in duplicate ($p < 0.05$). (C) In RSK2+/+ MEFs, 5-HT_{2A}-mediated Ca^{2+} responses elicited by an EC_{50} concentration of 5-HT (10 nM) were significantly attenuated following 30 min (○) and 60 min (▲) EGF treatments relative to untreated cells (■). Shown are normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of three to nine independent experiments ($p < 0.05$). (D) In RSK2+/+ MEFs, EGF attenuated 5-HT_{2A}-mediated Ca^{2+} responses with an IC_{50} of 1.3 ng/mL. Shown are the results (mean ± SEM) of four independent experiments performed in duplicate. (E) In RSK2−/− MEFs, CRCs for 5-HT were not significantly shifted following EGF treatments (30 min (○) and 60 min (▲)) relative to untreated cells (■). Shown are the results (mean ± SEM) of six independent experiments performed in duplicate ($p > 0.05$). (F) In RSK2−/− MEFs, 5-HT_{2A}-mediated Ca^{2+} responses elicited by an EC_{50} concentration of 5-HT (10 nM) were not decreased after 30 min (○) and 60 min (▲) EGF treatments relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of three to six independent experiments ($p > 0.05$).

fluorescence unit (RFU) values (excitation 470−495, emission 515−575 nm). RFU values were collected every second for 5 min, and the average baseline values were subtracted from maximum RFU values. Values were expressed relative to the maximal untreated response in each cell line and analyzed by nonlinear regression to generate fit parameters of potency (EC_{50}) and maximal signaling (E_{max}) (Graphpad software). The *F* test was used to determine the statistical significance (defined as $p < 0.05$) of the fit parameters in growth factor-treated vs untreated cells.

Analysis of Intracellular Ca^{2+} Release in Primary Cortical Neurons. Cortical neurons were isolated, cultured, and imaged as described previously (*35*, *36*). In brief, 48 h after lentivirus infection each well was imaged for total GFP fluorescence using the BD Pathway 855 high content imaging microscope equipped with environmental control. Ca^{2+} flux was then determined using the FLIPR Ca^{2+} assay kit (Molecular Probes) as detailed by the manufacturer. In brief, prior to live cell imaging, cells were washed 1× with phosphate-buffered saline followed by 60 min incubation with Ca^{2+} assay buffer (20 mM

Table 1: Effects of Various Growth Factors on the Signaling of GPCR Ligands in MEFs

		RSK2+/+ MEFs at time treated with growth factor						RSK2−/− MEFs at time treated with growth factor					
		0 min		30 min		60 min		0 min		30 min		60 min	
RTK ligand	GPCR ligand	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]
EGF	5-HT	8.45 ± 0.06	99.3 ± 2.1	8.05 ± 0.09[d]	97.9 ± 3.5	7.83 ± 0.09[d]	93.8 ± 3.5	7.61 ± 0.07	102 ± 3.0	7.45 ± 0.05	102 ± 2.0	7.78 ± 0.10	94.3 ± 3.4
EGF	5-methoxy-DMT	7.59 ± 0.10	86.7 ± 3.3	ND[e]	ND	7.17 ± 0.07[d]	79.6 ± 2.2	7.05 ± 0.06	80.4 ± 2.0	ND[e]	ND	7.05 ± 0.07	79.1 ± 2.3
EGF	lisuride	6.57 ± 0.09	54.8 ± 2.1	ND	ND	6.49 ± 0.11	40.5 ± 1.8[d]	5.63 ± 0.14	42.4 ± 2.7	ND	ND	5.90 ± 0.20	37.2 ± 3.4
TGF-α	5-HT	8.65 ± 0.05	99.9 ± 1.8	8.40 ± 0.07[d]	94.4 ± 2.6	8.23 ± 0.04[d]	91.8 ± 1.6[d]	8.07 ± 0.06	100 ± 2.0	8.08 ± 0.05	99.2 ± 1.8	8.01 ± 0.05	100 ± 1.8
IGF-1	5-HT	8.53 ± 0.09	100 ± 3	8.46 ± 0.09	96.8 ± 3.4	8.54 ± 0.07	93.7 ± 2.4	8.26 ± 0.05	100 ± 2	8.54 ± 0.08[d]	93.4 ± 2.2[d]	8.53 ± 0.08[d]	91.7 ± 2.2[d]
EGF	ATP	5.16 ± 0.05	99.7 ± 2.0	4.95 ± 0.06[d]	88.4 ± 2.5[d]	4.88 ± 0.09[d]	82.5 ± 3.2[d]	5.37 ± 0.09	98.6 ± 3.3	5.34 ± 0.09	93.4 ± 3.2	5.30 ± 0.09	100 ± 3.5

[a]Agonist-induced Ca^{2+} responses were quantified via FLIPR assays in untreated and growth factor-treated RSK2+/+ and RSK2−/− MEFs. The fit parameters of potency (EC_{50}) and maximal signaling (E_{max}) were obtained from nonlinear regression (GraphPad software) and represent the mean ± SEM of at least three independent experiments performed in duplicate. [b]pEC_{50} values are represented as −log of EC_{50} in M. [c]The maximum response of agonist (E_{max}) in untreated cells was set equal to 100%. [d]The *F* test was used to determine the statistical significance (defined as $p < 0.05$) of the fit parameter compared to untreated cells. [e]ND, not determined.

HEPES, 1× HBSS, 2.5 mM probenecid, 0.57 mM ascorbic acid, and Ca^{2+} assay reagent, pH 7.4). Assays using growth factors were performed similarly except that growth factor was added during the dye loading step. Cells were maintained at 37 °C during the entire period of observation and were imaged for 20 s prior to drug addition to obtain baseline dye fluorescence. The liquid handling capability of the BD Pathway 855 was used to add 10× drug, and then fluorescence images were obtained for 120 s. To control for subtle differences in receptor expression, Ca^{2+} responses were normalized to GFP intensity/well using custom written macros for Excel (Microsoft, Redmond, WA) and Image J (National Institutes of Health, Bethesda, Maryland). Values were expressed as fold over baseline and a two-tailed, paired *t* test was used to determine the statistical significance (defined as $p < 0.05$) of responses in growth factor-treated vs untreated cells.

RESULTS

RSK2 Is Required for EGF-Induced Attenuation of 5-HT$_{2A}$ Receptor Signaling. We recently determined that RSK2 interacts with the 5-HT_{2A} serotonin receptor and attenuates signaling via direct receptor phosphorylation (*13*, *14*). Moreover, preliminary data suggested that EGFR activation attenuates 5-HT_{2A} receptor signaling, presumably by activating RSK2. Considering the potential for describing how 5-HT_{2A} receptors are regulated in cells, and perhaps *in vivo*, we applied pharmacological and genetic approaches to determine if various RTKs, including members of the EGFR family, require RSK2 for attenuating 5-HT_{2A} receptor signaling.

First, we activated the EGFR (also known as ErbB1) in RSK2+/+ and RSK2−/− MEFs with two canonical agonists (i.e., EGF and TGF-α) and monitored 5-HT_{2A} receptor responsiveness using 5-HT_{2A} agonists of varying intrinsic efficacies. Consistent with our initial EGFR findings (*14*), EGF significantly attenuated 5-HT_{2A} receptor signaling in RSK2+/+ MEFs. As shown in Figure 1A, 100 ng/mL EGF maximally activated the EGFR and RSK2 in these cells. When receptor signaling was assayed, we found that EGF pretreatment resulted in significant rightward shifts in 5-HT concentration−response curves (CRCs) as early as 30 min, with maximal effects reached within 60 min (Figure 1B, Table 1). According to classic concepts of receptor pharmacology, these rightward shifts in 5-HT CRCs denoted decreases in agonist potency, most likely resulting from attenuation of receptor signaling given the short time scale of the experiment (*38*). To best illustrate this decrease in 5-HT potency, we compared the 5-HT_{2A}-mediated Ca^{2+} responses elicited by a submaximal, EC_{50} concentration of 5-HT (i.e., 10 nM). As shown in Figure 1C, 10 nM 5-HT elicited significantly lower Ca^{2+} responses after treating with EGF for 30 min (peak Ca^{2+} release = 99.7 ± 0.1% vs 79.7 ± 3.1% in untreated and EGF-treated RSK2+/+ MEFs, respectively; $N = 3-9$, $p < 0.05$) and 60 min (peak Ca^{2+} release = 99.7 ± 0.08% vs 63.7 ± 6.0% in untreated and EGF-treated RSK2+/+ MEFs, respectively; $N = 7-9$, $p < 0.05$). Although it was clear that EGF decreased 5-HT signaling, these results could be explained by nonspecific effects associated with a single, supramaximal concentration of EGF. To address this concern, we determined that EGF attenuated 5-HT_{2A} receptor signaling with an IC_{50} of 1.3 ng/mL (Figure 1D), a value that is within the concentration range typically observed for EGFR-mediated signaling events (*39*).

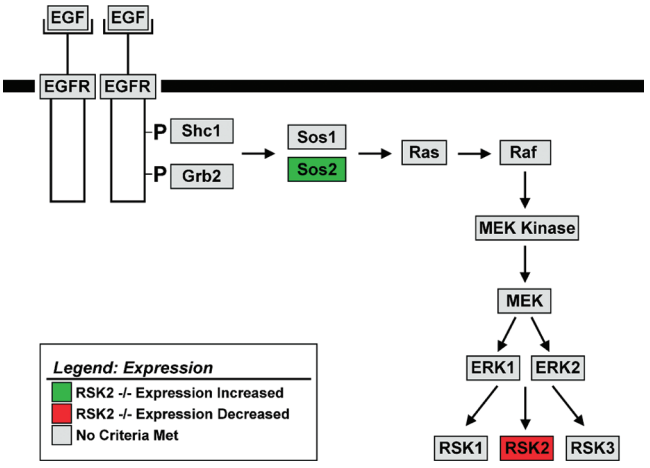

FIGURE 2: Genes involved in EGFR signal transduction are expressed similarly in RSK2+/+ and RSK2−/− MEFs. The microarray data quantifying gene expression in RSK2+/+ and RSK2−/− MEFs were published previously by Sheffler et al. (*13*). Here we overlaid the mRNA expression levels of EGFR signal transduction genes in RSK2+/+ and RSK2−/− MEFs with gene expression color criterion and fold changes from the programs GenMAPP and MAPPFinder. Gray colored genes are equally expressed in RSK2+/+ and RSK2−/− MEFs. Green colored genes show greater than a 2-fold increase in expression in RSK2−/− MEFs compared to RSK2+/+ fibroblasts. Red colored genes show greater than a 2-fold decrease in expression in RSK2−/− MEFs compared to RSK2+/+ fibroblasts.

In contrast to these results in RSK2+/+ MEFs, EGF cannot attenuate 5-HT_{2A} receptor signaling in RSK2−/− MEFs. Specifically, EGF did not significantly alter 5-HT potency in RSK2−/− MEFs as evidenced by superimposed 5-HT CRCs (Figure 1E and Table 1). Moreover, 5-HT_{2A}-mediated Ca^{2+} responses elicited by an EC_{50} concentration of 5-HT were not significantly decreased after treating with EGF for 30 min (peak Ca^{2+} release = 99.7 ± 0.2% vs 93.9 ± 4.2% in untreated and EGF-treated RSK2−/− MEFs, respectively; $N = 3-6$, $p > 0.05$) and 60 min (peak Ca^{2+} release = 99.7 ± 0.2% vs 96.8 ± 6.3% in untreated and EGF-treated RSK2−/− MEFs, respectively; $N = 6$, $p > 0.05$) (Figure 1F).

It was conceivable that differences in EGFR signal transduction between RSK2+/+ and RSK2−/− MEFs could account for the differential effects of EGF. To evaluate this possibility, we compared the mRNA expression profiles of genes constituting the EGFR signal transduction pathway in RSK2+/+ and RSK2−/− MEFs. As shown in Figure 2, analysis of microarray studies revealed no substantial differences in gene expression profiles between RSK2+/+ and RSK2−/− MEFs that could account for lack of attenuation in RSK2−/− MEFs. Only two differences were apparent in RSK2−/− cells: (1) a decrease in RSK2 mRNA (as predicted in knockout cells) and (2) an increase in Sos2 mRNA. Additionally, at the protein level we found that EGFR activation was similar between RSK2+/+ and RSK2−/− MEFs after 5 min of EGF treatment (472 ± 131% vs 491 ± 96% for EGFR phosphorylation in RSK2+/+ and RSK2−/− MEFs, respectively; $N = 3$, $p > 0.05$) (Figure 1A). Taken together, these findings indicate (1) that RSK2 is a critical mediator of inhibitory cross-talk between EGF and 5-HT_{2A} receptors in MEFs and (2) that the effects are not due to compensatory changes in expression of EGFR signaling partners.

We further confirmed that RSK2 was required for inhibitory cross-talk by activating the EGFR in RSK2+/+ and RSK2−/− MEFs with another selective and potent EGFR agonist, TGF-α. Similar to our results with EGF, TGF-α attenuated 5-HT_{2A} receptor signaling in RSK2+/+ MEFs. Specifically, 1 h treatment with TGF-α decreased 5-HT potency, as illustrated by significant rightward shifts in 5-HT CRCs (Figure 3A and Table 1) and significant decreases in 5-HT_{2A}-mediated Ca^{2+} release elicited by an EC_{50} concentration of 5-HT (peak Ca^{2+} release = 99.7 ± 0.2% vs 76.9 ± 2.2% in untreated and TGF-α-treated RSK2+/+ MEFs, respectively; $N = 5$, $p < 0.05$) (Figure 3B). Moreover, TGF-α attenuated 5-HT_{2A} receptor signaling in RSK2+/+ MEFs with an IC_{50} of 4.1 ng/mL (Figure 3C), again consistent with EGFR-mediated signaling events. In agreement with our previous experiments using EGF, TGF-α treatment did not attenuate 5-HT_{2A} receptor signaling in RSK2−/− MEFs (Figure 3D and Table 1). Moreover, we did not detect large decreases in 5-HT_{2A}-mediated Ca^{2+} release elicited by an EC_{50} concentration of 5-HT (peak Ca^{2+} release = 99.8 ± 0.2% vs 91.7 ± 1.2% in untreated and TGF-α-treated RSK2−/− MEFs, respectively; $p < 0.05$) (Figure 3E).

In addition to full agonists, partial agonists display characteristic and predictable signaling behaviors under conditions of decreased receptor responsiveness. Notably, in this context, full agonists commonly exhibit effects on potency but lesser effects on maximal signaling, while partial agonists commonly display decreases in maximal signaling (i.e., large downward shifts in CRCs) (*38*). Thus, we predicted that EGF treatment would decrease the maximal signaling of the weak partial agonist lisuride with minimal effects on potency, whereas the potency of the strong partial agonist 5-methoxy-DMT would be significantly decreased. Consistent with these predictions, EGF treatment significantly decreased the maximal signaling of lisuride in RSK2+/+ MEFs (Figure 4A, Table 1). Furthermore, 5-methoxy-DMT displayed behaviors intermediate between the full agonist 5-HT and the weak partial agonist lisuride, exhibiting a minor decrease in maximal signaling and a significant decrease in potency (Figure 4C, Table 1). In agreement with a requirement for RSK2, we did not observe significant shifts in the CRCs of either partial agonist in RSK2−/− MEFs (Figure 4B,D, Table 1). Taken together, these pharmacological and genetic approaches strongly support the hypothesis that EGFRs act via RSK2 to attenuate 5-HT_{2A} receptor signaling in MEFs.

RSK2 Is Required for PDGFR-Mediated Attenuation of Endogenous 5-HT_{2A} Receptor Signaling in Primary mVSMCs. The growth factor PDGF is a potent mitogen, chemoattractant, and survival factor that activates RSKs downstream of PDGFR activation in VSMCs (*40*, *41*). VSMCs also endogenously express 5-HT_{2A} receptors, which produce measurable Ca^{2+} responses in FLIPR assays (Figure 5A) (*42*). Therefore, VSMCs isolated from RSK2+/+ and RSK2−/− mice represented an intact model system whereby we could test (1) whether inhibitory cross-talk occurs between additional RTKs and endogenously expressed 5-HT_{2A} receptors and (2) to what extent this requires RSK2.

In these studies we activated PDGFRs with PDGF-AB and PDGF-BB, the principal PDGF ligands in serum (*43*). As evidenced by significant downward shifts in 5-HT CRCs following 60 min treatments with PDGF-AB (Figure 5B) and PDGF-BB (Figure 5D), activation of the PDGFR resulted in attenuation of 5-HT_{2A} receptor signaling (Table 2). To best illustrate this, we showed that PDGF-BB treatment significantly decreased 5-HT_{2A}-mediated Ca^{2+} responses in RSK2+/+ mVSMCs elicited by a saturating concentration of 5-HT (i.e., 10 μM) (peak Ca^{2+} release = 90.2 ± 0.7% vs 67.0 ± 8.5% in untreated and

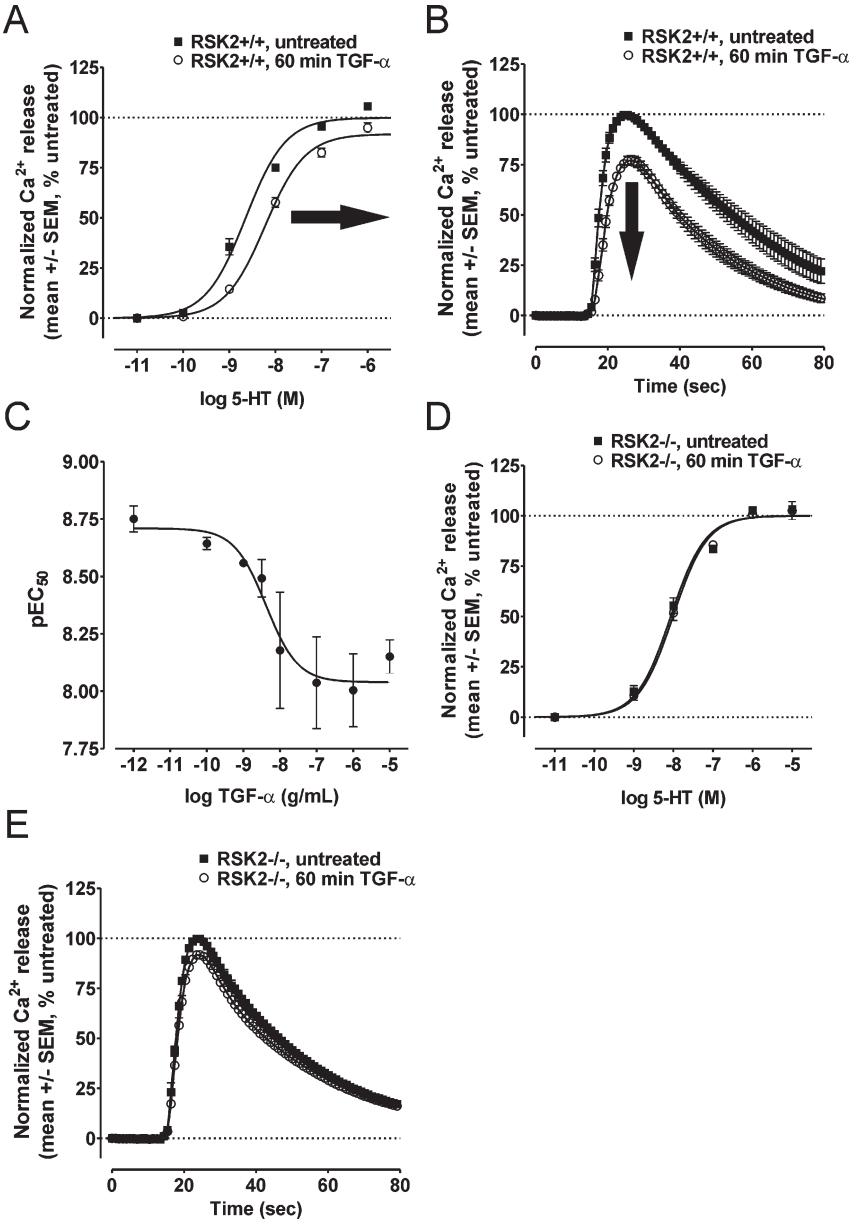

FIGURE 3: RSK2 is required for TGF-α-induced attenuation of 5-HT_{2A} receptor signaling. The EGFR was activated with 100 ng/mL TGF-α, and then 5-HT_{2A}-mediated Ca^{2+} responses were measured via $FLIPR^{tetra}$ assays in RSK2+/+ (A−C) and RSK2−/− (D, E) MEFs. (A) In RSK2+/+ MEFs, the CRC for 5-HT was significantly shifted rightward (i.e., decreased potency, bold arrow) and downward (i.e., decreased maximal signaling, bold arrow) following 60 min (○) TGF-α treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of five independent experiments performed in duplicate ($p < 0.05$). (B) In RSK2+/+ MEFs, activation of 5-HT_{2A} receptors with an EC_{50} concentration of 5-HT (10 nM) was significantly attenuated following 60 min (○) TGF-α treatment relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of five independent experiments ($p < 0.05$). (C) In RSK2+/+ MEFs, TGF-α attenuated 5-HT_{2A}-mediated Ca^{2+} responses with an IC_{50} of 4.1 ng/mL. Shown are the results (mean ± SEM) of three independent experiments performed in duplicate. (D) In RSK2−/− MEFs, the CRC for 5-HT was not significantly shifted following 60 min (○) TGF-α treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p > 0.05$). (E) In RSK2−/− MEFs, activation of 5-HT_{2A} receptors with an EC_{50} concentration of 5-HT (10 nM) was not significantly decreased following 60 min (○) TGF-α treatment relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of three independent experiments ($p > 0.05$).

PDGF-BB-treated RSK2+/+ mVSMCs, respectively; $N = 4$, $p < 0.05$) (Figure 5E). As expected, PDGF treatments did not significantly reduce the maximal signaling of 5-HT in RSK2−/− mVSMCs (Figure 5C,F). Moreover, PDGF-BB treatment failed to significantly decrease 5-HT_{2A}-mediated Ca^{2+} responses in RSK2−/− mVSMCs elicited by a saturating concentration of 5-HT (peak Ca^{2+} release = 90.3 ± 3.8% vs 77.9 ± 7.6% in untreated and PDGF-BB-treated RSK2−/− mVSMCs, respectively; $N = 4$, $p > 0.05$) (Figure 5G). Together with our results using two different EGFR agonists, these results strongly suggest that RSK2 is required for inhibitory cross-talk between multiple growth factor signaling pathways and the 5-HT_{2A} receptor.

IGF-1 Weakly Activates RSK2 in MEFs and Does Not Attenuate 5-HT_{2A} Receptor Signaling. We have demonstrated using various RTK agonists (i.e., EGF, TGF-α, and PDGF), cell lines (MEFs and VSMCs), and GPCR ligands (i.e., 5-HT, 5-methoxy-DMT, and lisuride) that RTKs require RSK2 to attenuate 5-HT_{2A} receptor signaling. However, it was unknown whether insulin or IGF-1, which have been shown to attenuate the signaling of GPCRs including the closely related

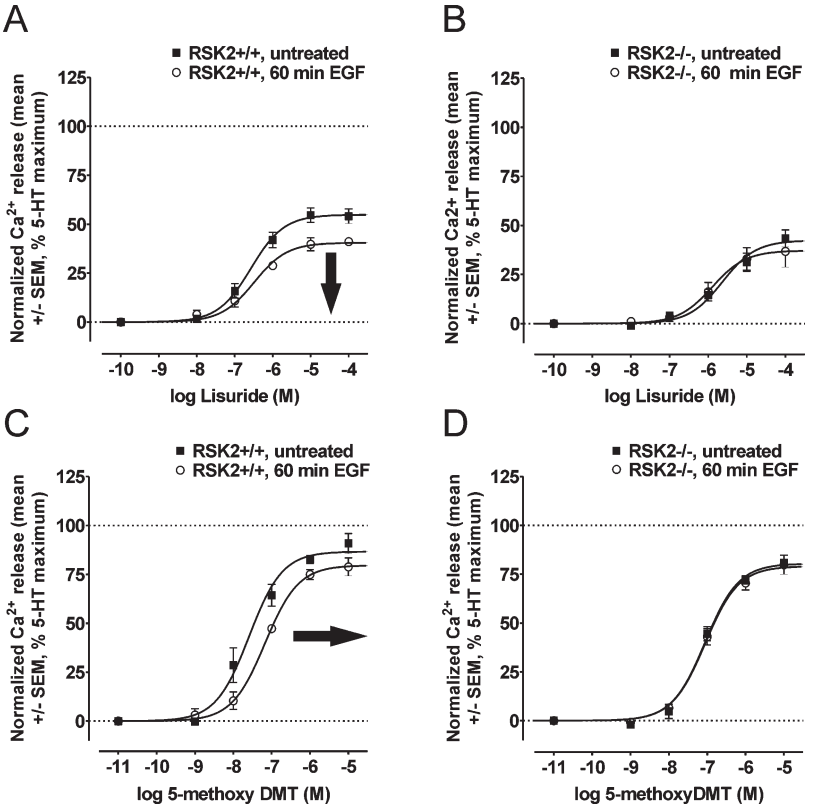

FIGURE 4: The unique pharmacology of partial agonists shows that RSK2 is required for EGFR-mediated attenuation of 5-HT_{2A} receptor signaling. The EGFR was activated with 100 ng/mL EGF, and then 5-HT_{2A}-mediated Ca^{2+} responses elicited by the weak partial agonist lisuride (A, B) and the strong partial agonist 5-methoxy-DMT (C, D) were measured via $FLIPR^{tetra}$ assays in RSK2+/+ and RSK2−/− MEFs. (A) In RSK2+/+ MEFs, the CRC for lisuride was significantly shifted downward (i.e., decreased maximal signaling, bold arrow) following 60 min (○) EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p < 0.05$). (B) In RSK2−/− MEFs, the CRC for lisuride was not significantly decreased following EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p > 0.05$). (C) In RSK2+/+ MEFs, the CRC for 5-methoxy-DMT was significantly shifted rightward (i.e., decreased potency, bold arrow) following 60 min (○) EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p < 0.05$). (D) In RSK2−/− MEFs, the CRC for 5-methoxy-DMT was not significantly shifted following EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p > 0.05$).

5-HT_{2C} receptor (*22*), also attenuate 5-HT_{2A} receptor signaling.

In initial experiments testing insulin, we determined that insulin showed no effect on 5-HT_{2A} receptor signaling, despite modest activation of RSK2 (data not shown). However, upon closer examination we discovered that insulin receptors are not expressed at detectable levels in RSK2+/+ and RSK2−/− MEFs (Supporting Information Figure S1), suggesting that RSK2 activation is mediated via the IGF-1 R which has low affinity for insulin (*44*). Since both RSK2+/+ and RSK2−/− MEFs express equal amounts of IGF-1 R (Supporting Information Figure S1), we next determined if IGF-1 could attenuate 5-HT_{2A} receptor signaling. As shown in Figure 6A,B, IGF-1 treatment did not result in large shifts in 5-HT CRCs in RSK2+/+ or RSK2−/− MEFs (Table 1), identical to our results with insulin. Importantly, these results could not be explained by a general deficiency in RTK signaling since a 1 h treatment with EGF attenuated 5-HT_{2A} signaling in parallel control experiments (peak Ca^{2+} release = 99.9 ± 0.1% vs 80.7 ± 2.3% in untreated and EGF-treated RSK2+/+ MEFs, respectively; $N = 3$, $p < 0.05$) (Figure 6C). Additional experimentation showed that the IGF-1R is activated by IGF-1 (Supporting Information Figure S2). Together, these findings suggested that the mechanism(s) underlying inhibitory cross-talk between an RTK and a GPCR (e.g., RSK2 activation) engender(s) some level of specificity. One such possibility was that IGF-1 treatment only modestly activated RSK2 (*24*). Indeed, as shown in Figure 6D and quantified in Figure 6E, maximal activation of RSK2 by IGF-1 was significantly less when compared to EGF ($0.546 \times 10^6 \pm 0.107 \times 10^6$ vs $1.25 \times 10^6 \pm 0.25 \times 10^6$ for IGF-1 and EGF, respectively; $N = 3$, $p < 0.05$). Thus, robust activation of RSK2 by RTKs seems to be required for inhibitory cross-talk with the 5-HT_{2A} receptor. Regardless of the mechanism, it is likely that some degree of specificity exists given the potential physiological importance of RTK-GPCR cross-talk.

RSK2 Is Required for EGF-Mediated Attenuation of Endogenous P2Y Purinergic Receptor Signaling. RSK2 attenuates the signaling of additional GPCRs endogenously expressed in MEFs, including P2Y purinergic receptors (*13*). Therefore, we hypothesized that EGFR activation, in addition to regulating 5-HT_{2A} receptors, could also attenuate P2Y receptor signaling in a RSK2-dependent manner. By testing this hypothesis, we could begin to address whether this novel regulatory mechanism is conserved across multiple GPCRs that show sensitivity to RSK2 regulation.

As shown in Figure 7A, EGF treatment significantly reduced ATP signaling in RSK2+/+ MEFs (Table 1). Specifically, we observed significant decreases in ATP maximal signaling (E_{max} = 99.7 ± 2.0% vs 82.5 ± 3.2% in untreated and EGF-treated RSK2+/+ MEFs, respectively; $N = 5$, $p < 0.05$) and potency

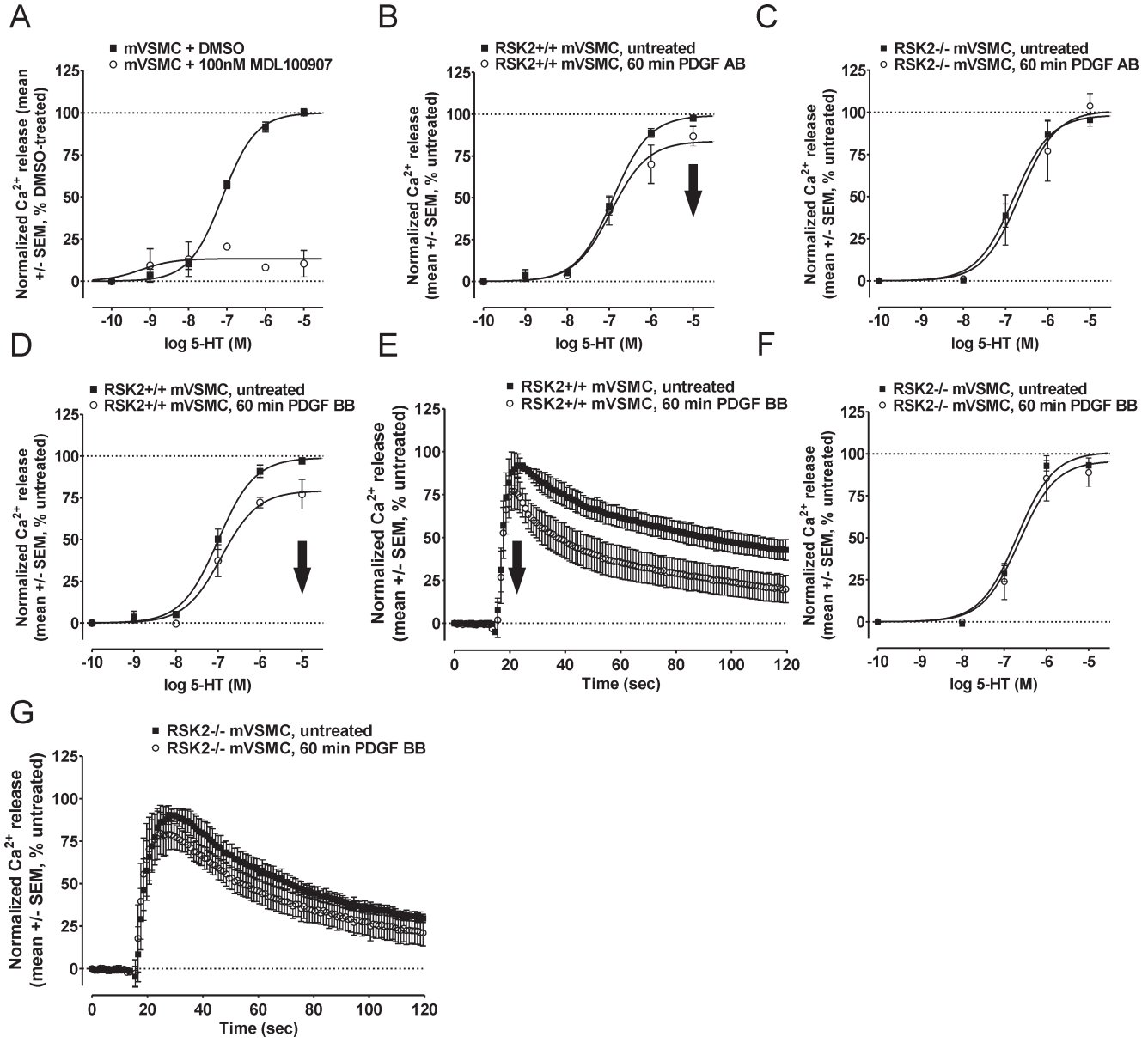

FIGURE 5: RSK2 is required for inhibitory cross-talk between the PDGFR and the 5-HT_{2A} receptor. 5-HT_{2A}-mediated Ca^{2+} responses were measured via $FLIPR^{tetra}$ assays (A) in RSK2+/+ and RSK2−/− mVSMCs following treatment with the PDGF ligands PDGF AB (B, C) and PDGF BB (D−G). (A) 5-HT elicits robust Ca^{2+} responses in RSK2+/+ mVSMCs which can be blocked with the 5-HT_{2A}-specific antagonist MDL100907 (100 nM). Shown are the results (mean ± SEM) of two independent experiments performed in duplicate. (B) In RSK2+/+ mVSMCs, the CRC for 5-HT was significantly shifted downward (i.e., decreased maximal signaling, bold arrow) following 60 min (○) PDGF AB treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of four to six independent experiments performed in duplicate ($p < 0.05$). (C) In RSK2−/− mVSMCs, the CRC for 5-HT was not significantly shifted following PDGF AB treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of four independent experiments performed in duplicate ($p > 0.05$). (D) In RSK2+/+ mVSMCs, the CRC for 5-HT was significantly shifted downward (i.e., decreased maximal signaling, bold arrow) following 60 min (○) PDGF BB treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of four to six independent experiments performed in duplicate ($p < 0.05$). (E) In RSK2+/+ mVSMCs, 5-HT_{2A}-mediated Ca^{2+} responses elicited by a maximal concentration of 5-HT (10 μM) were significantly attenuated following 60 min (○) PDGF BB treatment relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of four independent experiments ($p < 0.05$). (F) In RSK2−/− mVSMCs, the CRC for 5-HT was not significantly shifted following PDGF BB treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of four independent experiments performed in duplicate ($p > 0.05$). (G) In RSK2−/− mVSMCs, 5-HT_{2A}-mediated Ca^{2+} responses elicited by a maximal concentration of 5-HT (10 μM) were not significantly decreased following PDGF BB treatment relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of four independent experiments ($p > 0.05$).

(7.0 vs 13 μM for untreated and hEGF-treated, respectively; $N = 5$, $p < 0.05$) following 60 min EGF treatment. Similar to our observations in mVSMCs (Figure 5), decreased maximal signaling of the full agonist ATP was consistent with desensitization of endogenously expressed P2Y receptors. However, treating RSK2−/− MEFs with EGF failed to significantly decrease ATP maximal signaling or potency (Figure 7B, Table 1). These data are important because they provide the first evidence for a common regulatory mechanism whereby RTKs act via RSK2 to regulate the signaling of multiple GPCRs.

Growth Factors Essential for Normal Brain Function Attenuate 5-HT_{2A} Receptor Signaling in Cortical Neurons. We have presented multiple lines of evidence to show that 5-HT_{2A} signaling is indeed attenuated following activation of several

Table 2: Effects of PDGFR Agonists on 5-HT_{2A} Receptor Signaling in mVSMCs

		RSK2+/+ mVSMCs at time treated with growth factor						RSK2−/− mVSMCs at time treated with growth factor					
		0 min		30 min		60 min		0 min		30 min		60 min	
RTK ligand	GPCR ligand	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]	pEC_{50} ± SEM[a,b]	E_{max} ± SEM, %[c]
PDGF AB	5-HT	6.91 ± 0.05	99.4 ± 2.2	6.80 ± 0.13	90.5 ± 5.4	6.95 ± 0.15	83.9 ± 5.6[d]	6.80 ± 0.10	98.3 ± 4.4	6.62 ± 0.14	96.9 ± 6.4	6.66 ± 0.22	101 ± 10.8
PDGF BB	5-HT	7.00 ± 0.06	99.0 ± 2.7	6.88 ± 0.17	94.5 ± 7.3	6.92 ± 0.14	79.2 ± 5.1[d]	6.70 ± 0.08	101 ± 3.5	6.57 ± 0.14	108 ± 7.8	6.64 ± 0.19	95.8 ± 7.7

[a]Agonist-induced Ca^{2+} responses were quantified via FLIPR assays in untreated and growth factor-treated RSK2+/+ and RSK2−/− mVSMCs. The fit parameters of potency (EC_{50}) and maximal signaling (E_{max}) were obtained from nonlinear regression (GraphPad software) and represent the mean ± SEM of at least three independent experiments performed in duplicate. [b]pEC_{50} values are represented as −log of EC_{50} in M. [c]The maximum response of agonist (E_{max}) in untreated cells was set equal to 100%. [d]The *F* test was used to determine the statistical significance (defined as $p < 0.05$) of the fit parameters in growth factor-treated and untreated cells.

endogenous RTKs in multiple cell types. In addition to expression in peripheral tissues, RTKs are widely expressed throughout the brain (e.g., in the cortex) and are activated by endogenous ligands such as EGF and NRG-1 (*45*). Since 5-HT_{2A} receptors are also highly expressed in the cortex (*46*), it was tempting to speculate that cross-talk between RTKs and 5-HT_{2A} receptors could explain how 5-HT_{2A} receptors are regulated in cortical neurons.

To test this possibility, we developed a live cell imaging technique to measure 5-HT_{2A} receptor signaling in cortical neurons in the presence and absence of growth factors. As shown in Figure 8A,B, uninfected neurons were unresponsive to the 5-$HT_{2A/2C}$ selective agonist DOI, despite robust Ca^{2+} responses following depolarization with 80 mM KCl (Figure 8C). However, DOI elicited measurable Ca^{2+} responses in neurons only after infection with GFP-tagged 5-HT_{2A} receptors (Figure 8D,E), thus ensuring specificity of the DOI response. We then quantified these DOI-induced responses in untreated (Figure 8E) and growth factor-treated (Figure 8H) neurons via manual segmenting (Figure 8F,I). As shown in Figure 8J and quantified in Table 3, treatment with either EGF or NRG-1 significantly reduced the Ca^{2+} response elicited by DOI. In these studies we present the first evidence that inhibitory cross-talk occurs between RTKs and GPCRs in neurons. Most importantly, our data show that RTKs attenuate 5-HT_{2A} signaling in neurons, a finding with enormous potential for explaining how 5-HT_{2A} receptors are regulated in the brain.

DISCUSSION

The three major findings in this paper are (1) multiple endogenous RTK receptors and their ligands attenuate 5-HT_{2A} receptor responsiveness in several physiologically relevant cell types, (2) RSK2 is required for RTK-mediated attenuation of 5-HT_{2A} receptor signaling, and (3) RTK activation similarly attenuates P2Y purinergic signaling in a RSK2-dependent manner. By directly testing multiple endogenous growth factors/RTK pathways and multiple Gq-coupled GPCRs, we have now established a cellular mechanism whereby RTK signaling cascades attenuate GPCR signaling through RSK2. Importantly, these findings support a novel paradigm of inhibitory cross-talk between RTKs and GPCRs and extend it to include a larger mechanism whereby RTKs act via RSK2 to regulate the signaling of multiple GPCRs.

RSK2 Is Required for Inhibitory Cross-Talk between RTKs and the 5-HT_{2A} Receptor in a Variety of Cell Types. Consistent with evidence for inhibitory cross-talk between RTKs and select GPCRs (i.e., the β_1-, β_2-, α_{1B}-, and α_{1D}-adrenergic receptors and 5-HT_{2C} receptor) (*16*−*22*), our data demonstrate that activation of the EGFR attenuates 5-HT_{2A} receptor signaling in MEFs, VSMCs, and cortical pyramidal neurons. Moreover, we discovered that this novel regulatory pathway requires RSK2. We verified that the EGFR requires RSK2 to attenuate 5-HT_{2A} receptor signaling by observing the signaling of both full and partial 5-HT_{2A} agonists in RSK2+/+ and RSK2−/− cells. Since changes to GPCR responsiveness affect each agonist class differently, this approach allowed us to unambiguously identify RTK-mediated effects on receptor signaling. Explicitly, full agonists have a large receptor reserve and are resistant to changes in the population of functional receptors (i.e., resulting from receptor desensitization or downregulation). As a result, full agonists signal maximally but with lower potency under conditions of receptor desensitization in cells overexpressing a GPCR

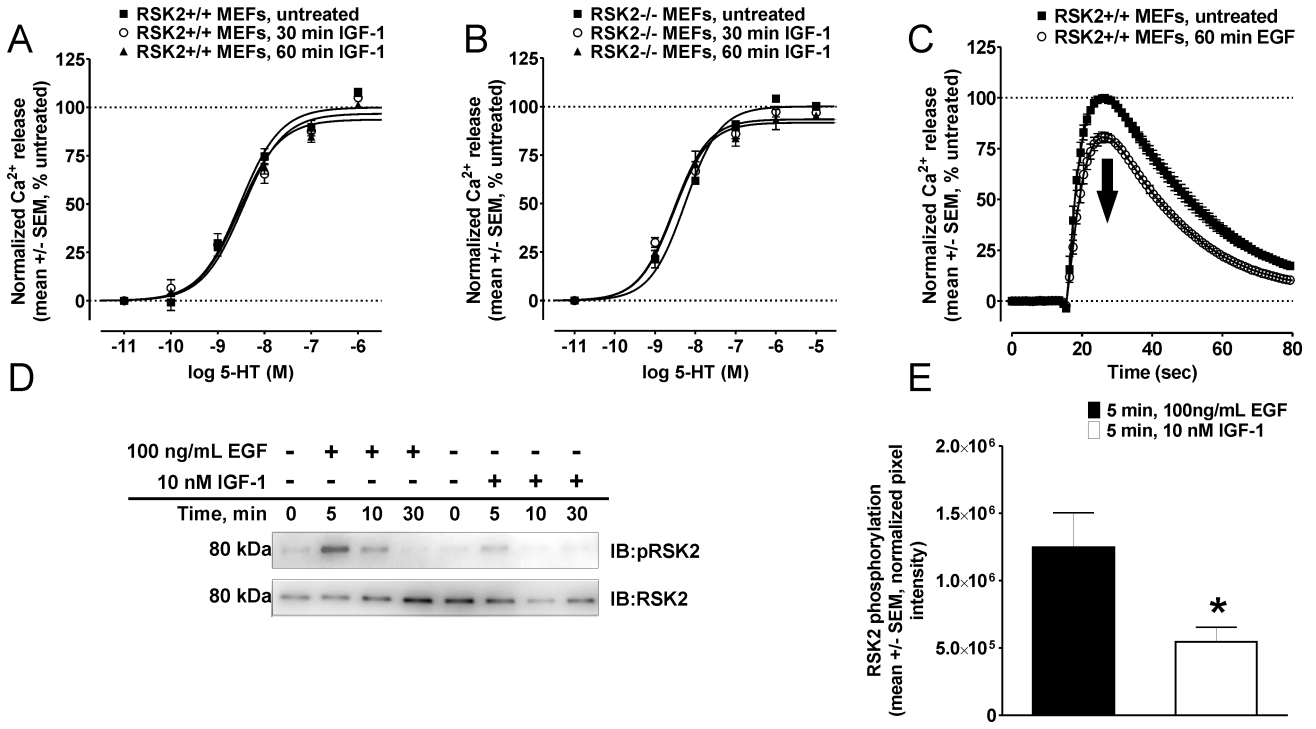

FIGURE 6: IGF-1 weakly activates RSK2 and does not attenuate 5-HT_{2A} receptor signaling. 5-HT_{2A}-mediated Ca^{2+} responses were measured via $FLIPR^{tetra}$ assays in RSK2+/+ and RSK2−/− MEFs following treatment with 10 nM IGF-1 (A, B) or 100 ng/mL EGF (C). For Western blotting, a phospho-specific antibody was used to detect activation of RSK2 (D, E). (A, B) In RSK2+/+ and RSK2−/− MEFs, CRCs for 5-HT were not significantly shifted following 30 min (○) and 60 min (▲) IGF-1 treatments relative to untreated cells (■). Shown are the results (mean ± SEM) of three independent experiments performed in duplicate ($p > 0.05$). (C) Control experiments in RSK2+/+ MEFs showed that 5-HT_{2A}-mediated Ca^{2+} responses elicited by an EC_{50} concentration of 5-HT (10 nM) were significantly attenuated following 60 min (○) 100 ng/mL EGF treatment relative to untreated cells (■). Shown are the normalized Ca^{2+} traces (untreated set to 100%, mean ± SEM) of three independent experiments performed in duplicate ($p < 0.05$). (D) Immunoblot showing that EGF robustly activated RSK2 (Ser(P)-386) in RSK2+/+ MEFs, whereas IGF-1 weakly activated RSK2 in RSK2+/+ MEFs. Shown are representative data from three independent experiments. (E) Quantification of immunoblots in (D) showing that maximal activation of RSK2 at 5 min was significantly greater after EGF treatment than after IGF-1 treatment (*, $p < 0.05$). Shown are the results (sum pixel intensity normalized to total RSK2, mean ± SEM) of three independent experiments.

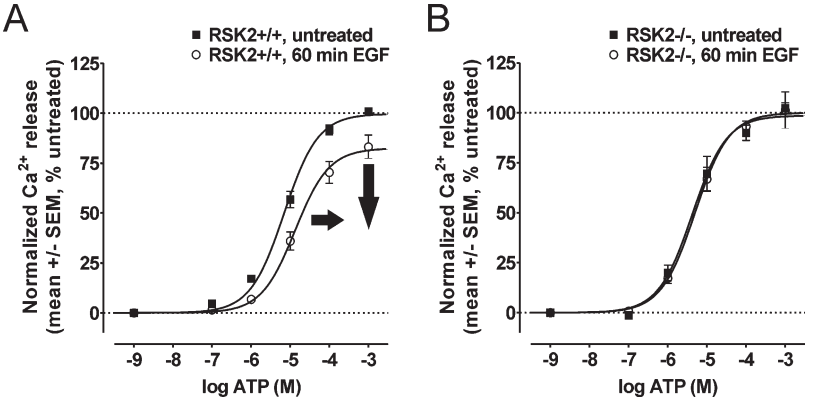

FIGURE 7: RSK2 is required for inhibitory cross-talk between the EGFR and the P2Y purinergic receptor. The EGFR was activated with 100 ng/mL EGF, and then P2Y-mediated Ca^{2+} responses were measured via $FLIPR^{tetra}$ assays in RSK2+/+ (A) and RSK2−/− (B) MEFs. (A) In RSK2+/+ MEFs, the CRC for ATP was significantly shifted rightward (i.e., decreased ATP potency, bold arrow) and downward (ie., decreased maximal signaling, bold arrow) following 60 min (○) EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of five independent experiments performed in duplicate ($p < 0.05$). (B) In RSK2−/− MEFs, the CRC for ATP was not significantly shifted following EGF treatment relative to untreated cells (■). Shown are the results (mean ± SEM) of five independent experiments performed in duplicate ($p > 0.05$).

(i.e., CRCs are right-shifted) (*38*). However, both the maximal signaling and potency of full agonists are decreased under conditions of receptor desensitization in cells with endogenous GPCR expression (i.e., CRCs are predominantly shifted downward with minor rightward shifts). Partial agonists, on the other hand, have low receptor reserve and are more sensitive to changes in the population of functional GPCRs. As a result, partial agonists typically signal with lower efficacy and potency under conditions of receptor desensitization irrespective of receptor expression (*38*). In line with these predictions, we observed that EGF significantly decreased full agonist (i.e., 5-HT) potency and partial agonist (i.e., lisuride) efficacy in high-expressing RSK2+/+ MEFs. Additionally, growth factor treatment significantly decreased the maximal signaling of full agonists (i.e., 5-HT and ATP) when their cognate receptors were expressed at endogenous levels in RSK2+/+ mVSMCs and MEFs. Taken together, our

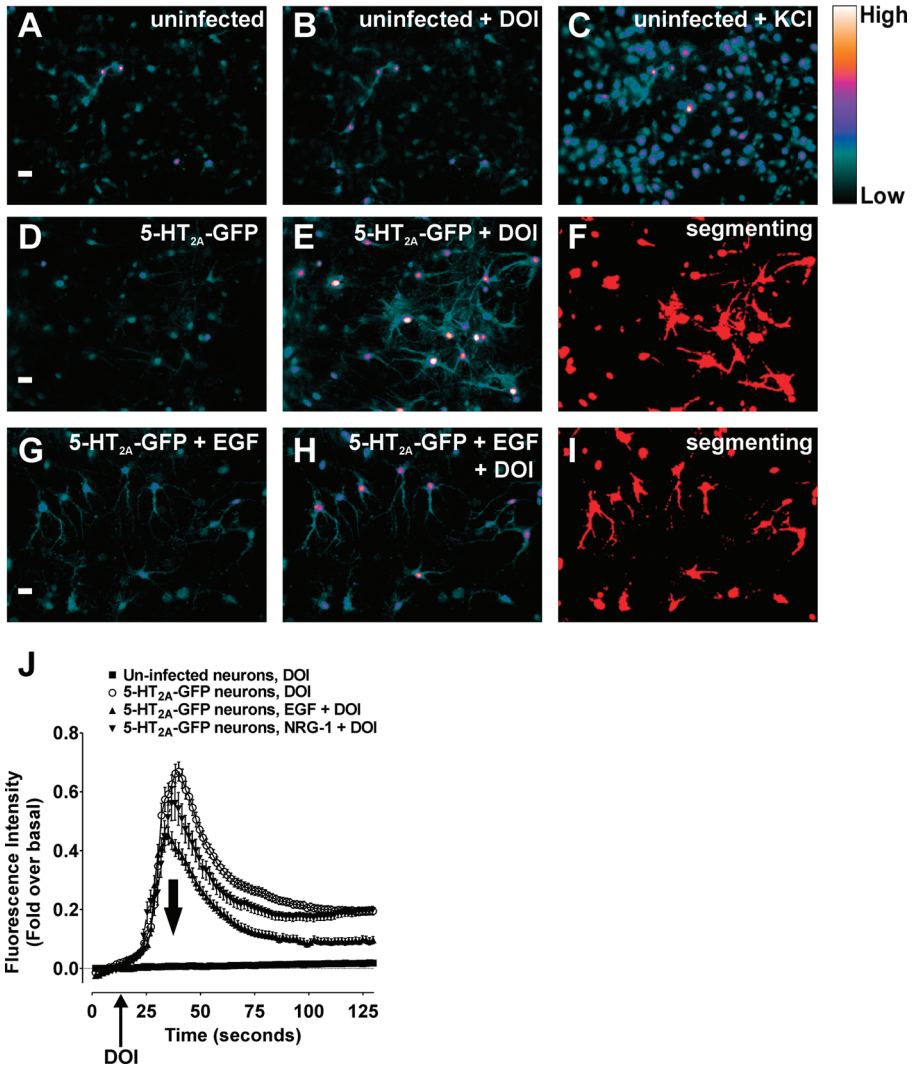

FIGURE 8: Growth factors essential for normal brain function attenuate 5-HT_{2A} receptor signaling in cortical neurons. Cortical neurons were isolated from RSK2+/+ mice and cultured for ~14 days *in vitro* (scale bar is 20 μm). These neurons were loaded with Ca^{2+} dye for 60 min prior to stimulation with the 5-HT_{2A}-selective agonist DOI (1 μM), and 5-HT_{2A}-mediated Ca^{2+} responses (see color scale on right) were measured by live cell fluorescence microscopy in uninfected cortical neurons (A−C) and cortical neurons infected with GFP-tagged 5-HT_{2A} receptors (D−I). Growth factor-mediated changes in signaling were detected by comparing Ca^{2+} responses in untreated neurons (D, E) and neurons treated with 100 ng/mL EGF (G−I) and 100 ng/mL NRG-1 (images not shown). Ca^{2+} responses were quantified by segmenting the total area of responding cells and assessing the mean fluorescence intensity/area (F, I, J). (A, B) The 5-HT_{2A}-selective agonist DOI did not produce measurable Ca^{2+} responses in uninfected neurons. (C) The uninfected neurons produced measurable Ca^{2+} responses upon depolarization with 80 mM KCl. (D) Shown are cortical neurons that were infected with lentivirus encoding GFP-tagged 5-HT_{2A} receptors prior to stimulation with DOI. (E, F) DOI produced robust Ca^{2+} responses in infected neurons which were quantified via manual segmenting. (G) Shown are cortical neurons infected with GFP-tagged 5-HT_{2A} receptors loaded with Ca^{2+} imaging dye containing 100 ng/mL EGF for 60 min prior to stimulation with DOI. (H, I) The 5-HT_{2A}-selective agonist DOI produced weak Ca^{2+} responses in infected neurons treated with EGF. (J) The DOI-induced 5-HT_{2A} Ca^{2+} responses in infected neurons (○) was significantly greater than those measured from neurons pretreated with EGF (▲) or NRG-1 (▼) ($p < 0.05$). No response was detected for uninfected neurons (■). Quantified results (mean ± SEM) are shown from three independent experiments.

results in RSK2+/+ cells are consistent with RTK-mediated attenuation of 5-HT_{2A} receptor signaling. Importantly, none of these predicted effects were observed in RSK2−/− MEFs, thus supporting the hypothesis that RTKs act via RSK2 to attenuate 5-HT_{2A} receptor signaling.

Alternatively, these results could be explained by differences in gene expression profiles between RSK2+/+ and RSK2−/− cells. However, microarray data show that the expression of genes required for EGFR signal transduction are not significantly different between RSK2+/+ and RSK2−/− MEFs. Thus, the simplest explanation for our results remains that RSK2 is a critical mediator of cross-talk between the EGFR and 5-HT_{2A} receptor.

In addition to our results in RSK2+/+ MEFs, we observed that 5-HT_{2A} signaling was significantly decreased following activation of endogenous RTKs in mVSMC and cortical neuron primary cell lines. Considering the physiological importance of inhibitory cross-talk, it is attractive to speculate that growth factor signaling may be relevant for regulating the 5-HT_{2A} receptor in the CNS. In support of this, members of the EGFR family are widely expressed throughout the brain and regulate a variety of functions including proliferation, differentiation, maturation, and survival of a variety of neurons (*45*). Interestingly, the ErbB4 neuregulin receptor, which is a member of the EGFR family, is expressed throughout the mature brain and is known to reside in some of the same cortical layers (*47*) as the 5-HT_{2A} receptor (*48*). Moreover, ErbB4 interacts with PSD-95, a postsynaptic density protein that associates with and regulates 5-HT_{2A} receptor signaling and trafficking *in vitro* and *in vivo* (*26*, *29*, *35*, *49*).

Table 3: Effects of EGFR Family Agonists on 5-HT_{2A} Receptor Signaling in Cortical Neurons

RTK ligand	GPCR ligand	peak Ca^{2+} release[a]	area under curve
none	DOI	0.67 ± 0.03	33.7 ± 1.3
EGF	DOI	0.45 ± 0.03[b]	20.9 ± 1.2[b]
NRG-1	DOI	0.56 ± 0.06	28.6 ± 1.7[b]

[a]Agonist-induced Ca^{2+} responses in untreated and growth factor-treated cortical neurons were quantified using the FLIPR Ca^{2+} assay kit and BD Pathway 855 high content imaging microscope. To control for subtle differences in 5-HT_{2A}-GFP receptor expression, Ca^{2+} responses were normalized to GFP intensity/well using custom written macros for Excel (Microsoft) and Image J (National Institutes of Health). Values were expressed as fold over baseline and represent the mean ± SEM of three independent experiments. [b]A two-tailed, paired *t* test was used to determine the statistical significance (defined as $p < 0.05$) of responses in growth factor-treated vs untreated cells.

Thus, considering the pervasiveness of growth factor signaling in the brain, as well as its overlapping expression with 5-HT_{2A} receptors, RTK signaling could modulate 5-HT_{2A} receptor signaling *in vivo*. Intriguingly, aberrant signaling of both RTKs and 5-HT_{2A} receptors has been associated with neuropsychiatric disorders such as depression and schizophrenia (*47*, *50*−*52*). Together, these findings suggest that a more complete understanding of the mechanism(s) underlying inhibitory cross-talk between RTKs and 5-HT_{2A} receptors is of considerable therapeutic importance.

IGF-1 Fails To Robustly Activate RSK2 and Does Not Attenuate 5-HT_{2A} Receptor Signaling. In stark contrast to our results using EGF, PDGF, and ErbB4 receptor agonists, we discovered that IGF-1 did not attenuate 5-HT_{2A} receptor signaling in either RSK2+/+ or RSK2−/− MEFs. In order to interpret these negative results, we showed that EGF treatment retained the ability to attenuate 5-HT_{2A}-mediated Ca^{2+} release in parallel control experiments. These data suggest that, unlike EGF receptor activation, IGF-1 signaling does not desensitize 5-HT_{2A} receptors. The reasons for this are unknown, although our data showing that IGF-1 weakly activates RSK2 when compared to EGF suggest that a threshold level of RSK2 activation must be reached in order to elicit 5-HT_{2A} receptor desensitization.

Other mechanisms have been proposed to explain IGF-1-induced GPCR desensitization including phosphorylation of tyrosine residues in the second intracellular loop of the β_2-adrenergic receptor and Akt-mediated phosphorylation of the β_1-adrenergic receptor (*16*, *19*). However, 5-HT_{2A} receptors are not known to be phosphorylated on tyrosine residues and are not substrates for Akt, perhaps explaining why IGF-1 has no effect on 5-HT_{2A} receptor signaling.

RSK2 Is Required for Growth Factor-Mediated Regulation of Multiple GPCRs: Evidence from P2Y Purinergic Receptors. In addition to the 5-HT_{2A} receptor, endogenous P2Y purinergic receptor signaling is regulated by RSK2 (*13*). Here we provide the first evidence showing that, like 5-HT_{2A} receptors, EGFR activation attenuates P2Y-purinergic receptor signaling in a RSK2-dependent manner. Thus, it appears that RSK2 is a critical mediator of inhibitory cross-talk between multiple RTKs and GPCRs. Interestingly, the β_1-adrenergic and PAR-1 thrombinergic receptors are also regulated by RSK2 (*13*), and it remains to be determined if these receptors are regulated by RTKs in a RSK2-dependent manner. This is an especially intriguing question for the β_1AR since it is already known that activation of the IGF-1R regulates β_1-adrenergic receptor signaling through activation of PI3 kinase and Akt (*16*).

A question of important physiological relevance is whether specific RTK signaling pathways influence the signaling of all or only select groups of GPCRs. Our results, along with those of others, indicate that signaling from some Gq-coupled receptors (i.e., $5HT_{2A}$, P2Y, α_{1b}-adrenergic, and α_{1d}-adrenergic) are similarly attenuated by one RTK, the EGFR (*20*, *21*). Other RTKs, such as insulin and IGF-1 receptors, are well-known to decrease the signaling of some Gs-coupled GPCRs such as β_1- and β_2-adrenergic receptors (*16*, *53*). However, insulin and IGF-1 receptors attenuate signaling from only some (i.e., 5-HT_{2C}), but not all (i.e., M1 muscarinic or 5-HT_{2A}) Gq-coupled GPCRs (*22*) (Figure 6). Therefore, a robustness of this signaling cross-talk is evident, and RTK inhibitory cross-talk to GPCRs will likely emerge as a receptor-specific phenomenon. Ultimately, further studies testing many RTKs and GPCRs will help to elucidate if this cross-talk is a conserved phenomenon.

In summary, multiple lines of evidence suggest that RSK2 is a critical mediator of inhibitory cross-talk between RTKs and the 5-HT_{2A} receptor. Specifically, this study presents the first evidence that 5-HT_{2A} receptor signaling is attenuated by the growth-factor-mediated activation of RTKs endogenously expressed in multiple cell types including physiologically relevant mVSMCs and cortical neurons. Moreover, genetic deletion of RSK2 was sufficient to block these effects, thus demonstrating that RSK2 is required for the inhibitory cross-talk between RTKs and 5-HT_{2A} receptors in all relevant cell types examined. Intriguingly, we discovered that the P2Y purinergic receptor, whose signaling is also regulated by RSK2, is similarly attenuated following EGFR activation in RSK2+/+ MEFs. Taken together, these findings provide the initial framework for a conserved regulatory mechanism whereby multiple RTKs act via the ERK/MAPK effector RSK2 to attenuate GPCR signaling. Most importantly, inhibitory cross-talk between RTKs and 5-HT_{2A} receptors could provide insight into how these receptors are regulated *in vivo*.

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SUPPORTING INFORMATION AVAILABLE

(1) Microarray analysis of genes involved in IR and IGF-1 R signaling pathways in RSK2+/+ and RSK2−/− MEFs and (2) IGF-1 activation of the IGF-1 R. This material is available free of charge via the Internet at http://pubs.acs.org.

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