p38 MAPK Activation Elevates Serotonin Transport Activity via a Trafficking-independent, Protein Phosphatase 2A-dependent Process*

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The indoleamine 5-hydroxytryptamine (serotonin, 5-HT) plays a pivotal, modulatory role in a variety of centrally controlled physiological processes, including respiration, arousal, aggression, and mood, and in the periphery supports gastrointestinal, platelet, and placental function (1). 5-HT is inactivated following vesicular release by a presynaptic, antidepressant-sensitive 5-HT transporter (SERT, 5-HTT), a member of the Na+/Cl- -dependent solute transporter family (SLC6A4) (2–4). SERT knock-out mice display altered presynaptic 5-HT homeostasis, modified 5-HT receptor sensitivities, and stress-dependent behavioral modulation as well as altered responses to psychostimulants (5). In humans, altered SERT gene expression and/or transport function have been linked to multiple disorders including autism, obsessive-compulsive disorder, depression, and suicide (6–11).

Previous studies have demonstrated that both genetic and posttranscriptional processes regulate SERT activity (12, 13). SERT activity in native cells and transfected models can be rapidly (in minutes) modulated by multiple signaling pathways (14, 15). Observations with transfected HEK cells expressing human SERT (hSERT) demonstrated that protein kinase C activators or protein phosphatase 2A inhibitors trigger hSERT phosphorylation and a parallel decrease in hSERT cell surface density, effects that can be attenuated by SERT substrates (16–18). Protein kinase A and protein kinase G (PKG) activation can also trigger SERT phosphorylation (17), although the functional consequences of these stimuli are only beginning to be appreciated. SERTs appear to form homomultimers at the plasma membrane (19) and also interact with a growing list of associated proteins, including syntaxin 1A (20–22) and the catalytic subunit of protein phosphatase 2A (PP2A) (23). Notably, syntaxin 1A and PP2A/SERT interactions are destabilized by protein kinase C-activating phorbol esters as well as by protein phosphatase 1/2A inhibitors, such as okadaic acid and calyculin A, suggesting that signaling

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1 The abbreviations used are: 5-HT, 5-hydroxytryptamine, serotonin; SERT/5-HTT, serotonin transporter; hSERT, human SERT, NET, norepinephrine transporter; hNET, human NET; DAT, dopamine transporter; hDAB, human DAT; AR, adenosine receptor; PKG, protein kinase G; GBR 12935, 1-(2-diphenylmethoxyethyl)-4-(3-phenylpropyl)piperazine; NECA, 5′-N-ethylcarboxamidoadenosine; ODQ, 1H-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one; RTI-55, (3-(4-iodophenyl)-tropane-2β-carboxylic acid methylester tartrate; RBL-2H3, rat basophilic leukemia 2H3 cell line; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; CHO, Chinese hamster ovary; N8, N′-[methylaminomethyl]-5-isouquinoline sulfonamide; MEM, minimal essential medium; KRH, Krebs-Ringer HEPES; DA, dopamine; NE, norepinephrine; PBS, phosphate-buffered saline; siRNA, small interfering RNA; ANOVA, analysis of variance.
networks converge on SERT protein complexes to influence plasma membrane transport capacity.

Whereas important observations of SERT regulation have arisen using heterologous expressed SERTs, reports of receptor-linked modulation of native SERT activity provide important evidence for physiologically relevant SERT regulation. For example, in neurons, acute modulation of presynaptic 5-HT1-class receptors alters hippocampal 5-HT clearance \textit{in vivo} (24). We have described rapid down-regulation of SERT in brain preparations \textit{in vitro} and \textit{in vivo} following treatments with the \alpha2-adrenergic agonist, UK14304 (25). In a rat basophilic leukemia cell line (RBL-2H3) (26) and in platelets (27), adenosine receptors (ARs) and histamine receptors, respectively, induce a cGMP-linked increase in 5-HT transport. In examination of the receptors (ARs) and histamine receptors, respectively, induce a cGMP-linked increase in 5-HT transport. In examination of the RBL-2H3 cell model, as well as AR/SERT co-transfected Chinese hamster ovary (CHO) cells, we (28, 29) recently defined SERT stimulation as supported by distinct trafficking-dependent and independent pathways, activated downstream of AR- or independent pathways, activated downstream of AR- or transfected model systems, is sensitive to stimuli (anisomycin, H2O2, and UV irradiation) known to trigger both p38 MAPK phosphorylation and activation and whether ensuing modulation of 5-HT transport parallels p38 MAPK activation and is sensitive to specific p38 MAPK inhibitors. Using transfected cells, we asked whether SERT regulation is shared by the homologous biogenic amine transporters, NET and DAT, or exhibits transporter specificity. Using RBL-2H3 cells, as well as the serotoninergic neuroblastoma RN46A (32), we asked whether p38 MAPK-linked SERT regulation arises from catalytic modulation, changes in transporter surface density, or both and, by using competition binding assays, whether 5-HT affinity is altered. To test an emerging model favoring p38 MAPK as downstream of PKG activation where p38 MAPK may be capable of targeting surface-inserted SERTs, we tested the sensitivity of anisomycin modulation of SERT activity to PKG inhibitors and explored the impact on regulation of surface SERT-inactivating methanethiosulfonates, respectively. Finally, we sought to determine whether the SERT-associated phosphatase PP2A, whose inhibitors block AR stimulation of 5-HT uptake (28), contributes to SERT exocytosis versus catalytic activation. We discuss our findings with respect to a model for acute SERT regulation dictated by complementary yet resolvable pathways that differentially impact SERT trafficking and catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Constructs—**Anisomycin, hydrogen peroxide (H2O2), fosfomycin, calcium A, eurexin, 1H-(1,2,4)-oxadiazole-3,4-qlquinoxa- lin-1-one (ODQ), NECA, N-[2-(methylamino)ethyl]-5-isoquinoline sulfonyamide (HS), paroxetine, and desipramine were purchased from Sigma. GBR 12935 dihydrochloride is a product of Torcis (Elliaville, MO). SB203580 and SB202190 were obtained from Alexis Biochemicals (San Diego, CA). SB202474 dihydrochloride was purchased from Calbiochem. [2-Trimethylammonium]ethyl)methanethiosulfonate (MTSET) was obtained from Toronto Research Chemicals Inc. (North York, Canada). [3H]-5-HT (5-hydroxy-[3H]tryptamine trifluoroacetate; 102 Ci/mmol) was purchased from Amersham Biosciences; [3H]-(4-toludinyl)-tropane-2J-carboxylic acid methylester tartrate ([3H]RTI-55; 2200 Ci/mmol) was purchased from PerkinElmer Life Sciences. Trypsin-EDTA, glutamine, and ampicillin/streptomycin were purchased from Invitrogen; modiﬁed Eagle’s medium (MEM) and Dulbecco’s MEM were derived from Invitrogen and reagents and prepared in the Vanderbilt Media Core. Membrane-permeant PKG-inhibitor peptide DT-2 was synthesized as previously described (33). hSERT (4), hNET (34), and hDAT (35) cDNAs have been described. hDAT was a gift from Dr. Marc Caron (Duke University, Durham, NC). Anti-total and phosphorylated p38 MAPK antibodies were purchased from Cell Signaling (La Jolla, CA). siGENOME SMART pool® siRNA for rat p38 MAPK was a product of Dharmacon (Chicago, IL). Antibodies to β-actin were obtained from Sigma.

**Cell Culture and Transfection—**RBL-2H3 cells (ATCC, Manassas, VA) were maintained at 37 °C in MEM containing 15% fetal bovine serum (Invitrogen), 1% L-glutamine (L-Glu), 100 μg/ml penicillin, and 100 μg/ml streptomycin. RN46A cells (provided by Dr. Whitemore, University of Miami School of Medicine, Miami, FL) were cultured at 33 °C with Dulbecco’s MEM/F-12 (1:1 in volume) containing 250 mg/liter G418, 10% fetal bovine serum, and 100 μg/ml penicillin/streptomycin (32). CHO cells (ATCC, Manassas, VA) were maintained at 37 °C in Dulbecco’s MEM containing 10% fetal bovine serum, 1% 1-glutamine (L-Glu), 100 μg/ml penicillin, and 100 μg/ml streptomycin. RN46A cells were plated at 200,000 cells/well 24 h before the 5-HT uptake assay. Medium was removed by aspiration, and cells were washed once with Krebs-Ringer HEPES (KRH) buffer containing 130 mM NaCl, 1.3 mM KH2PO4, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.8 g/liter glucose, 10 mM HEPES, pH 7.4. Cells were incubated in triplicate at 37 °C in KRH buffer (0.2 ml/well) containing 100 μM paroxetine, 100 μM l-ascorbic acid, and/or 1.0 mM tropolone (Sigma), with or without modifiers. After a 10-min incubation with [3H]-5-HT (brought to 0.5 Ci/mmol using unlabeled 5-HT), and again, [3H]DA (50 nM), or [3H]NE (50 nM) at 37 °C, buffer was aspirated, and the uptake assay was performed 24 h following transfection. RN46A cells were plated at 200,000 cells/well 24 h before the 5-HT uptake assay. Medium was removed by aspiration, and cells were washed once with Krebs-Ringer HEPES (KRH) buffer containing 130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.8 g/liter glucose, 10 mM HEPES, pH 7.4. Cells were incubated in triplicate at 37 °C in KRH buffer (0.2 ml/well) containing 100 μM para- tophenol, 0.1% FCS, 0.1% L-glutamine, and/or 1.0 mM tropolone (Sigma), with or without modifiers. After a 10-min incubation with [3H]-5-HT (100 mM for RN46A and RN46A cells, 20 mM for SERT-transfected CHO cells), [3H]DA (50 mM), or [3H]NE (50 mM) at 37 °C, buffer was aspirated, and the cells were washed three times with ice-cold KRH buffer. Cells were solubilized with 0.5 ml of Microtint 20 (PerkinElmer Life Sciences), and tritium-labeled monoamine accumulation was quantitated using a TopCount plate scintillation counter (PerkinElmer Life Sciences). Specific 5-HT, DA, and NE uptake was determined by subtracting the amount of [3H]-5-HT, [3H]DA, and [3H]NE accumulated in the presence of 10 μM paroxetine, GBR 12935, and desipramine, respectively. 5-HT saturation kinetics in RBL-2H3 and RN46A cells were defined as described for standard transport assays, using varying concentrations of [3H]-5-HT (brought to 0.5 Ci/mmol using unlabeled 5-HT), and again, nonspecific uptake was defined with 10 μM paroxetine. For studies seeking to inactivate surface SERTs in RBL-2H3 cells, we treated cells with the membrane-impermeant, cytochrome-specific alkylating reagent MTSET (36), as previously described (28). Cells were treated either with vehicle or MTSET (10 mM) for 10 min on ice prior to washing, and cells were then re-equilibrated in normal medium at 37 °C with anisomycin-stimulated 5-HT transport determined as described above.

**Platelet Isolation and Transport Assays—**Human platelet-rich plasma was obtained from the American Red Cross. Approximately 2 × 109 platelets were incubated with the appropriate pharmacological reagents for 10 min at 37 °C and collected by centrifugation at 2000 rpm for 30 s. Platelets were then washed twice with 1X PBS and resuspen- sed in 130 mM NaCl, 1.5 mM KH2PO4, 2.68 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.8 g/liter glucose, 100 μM HEPES, 100 μM pargyline, 100 μM ascorbic acid, and 1 mM tropolone. After a 5-min incubation with 50 nM [3H]-5-HT, uptake was terminated by filtration through GF/F Whatman paper. Filters were washed three times with ice-cold KRH buffer, immersed in scintillation liquid for 8 h, and counted by scintillation spectrometry. The counts obtained from the filtered samples were corrected for the nonspecific binding/uptake ob-
tained using parallel samples incubated with paroxetine (1 μM). Experiments were performed in duplicate and replicated at least three times.

**[^51][51]RTI-55 Binding Assays—**To assess SERT surface density, we quantitated the binding of the high affinity cocaine analog[^51]RTI-55 (5 nM) to intact cells at 4 °C for 45 min in PBS buffer (phosphatase- and proteinase-inhibitor cocktail) to eliminate non-specific binding. Values were normalized to cell protein content. Binding assays were performed with GraphPad Prism (rectangular hyperbola, one-site binding model).

**Assays of p38 MAPK Activation—**To monitor activation of p38 MAPK by anisomycin in intact cells, RBL-2H3 cells were seeded in 96-well plates (10^4 cells/well) and cultured in MEM containing 15% fetal bovine serum in a 37 °C incubator with 5% CO_2 for 16–24 h. Medium was removed by aspiration, and cells were washed once with KRH buffer and then incubated in triplicate at 37 °C in KRH buffer with or without p38 MAPK activators for 5–10 min. Assay buffer was removed, and cells were immediately fixed with fresh 4% formaldehyde (Sigma) in PBS for 20 min at room temperature and washed four times with 1× PBS containing 0.1% Triton X-100 (Sigma) (5 min per wash) prior to 1 h of blocking with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). RBL-2H3 cells were subsequently probed with dual phosphorylated (Thr180 and Tyr182) p38 MAPK polyclonal antibody (Cell Signaling; 1:400 in Odyssey blocking buffer with gentle mixing for 2 h at room temperature. Cells were then washed four times with 1× PBS containing 0.1% Tween 20 (Sigma) for 5 min. Bound antibody was detected with fluorescence-labeled secondary antibody-Alexa Fluor® 680 (avoiding exposure to light; 1:200 in Odyssey Blocking Buffer, Molecular Probes, Inc., Eugene, OR) for 1 h. After four washes with 1× PBS, 0.1% Tween 20, the plate was scanned, and the captured image of the signal was processed and quantified with the Odyssey™ Infrared Imaging System (Li-Cor Biosciences). For UV radiation to activate p38 MAPK, 100 μM 5-HT was placed on a 1.0 μM p38 MAPK polyclonal antibody (Cell Signaling; 1:400 in Odyssey blocking buffer with gentle mixing for 2 h at room temperature.

**RESULTS**

Stimulation of 5-HT Uptake in RBL-2H3 Cells by p38 MAPK Activators—We previously demonstrated that AR agonists such as NECA rapidly augment p38 MAPK phosphorylation and elevate 5-HT uptake in RBL-2H3 cells in an SB203580-sensitive manner (28). Similar findings were obtained with ARhSERT co-transfected CHO cells. These results, as well as the novelty of drug-modulated SERT catalytic function, warranted a more in depth evaluation of p38 MAPK-dependent SERT stimulation. To accomplish this, we first explored the effects of the p38 MAPK activator, anisomycin (37), on 5-HT uptake in RBL-2H3 cells. In these experiments (Fig. 1, A and B), we found that anisomycin exerts a dose- and time-dependent stimulation of 5-HT transport activity. At 1 μM, anisomycin stimulation of 5-HT transport was transient, peaking at 10 min and declining to basal levels by 30 min. Using a 10-min pre-treatment, anisomycin stimulation peaked at 1 μM and became inhibitory at 25 μM, effects that could represent toxicity, although this was not pursued further. Consistent with previous findings (28), the effects of anisomycin on 5-HT uptake were blocked by pretreatment with the specific p38 MAPK inhibitor, SB203580 (Fig. 1C). Moreover, the effects of anisomycin could be mirrored by other p38 MAPK activators. Reactive oxygen species, such as H_2O_2, and UV radiation are known to trigger p38 MAPK activation in a wide range of cells (38, 39). Both H_2O_2 (20 μM) and UV light (4 × 10^4 μJ/cm^2) stimulated 5-HT uptake in RBL-2H3 cells.

**Statistical Analyses—**Statistical analyses, comparing base-line and compound-modified uptake, antagonist binding, or p38 MAPK activation, were performed with GraphPad Prism (GraphPad, San Diego, CA) using one- and two-way analyses of variance (ANOVA) with subsequent planned comparisons (Dunnett, Bonferroni) as well as t tests as noted in the legends to Figs. 5 and 6. Saturation kinetic and competition binding data were fit using GraphPad Prism (rectangular hyperbola, one-site binding isotherm).
transport to a level comparable with 1 μM anisomycin, and as with anisomycin, these effects were abolished by SB203580 (Fig. 1C). Additionally, other potent p38 MAPK inhibitors, SB202190 and PD169316 (data not shown), also blocked anisomycin stimulation of SERT, whereas the inactive analog, SB202474, and c-Jun NH2-terminal kinase inhibitor, curcumin, lacked antagonistic activity (Fig. 1D). PD98059 (10 μM), a selective inhibitor of extracellular signal-regulated kinase 1/2, however, failed to block anisomycin-stimulated SERT activity (data not shown).

p38 MAPK must be dually phosphorylated to achieve catalytic activation (39). To verify that our anisomycin treatments of RBL-2H3 cells trigger p38 MAPK activation, we monitored the dual phosphorylation status of the kinase monitoring total and phosphor-specific p38 MAPK antibodies using an In-Cell Western format. NECA and 8-bromo-cGMP (data not shown) were used as positive controls, since we previously demonstrated these two agents activate p38 MAPK, with no change in total p38 MAPK (28). In the experiments shown, RBL-2H3 cells were treated with vehicle, NECA, or anisomycin (0.5 and 1.0 μM) for 5 min in KRH assay buffer in the absence/presence of the p38 MAPK inhibitor, SB203580. Similar to NECA, anisomycin stimulates a 2–3-fold increase in phosphorylated p38 MAPK, effects that are blocked by pretreatment with SB203580 (Fig. 2, A and B). The level of stimulation by NECA and anisomycin was comparable to stimulation achieved with 8-bromo-cGMP. No changes were observed in total p38 MAPK levels (data not shown).

In order to validate a role for p38 MAPK in anisomycin-triggered SERT up-regulation and to gain insight into specific p38 MAPK isoforms involved in SERT modulation, we examined the impact of siRNA-mediated suppression of p38 MAPK-α on anisomycin stimulation. Treatment of RBL-2H3 cells with p38 MAPK-α siRNA (100 nM) abolished the stimulation of 5-HT transport activity (Fig. 3A). Cells treated with transfection reagents alone retained up-regulation. Western blot analyses confirmed a down-regulation of p38 MAPK protein by these treatments (Fig. 3, B and C).

**Fig. 2.** Anisomycin triggers p38 MAPK phosphorylation in RBL-2H3 cells in an SB203580-sensitive manner. RBL-2H3 cells were seeded in a 96-well plate and cultured overnight. The cells were then treated with vehicle (assay buffer) or SB203580 (10 μM) for 15 min followed by another 5-min treatment with anisomycin or NECA at the concentration indicated. The cells were fixed, and an in-cell Western assay was conducted as detailed under “Experimental Procedures.” Shown here is the average intensity of fluorescence for labeling activated p38 MAPK. Values are expressed as the mean ± S.E. (n = 3). *p < 0.05; **p < 0.01 versus vehicle control (one-way ANOVA, Bonferroni). ANS, anisomycin; SB, SB203580.

**Fig. 3.** Effect of p38 MAPK siRNA transfection on SERT activity and p38 MAPK protein expression. RBL-2H3 cells were transfected with vehicle, transfection reagent (Trans-It), or siRNA (100 nM). 5-HT uptake and Western blots were performed 48 h following the transfection. A, siRNA transfection abolished anisomycin-induced 5-HT uptake without affect basal SERT activity (n = 4). B, Western blot (a representative from three experiments) showing the expression level of p38 MAPK. C, quantification of p38 MAPK band density (n = 3). Values are expressed as the mean ± S.E. **p < 0.01 versus vehicle control (one-way ANOVA, Bonferroni). ANS, anisomycin; SB, SB203580.
treatments significantly reduced the SERT Km versus 0.01 versus respective control (n = 5, one-way ANOVA, Bonferroni). ANS, anisomycin; SB, SB203580.

Next we sought evidence for a more general role of p38 MAPK in SERT modulation, examining anisomycin’s effects in both a neuronal cell model and in platelets. The RN46A line is a serotonergic neuronal cell line, derived from rat E13 raphe nucleus, that expresses native SERT (32). As observed in RBL-2H3 cells, treatment with 8-bromo-cGMP induces a rapid, PKG antagonist (DT-2 (33))-sensitive increase in 5-HT transport (Fig. 5A). Anisomycin also dose-dependently stimulated 5-HT uptake in the RN46A cells, effects completely blocked by co-incubation with SB203580 (Fig. 5B). Platelets are a readily accessible and well studied model featuring native SERT expression (40). As found in nonneuronal and neuronal cell models, anisomycin also induced an SB203580-sensitive increase in 5-HT uptake in human platelets (Fig. 5C).

Anisomycin Treatment of RBL-2H3 Cells Selectively Reduces the 5-HT Transport Kₐ and Increases 5-HT Potency for Inhibition of Antagonist Binding—Our previous study revealed that p38 MAPK inhibition blocked AR-triggered increases in SERT activity but failed to attenuate elevations in surface SERT density (28). These remarkable findings suggested to us that p38 MAPK-mediated SERT stimulation might not arise from altered surface trafficking but rather represents a form of catalytic activation. We took multiple approaches to explore this issue. First we performed saturation kinetic analyses of 5-HT uptake in RN46A and RBL-2H3 cells, with or without anisomycin treatment. As shown in Fig. 6, A and B, anisomycin treatments significantly reduced the SERT Km for 5-HT in RN46A cells (799 ± 97 versus 396 ± 80 nm, p < 0.05) and in RBL-2H3 (1057 ± 145 versus 628 ± 70 nm, p < 0.05) but effecte no significant change in maximal transport capacity (Vₘₐₓ) in either model. Second, we asked whether the reduction in 5-HT Km reflected a change in 5-HT affinity. 5-HT does not bind to SERT with high enough affinity to determine its KD value directly. However, 5-HT affinity can be estimated by deriving its apparent Km for displacement of a competitive antagonist. In studies examining 5-HT inhibition of whole cell [125I]RTI-55 binding (Fig. 6C), we observed that pretreatment of cells with 1 μM anisomycin for 10 min significantly reduced the apparent 5-HT Kᵢ for [125I]RTI-55 competition (1.31 ± 0.26 μM in the vehicle control, 0.24 ± 0.005 μM in the presence of anisomycin, p < 0.05). This effect was blocked by SB203580, which had no significant effect on 5-HT Kᵢ on its own.

The studies noted above support the idea that p38 MAPK-linked SERT modulation arises from surface trafficking but catalytic modulation of preexisting surface-resident transporters. We thus wished to evaluate directly possible changes in SERT total and surface density in the context of anisomycin stimulation. However, in the RBL-2H3 cell model, SERT protein levels do not permit biotinylation paradigms more suitable for heterologous expression systems. Instead, we monitored the extent of 5-HT (surface)-displaceable versus paroxetine (total)-displaceable [125I]RTI-55 binding to intact cells at 4 °C, as previously described (28). Consistent with the results of saturation kinetic studies, anisomycin does not impact total surface-SERT binding (Fig. 7A). As a positive control that our assay can detect surface increases by agents known to elevate SERT density, we demonstrate a significant increase in SERT surface density following NECA treatment (28). As expected for the acute nature of these treatments, total SERT (internal plus external pools), as defined by paroxetine-dis-
Catalytically Active PP2A Is Required for p38 MAPK-mediated SERT Stimulation—One can envision multiple mechanisms by which p38 MAPK activation modifies SERT activity, including a direct action on SERT as well as interactions with a growing list of SERT-associated proteins (20, 23, 41–43). We were particularly interested in PP2A, since the protein phosphatase 1/2A inhibitor, calyculin A, com-

sought to verify formally that p38 MAPK lies downstream of cGMP production, a second messenger that augments 5-HT transport in an SB203580-sensitive manner (28, 29). We therefore monitored the impact of the guanylyl cyclase inhibitor, ODQ, as well the PKG antagonists, H8 and DT-2, on anisomycin-stimulated 5-HT transport in RBL-2H3 and RN46A cells. Whereas LY83583 and ODQ completely abolished AR stimulation of SERT in RBL-2H3 cells (and in co-transfected models (28)), neither agent attenuated anisomycin stimulation of SERT activity in these models (Fig. 8, A and B). Additionally, both DT-2 and H8 blocked 8-Br-cGMP stimulation of SERT (data not shown) but failed to attenuate anisomycin's effects on SERT. Since AR stimulation triggers p38 MAPK activation (see Fig. 2), a lack of effect of ODQ, H8, and DT-2 on SB203580-sensitive, anisomycin-stimulated 5-HT uptake places p38 MAPK downstream of guanylyl cyclase and PKG in the SERT regulatory pathway. Moreover, both RBL-2H3 and RN46A cells appear to utilize these pathways (although with different quantifying effects) to regulate SERT activity.

Inhibition of cGMP/PKG Does Not Impact Anisomycin-induced SERT Stimulation—To begin to evaluate targets of activated p38 MAPK-supporting SERT stimulation, we next

Anisomycin (1 μM) or with vehicle for 10 min prior to transport assays. B, RBL-2H3 cells were preincubated with anisomycin (1 μM) or with vehicle for 10 min prior to transport assays. C, RBL-2H3 cells were preincubated with vehicle, SB203580 (10 μM), or anisomycin (1 μM) for 10 min prior to the RTI-55 binding assay. 5-HT at varying concentrations as indicated in the figure was used to compete with surface binding of RTI-55. Data were fit in both A and B to a Michaelis-Menten equation (single binding site) to derive 5-HT Km and Vmax values and fit in C to a single competition site equation to derive Ks. Values are expressed as mean values (n = 3) ± S.E. *p < 0.05 versus control (Student’s t test). ANS, anisomycin; SB, SB203580.
RN46A cells were preincubated with vehicle, ODQ (10 μM), or DT-2 (1.0 μM) for 10 min, followed by the application of anisomycin (1 μM) or NECA (1 μM, as positive control). Both ODQ and H8/DT-2 abolish NECA-induced 5-HT uptake but do not impact anisomycin-stimulated uptake (n = 5). B, RN46A cells were preincubated with vehicle, ODQ (10 μM), H8 (0.1 μM), DT-2 (1.0 μM), or SB203580 (10 μM) for 10 min, followed by the application of anisomycin (1 μM). Only SB203580 blocks anisomycin-induced 5-HT uptake (n = 3). Values are expressed as mean ± S.E. *, p < 0.05; **, p < 0.01 versus vehicle control (one-way ANOVA, Dunnett).

To advance a role for p38 MAPK in SERT regulation, we used anisomycin, H2O2, and UV radiation as rapid p38 MAPK activators. Anisomycin, a protein synthesis inhibitor derived from Streptomyces griseolus, has been shown to trigger p38 MAPK phosphorylation in a variety of cells, including neutrophils (55), mast cells (37), and myocardial cells (56). At higher concentrations and with longer incubations, anisomycin is also a protein synthesis inhibitor (57). Anisomycin is also a potent c-Jun NH2-terminal kinase stimulator (58). Since we did not find comparable SERT regulation using treatments with cyclohexamide (data not shown), a protein synthesis inhibitor that does not activate p38 MAPK (58), we do not believe that translational suppression plays a role in the effects of anisomycin reported here. Additionally, our anisomycin effects are quite rapid relative to the time needed to synthesize, process, and traffic biogenic transporters (59). Importantly, we found SERT modulation by anisomycin to be blocked by the specific p38 MAPK inhibitors SB203580 and SB202190 but not by the c-Jun NH2-terminal kinase inhibitor, curcumin, nor the extracellular signal-regulated kinase 1/2 inhibitor, PD98059, and we thus attribute the activation of SERT to enhanced p38 MAPK activ-

DISCUSSION

SERTs are regulated through both transcriptional and posttranslational mechanisms (15, 46, 47). Whereas much evidence has arisen supporting altered gene expression linked to an hSERT promoter polymorphism (12) and its role as a possible risk factor in psychiatric disorders, disruption of posttranslational regulation may play an equally important role in setting disease risk. Indeed, disrupted PKG-linked posttranslational-regulation has been suggested to account for the linkage between a SERT polymorphic variant found in two families and the presence of obsessive-compulsive disorder and other severe psychiatric disorders (8, 9). We have recently provided evidence that both PKG and p38 MAPK signaling pathways are involved in rapid (in minutes) AR-induced SERT up-regulation in RBL-2H3 as well as in receptor/transporter co-transfected CHO cells. We were particularly intrigued by evidence of p38 MAPK involvement in SERT regulation, since the kinase had previously been implicated in catalytic activation of NET (30) and has drawn substantial attention as a critical determinant of insulin-modulated glucose uptake (48). p38 MAPK is generally categorized as a stress-activated protein kinase and is one of the members of the MAPK superfamily (49). p38 MAPK can be activated by numerous cytokines as well as by environmental and chemical stressors (38), in many cases triggering inflammatory and/or apoptotic sequelae. p38 MAPK can also be activated by hormones and growth factors (50–52) and has recently been implicated in both short and long term pathways of synaptic plasticity (53, 54).

FIG. 8. Inhibitors of guanylyl cyclase and PKG fail to block anisomycin-stimulated 5-HT uptake. A, RBL-2H3 cells were preincubated with vehicle, ODQ (10 μM), H8 (0.1 μM), or DT-2 (1.0 μM) for 10 min, followed by the application of anisomycin (1 μM) or NECA (1 μM, as positive control). Both ODQ and H8/DT-2 abolish NECA-induced 5-HT uptake but do not impact anisomycin-stimulated uptake (n = 5). B, RBL-2H3 cells were preincubated with vehicle, ODQ (10 μM), H8 (0.1 μM), DT-2 (1.0 μM), or SB203580 (10 μM) for 10 min, followed by the application of anisomycin (1 μM). Only SB203580 blocks anisomycin-induced 5-HT uptake (n = 3). Values are expressed as mean ± S.E. *, p < 0.05; **, p < 0.01 versus vehicle control (one-way ANOVA, Dunnett).
ity. Consistent with this idea, we demonstrated that anisomycin activates both p38 MAPK and SERT in an SB203580-sensitive manner and that the time course of p38 MAPK phosphorylation parallels that of SERT activation. We also found that MAPK-activated protein kinase 2, a direct substrate of p38 MAPK, is also activated following the anisomycin treatment (data not shown). Finally, two other modes of p38 MAPK stimulation, treatment with H$_2$O$_2$ and UV irradiation, also stimulate 5-HT uptake in an SB203580-sensitive manner.

Using siRNA-mediated suppression of p38 MAPK-α in RBL-2H3 cells, we were able to gather additional support for a role of this kinase in anisomycin’s effects. Similarly, Samuvel et al. (60) have recently reported that siRNA suppression of p38 MAPK blocks down-regulation of SERT by p38 MAPK inhibitors in HEK-293 cells. Under our conditions, p38 MAPK antagonists fail to suppress basal 5-HT uptake, although we do observe this behavior with higher concentrations or more chronic treatments (data not shown). We achieved ~50% reduction in total p38 MAPK protein with p38 MAPK-α siRNA. The p38 MAPK antibodies available to us do not differentiate the isoforms of p38 MAPK, suggesting that the remaining protein may represent nonsuppressed isoforms. Since SB203580 is specific for α and β isoforms, and our siRNA to the α isoform recapitulated the effect of SB203580, our findings suggest a specific role of the α isoform in SERT modulation.

We consistently observe a 20–40% stimulation of 5-HT uptake in nonneuronal cells, varying to some degree with batches of anisomycin and the model system under study. Regarding the degree of stimulation, we note that even higher levels of stimulation are evident with PKG activation, particularly in the presence of PDE5 antagonists such as sildenafil or zaprinast (29), yet all of this stimulation can be blocked by p38 MAPK inhibitors. SERT activity in serotonergic neuroblastoma RN46A appears particularly responsive to PKG and p38 MAPK-linked pathways (see Fig. 5), although lower basal uptake and p38 MAPK activity may explain these findings. Anisomycin at higher concentration (~20 μM) inhibits 5-HT transport, an effect that may not be linked solely to p38 MAPK nor specific to SERT. This might explain the discrepancy between our current study with human platelets and previous work reporting that anisomycin does not activate p38 MAPK in human platelets at a higher concentration (~300 μM) (61). The transient nature of anisomycin stimulation of SERT activity parallels the transient nature of p38 MAPK phosphorylation evident with anisomycin (61), an effect that is believed to arise from the induction of dual specificity p38 MAPK phosphatases that limit the dual phosphorylation state needed to maintain p38 MAPK activation (39).

To further analyze the anisomycin/p38 MAPK-stimulated SERT-activity, we conducted saturation kinetic assays in two different models and found that anisomycin induced a decrease of SERT $K_m$ without significant changes in $V_{max}$, suggesting that p38 MAPK participates in stimulating SERT intrinsic activity rather than impacting transporter trafficking, the predominant modulatory effect reported heretofore (16, 18, 23, 60, 62). Indeed, anisomycin effects appear to involve changes in the affinity of 5-HT for the transporter, as we also observe that 5-HT exhibited a greater potency in blocking antagonist binding. To our knowledge, these findings are the first that suggest that specific intracellular signals can regulate the affinity of SERT for an exogenous substrate.

Several additional findings indicate that p38 MAPK can target membrane-associated SERTs for activation. First, no change in surface density can be observed with p38 MAPK stimulation, although we can readily detect increases in surface-SERT density triggered by AR- and PKG-linked pathways. Second, the membrane-impermeant cysteine-modifying reagent MTSET, which inactivates surface SERTs but fails to attenuate regulation supported by transporter trafficking (36), abolishes the stimulation by anisomycin. These findings focus our inspection of possible SERT modulators responsible for SERT catalytic activation to either SERT itself or SERT-interacting proteins that are co-resident with SERT at the cell surface.

An important question now before us is how p38 MAPK activation leads to enhanced SERT activity. The lack of a stimulatory effect of anisomycin on hDAT suggests that SERT (and NET) modulation does not arise from a general change in ion gradients or membrane potential that drives or influences the transport process. Although the guanylyl cyclase inhibitor ODQ, the PKG inhibitors H8/DT-2, and SB203580 block AR-mediated stimulation of SERT, only SB203580 can antagonize anisomycin-induced SERT stimulation, consistent with p38 MAPK lying downstream of PKG in the SERT regulatory pathway (28). SERT is a phosphoprotein, both in transfected cells (16, 17) and in native tissues (60). Moreover, recent studies indicate that basal SERT phosphorylation is sensitive to p38 MAPK inhibitors (60). Thus, p38 MAPK may phosphorylate SERTs directly, triggering conformational changes that enhance 5-HT binding affinity. Alternatively, or in addition to direct actions, p38 MAPK may target SERT-associated proteins whose activity or physical interactions could impact SERT. Recently, SERT has been found to localize to lipid rafts (63) as also found for NET proteins (64). p38 MAPK phosphorylation of SERT-associated proteins could shift the distribution of SERTs within lipid microenvironments and bring about changes in substrate affinity and catalytic activity. Samuvel et al. (60) did not observe changes in lipid raft localization of SERT in rat brain synaptosomes treated with p38 MAPK inhibitors, although these effects may be distinct from changes arising from p38 MAPK activation. SERT associates with syntaxin 1A as well as with several other proteins, including Hic-5 (42) and the catalytic subunit of PP2A (23). For both the GAT1 GABA transporter (65) and antidepressant-sensitive hNET (22), phorbol ester-sensitive syntaxin 1A interactions have been linked to changes in transport activity. However, syntaxin 1A interactions with SERT (in Xenopus laevis oocytes) do not appear to alter intrinsic 5-HT uptake activity but rather influence ion conductance states (21). Whether Hic-5 associations affect transporter intrinsic activities is unknown, although studies with DAT proteins point more to an impact on surface trafficking (41).

With respect to PP2Ac, the phosphatase has been shown to be activated by p38 MAPK and to exhibit enhanced membrane targeting following kinase activation (44, 45). Our findings that treatments of cells with PP2A inhibitors fully block both AR- (28) and anisomycin-mediated stimulation of 5-HT uptake, under conditions where they fail to limit SERT plasma membrane trafficking, emphasize a more defined role for the phosphatase in SERT catalytic modulation. We speculate that p38 MAPK may either augment or stabilize SERT/PP2Ac associations, or, alternatively, p38 MAPK may enhance PP2A activity within membrane-resident, transporter-phosphatase complexes. Activated PP2Ac could then target SERT (or associated phosphoproteins) at sites that, when phosphorylated, reduce 5-HT affinity and SERT catalytic activity. We attempted to demonstrate changes in SERT/PP2A associations in RBL-2H3 cells via co-immunoprecipitation, following anisomycin treatments, but could not resolve these complexes, probably due to a low level of SERT protein in this model. Regardless, PP2A is likely to have multiple actions, only one of which we invoke when examining pathways downstream of PKG/p38 MAPK activation. Thus,
modulating serotonergic signaling within the central nervous system. As such, catalytic activation of this process by PKG-dependent or -independent processes may play a role in modulating serotonergic signaling within the central nervous system. The fact that p38 MAPK is activated by neuroimmune cytokines that are elevated under conditions of both acute and chronic psychological stress (for a review, see Ref. 69) raises the question of whether such activation could attenuate serotonergic signaling under these adverse circumstances. Given our findings that SERT is catalytically activated by p38 MAPK-linked pathways in heterologous, neuronal, and native preparations, it seems important to clarify specific mechanisms by which this kinase controls SERT activity and to address questions relating to the role of PKG- and neuroimmune-mediated activation of this kinase with native preparations, with the prospect that such investigations may have important physiologic and therapeutic implications.

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