Interdependent Serotonin Transporter and Receptor Pathways Regulate S100A4/Mts1, a Gene Associated With Pulmonary Vascular Disease

Allan Lawrie, Edda Speikerkoetter, Eliana C. Martinez, Noona Ambartsumian, W. John Sheward, Margaret R. MacLean, Anthony J. Harmar, Ann-Marie Schmidt, Eugene Lukandin, Marlene Rabinovitch

Abstract—Heightened expression of the S100 calcium–binding protein, S100A4/Mts1, is observed in pulmonary vascular disease. Loss of serotonin (5-hydroxytryptamine [5-HT]) receptors or of the serotonin transporter (SERT) attenuates pulmonary hypertension in animals, and polymorphisms causing gain of SERT function are linked to clinical pulmonary vascular disease. Because 5-HT induces release of S100β, we investigated the codependence of 5-HT receptors and SERT in regulating S100A4/Mts1 in human pulmonary artery smooth muscle cells (hPA-SMC). 5-HT elevated S100A4/Mts1 mRNA levels and increased S100A4/Mts1 protein in hPA-SMC lysates and culture media. S100A4/Mts1 was observed ubiquitously overexpressing S100A4/Mts1 develop occlusive pulmonary arterial lesions similar to those seen in patients with grade III to IV PVD.15 Additionally, 5% of transgenic mice

Key Words: smooth muscle cells □ pulmonary hypertension □ S100A4/Mts1 □ serotonin □ ERK1/2

A berrations in serotonin (5-hydroxytryptamine [5-HT])–mediated signaling events have been linked to pulmonary vascular disease (PVD).1,2 Studies in transgenic mice indicate that both the serotonin transporter (SERT)2–3 and 5-HT receptors4–6 are necessary in the development of pulmonary hypertension (PAH). An insertion/deletion polymorphism in the SERT promoter, resulting in 2- to 3-fold higher levels of SERT gene transcription, has been observed in 65% of idiopathic PAH (IPAH) versus 27% of control patients.7 Additional studies have shown that patients using the anorectic drug dexfenfluramine, a 5-HT uptake inhibitor, and iproniazid (monoamine oxidase-A inhibitor), blocked 5-HT–induced S100A4/Mts1. 5-HT signaling mediated phosphorylation (p) of extracellular signal–regulated kinase 1/2 (pERK1/2), but pERK1/2 nuclear translocation depended on SERT, monoamine oxidase activity, and reactive oxygen species. Nuclear translocation of pERK1/2 was required for pGATA-4–mediated transcription of S100A4/Mts1. These data provide evidence for a mechanistic link between the 5-HT pathway and S100A4/Mts1 in pulmonary hypertension and explain how the 5-HTβ receptor and SERT are codependent in regulating S100A4/Mts1. (Circ Res. 2005;97:227-235.)

GATA-4 and to heightened expression of cyclin D2, a gene expressed in proliferating cells.12 Transport of serotonin via SERT results in monoamine oxidase-A (MAO-A) activity, necessary for Rho kinase (ROCK)-mediated nuclear translocation of ERK1/2.13 How SERT interacts with 5-HT receptor signaling in regulating genes specifically associated with PVD has not been addressed.

In astrocytes, 5-HT mediates the release of S100β, a member of the S100 family of small calcium-binding proteins.14 We recently reported heightened expression of another S100 family member, S100A4/Mts1, in human pulmonary artery smooth muscle cells (hPA-SMC) within the neo-intima and adventitia of occlusive lesions in patients with grade III to IV PVD.15 Additionally, approximately 5% of transgenic mice ubiquitously overexpressing S100A4/Mts1 develop occlusive pulmonary arterial lesions similar to those seen in patients with PVD.15 S100A4/Mts1 has also been implicated in the heightened proliferation and motility of cancer cells,16 in angiogenesis17 and endothelial cell invasion,18 and in epithe-
lia–mesenchymal transformation in the lung. A specific S100A4/Mts1 receptor had not been defined. However, S100β mediates neurite outgrowth through the receptor for advanced glycation end products (RAGE). RAGE is up-regulated in vascular disease and is expressed on both endothelial and SMC surfaces.

These observations led us to hypothesize that 5-HT receptors and SERT cooperatively mediate signaling pathways required for gene expression and release of S100A4/Mts1, which, in turn, binds to RAGE, leading to vascular cell migration and proliferation, features associated with progressive PVD.

**Materials and Methods**

**Cell Culture and Functional Assays**

Before stimulation with 5-HT (Sigma Aldrich, St Louis, Mo), hPA-SMC (described in the online data supplement available at http://circres.ahajournals.org) were incubated in DMEM containing penicillin, streptomycin, amphotericin B solution and 0.2% FBS for 48 hours. Pretreatment with all inhibitors was performed for 30 minutes before 5-HT stimulation. Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) assay (American Type Culture Collection) and confirmed by Coulter Counting; measurement of migration was performed using a Boyden chamber assay as previously described. Conditioned media were concentrated using YM-3 filters (Chemicon, Temecula, Calif).

**5-HT- and S100A4/Mts1-Related Reagents**

5-HT- and S100A4/Mts1-related reagents are described in the online data supplement.

**TaqMan RT-PCR**

RNA was isolated with Trizol (Invitrogen, Carlsbad, Calif) from hPA-SMC or lung tissue and reverse transcribed using Superscript II (Invitrogen). Real-time polymerase chain reaction (PCR) was performed on a 7900HT Sequence Detection System with TaqMan

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**Figure 1.** 5-HT induces S100A4/Mts1 (Mts1) expression and release. Human PA-SMC were serum starved for 48 hours before stimulation with 10 μmol/L 5-HT. A, Representative Western immunoblot (top) and densitometric analysis (bottom) of S100A4/Mts1 (11 kDa) protein in cell lysates following stimulation with increasing concentrations of 5-HT for 24 hours. B, S100A4/Mts1 mRNA levels were measured by TaqMan PCR and normalized to β2-microglobulin (β2M) using the comparative C_{T} method. C, Representative Western immunoblot (top) and densitometric analysis (bottom) of S100A4/Mts1 (11 kDa) protein in cell lysates. Densitometry values are normalized to tubulin (50 kDa) as a loading control and expressed as a fold-increase over unstimulated cells at 0 hour. D, Secreted levels of S100A4/Mts1 (11 kDa) protein in concentrated CM at 24 hours, relative to control. Bars represent mean±SEM from 4 different experiments. *P<0.05 vs non-5-HT-treated control cells.
Assays on Demand gene expression probes (system and probes from Applied Biosystems, Foster City, Calif) for S100A4/Mts1 (human: assay ID Hs00243201_m1; mouse: assay ID Mm00803372_g1), SERT (assay ID Hs00169010_m1), and GATA-4 (assay ID Hs00171403_m1) using the comparative delta-CT method with /H2-microglobulin as the endogenous control.

Western ImmunobLOTS, Immunoprecipitation, and ELISA

Cell lysates and nuclear and cytoplasmic extracts were prepared for Western immunobLOTS as described in the online data supplement. Analysis of ERK1/2 phosphorylation by ELISA was performed using antibodies recognizing ERK1/2 pERK1/2 (pTpY185/187) and ERK1/2 (total) (Biosource International).

Immunofluorescence and Confocal Microscopy

hPA-SMC were seeded into 8-well chamber slides at 5 x 10^3 cells per well and stimulated as described above. The cells were fixed using 3% (weight/volume) paraformaldehyde before incubation with monoclonal mouse anti–phosphorylated ERK1/2 antibody (1:400) (Cell Signaling Technologies). Immunofluorescent detection was performed as described on the online data supplement.

Lentiviral Overexpression System

To overexpress human SERT, the full-length cDNA (a kind gift from Dr R. Blakely, Vanderbilt University, Nashville, Tenn), was cloned into the pLenti6/V5 expression plasmid via the Directional TOPO Cloning kit (Invitrogen). Lentivirus was propagated and cells transfected as described in the online data supplement. SERT expression was monitored at 48 hours by Western immunoblot and quantitative RT-PCR.

RNA-Interference Transfections

RNA-interference was induced by transient transfection using 100 nmol/L short-interfering RNA (siRNA) oligonucleotides complexed
Statistical Analyses

Statistical analysis was performed using a repeated-measure ANOVA followed by the Newman–Keuls post hoc test with a 95% confidence level where \( P < 0.05 \) was deemed statistically significant.

Results

5-HT Induces S100A4/Mts1 Expression and Release

Quiescent hPA-SMC were stimulated for 24 hours with a dose range of 0.001 to 10 \( \mu \text{mol/L} \) 5-HT. A significant elevation in S100A4/Mts1 mRNA level was seen at 10 \( \mu \text{mol/L} \), although incremental trends were apparent with all the lower doses (Figure 1A); 10 \( \mu \text{mol/L} \) 5-HT was used in all subsequent studies. The S100A4/Mts1 mRNA level, as measured by TaqMan PCR, was increased by 6 hours following 5-HT stimulation, with the maximal response (-3-fold) seen at 24 hours (Figure 1B). A comparable increase in S100A4/Mts1 protein was documented at the same time points by densitometric analysis of Western immunoblots from whole cell lysates (Figure 1C). The level of S100A4/Mts1 protein in concentrated conditioned media (CM) 24 hour following 5-HT stimulation, was similarly increased relative to unstimulated control cells (Figure 1D).

Conditioned Medium From 5-HT–Stimulated Cells Induces Proliferation and Migration of PA-SMC via S100A4/Mts1 and RAGE

An increase in cell counts 48 hours following stimulation with 500 ng/mL recombinant S100A4/Mts1 (rS100A4/Mts1) was repressed by preincubation with a soluble form of RAGE (sRAGE) (2.5 \( \mu \text{g/mL} \)), in contrast to a similar degree of cell proliferation observed in response to rS100A4/Mts1 or to the PDGF-BB (2.5 \( \mu \text{g/mL} \)) (Figure 2A). CM collected from PA-SMC 24 hour following 5-HT stimulation was similarly increased relative to unstimulated control cells (Figure 2B). CM from 5-HT stimulated cells but had no effect on the PDGF-BB mediated response (Figure 2B). Similar results were observed when we examined the effect of CM from 5-HT–stimulated hPA-SMC on hPA-SMC migration assessed in Boyden chamber assays (Figure 2C). These studies indicated that 5-HT–mediated production and release of S100A4/Mts1 induce proliferation and migration of hPA-SMC in a RAGE-dependent fashion.

5-HT induced SERT expression in pLenti-lacZ cells and further increased SERT expression in cells infected with pLenti-hSERT (Figure 3A). This correlated with an elevated S100A4/Mts1 protein level in the cell lysates of pLenti-hSERT–infected cells (Figure 3B) and a further increase following stimulation with 5-HT (Figure 3B). S100A4/Mts1 mRNA measured by TaqMan PCR in lungs of transgenic mice overexpressing human SERT (5HTT\(^{+/-}\))\(^{2} \) was increased 2-fold compared with littermate controls (Figure 3C).

SERT and 5-HT Receptors Are Both Required for 5-HT–Induced S100A4/Mts1 Expression

Human PA-SMC transfected with siRNA oligonucleotides targeting SERT reduced levels by ~90% as measured by TaqMan RT-PCR at 48 hours (data not shown) and to barely detectable levels in cell lysates 72 hours posttransfection (Figure 4A). 5-HT stimulation of the SERT siRNA–transfected cells failed to increase S100A4/Mts1 expression, in contrast to control hPA-SMC or cells transfected with a scrambled SERT siRNA sequence or a nonsilencing siRNA oligonucleotide encoding luciferase (Figure 4B). Further experiments showed that inhibi-

![Figure 4](https://example.com/figure4.png)

Figure 4. SERT and 5-HT receptors are both required for 5-HT–induced S100A4/Mts1 expression. A and B, hPA-SMC were transfected with 100 \( \text{nmol/L} \) scrambled siRNA (SCR si), luciferase siRNA (Luc si), or SERT siRNA (SERT si) oligos and serum starved for 48 hours before stimulation with serum for 24 hours. A, Representative Western immunoblot of SERT protein (70 kDa) following siRNA transfection. Ctrl indicates control. B, Representative Western immunoblot (top) and densitometric analysis (bottom) of S100A4/Mts1 (Mts1) protein (11 kDa). C, 5-HT–mediated S100A4/Mts1 (Mts1) (11 kDa) expression in cells treated with 10 \( \mu \text{mol/L} \) fluoxetine (Fluo), a SERT inhibitor, 10 \( \mu \text{mol/L} \) ketanserin (Ket), a 5-HT receptor antagonist, and 0.1 \( \text{mmol/L} \) iproniazid (IPR), an MAO inhibitor, and with DMSO (vehicle) alone. D, 5-HT–induced increase in S100A4/Mts1 protein expression in cell lysates is not blocked by ketanserin at 0.1 \( \mu \text{mol/L} \) (selective for 5-HT\(_{2A}\)) but is reduced by 1 \( \mu \text{mol/L} \) and 10 \( \mu \text{mol/L} \) (likely to antagonize 5-HT\(_{2A}\), 5-HT\(_{2B}\), and 5-HT\(_{1A}\)). This response is not blocked by 0.2 \( \mu \text{mol/L} \) SB204741 (selective for 5-HT\(_{2B}\), Sigma) but is blocked by 0.1 \( \mu \text{mol/L} \) SB224289 (selective for 5-HT\(_{1A}\), Sigma). Bars represent mean \pm SEM from 4 replicate experiments, normalized as described in Figure 1. \( P < 0.05 \) vs non-5-HT–treated control cells. Ctrl indicates control; Inc., increase.
tion of SERT activity using fluoxetine also failed to induce S100A4/Mts1 in response to 5-HT (Figure 4C). Inhibition of MAO-A with iproniazid demonstrated that suppression of events downstream of SERT, such as 5-HT breakdown by MAO-A activity, also prevented induction of S100A4/Mts1 in response to 5-HT (Figure 4C). A similar inability of 5-HT to induce production of S100A4/Mts1 was observed by blocking 5-HT receptors with ketanserin (Figure 4C), and selective inhibitors revealed the specific involvement of the 5-HT<sub>1B</sub> receptor in mediating production of S100A4/Mts1. Figure 4D shows that the 5-HT–induced increase in S100A4/Mts1 protein expression in cell lysates is not blocked by ketanserin at 0.1 μmol/L (selective for 5-HT<sub>2A</sub>) but is reduced by 1 μmol/L and 10 μmol/L (likely to antagonize 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>1B</sub>). This response is not blocked by 0.2 μmol/L SB204741 (selective for 5-HT<sub>2A</sub>; Sigma) but is blocked by 0.1 μmol/L SB224289 (Sigma, selective for 5-HT<sub>1B</sub>) (Figure 4D).

5-HT Receptor–Mediated ERK1/2 Phosphorylation Is Necessary to Induce S100A4/Mts1
Serotonin-mediated ERK1/2 phosphorylation (pERK) has been implicated in the induction of genes involved in SMC proliferation, such as cyclin D2. In keeping with these observations, concurrent treatment with 5-HT and the pERK inhibitor PD98059 (100 μmol/L) prevented the induction of S100A4/Mts1 mRNA (Figure 5A) and protein in cell lysates (Figure 5B) compared with treatment with 5-HT alone. A 4-fold increase in pERK was observed 5 minutes after 5-HT (10 μmol/L) stimulation, as assessed by ELISA and verified by Western immunoblot (Figure 5C). This increase in pERK was blocked by the 5-HT receptor antagonist ketanserin, but not by inhibitors of SERT (fluoxetine) or MAO-A activity (iproniazid), when compared with treatment with vehicle alone (DMSO) (Figure 5C).

The antibody used in the pERK ELISA identified tyrosine/threonine phosphorylation at amino acids 185/187. To rule out the possibility that the SERT pathway initiates phosphorylation of an alternate site, at amino acids 202/204, we compared our findings by ELISA with those on Western immunoblot using a monoclonal antibody recognizing pERK202/204, but results were similar (Figure 5D).

Blockade of SERT and MAO Reduces the Nuclear Translocation of pERK1/2
We therefore determined whether SERT, MAO-A activity, or generation of reactive oxygen species (ROS) mediate pERK1/2 nuclear translocation. Following 5-HT stimulation, pERK was significantly increased in the nuclear fraction (Figure 6), but this was prevented following treatment with fluoxetine, ketanserin, iproniazid, and the free radical (ROS) scavenger N-acetyl cysteine (NAC) (Figure 6A). No cytoplasmic pERK remained following 5-HT treatment alone or with the inhibitor vehicle (DMSO), consistent with nuclear translocation (Figure 6B), but treatment with fluoxetine, iproniazid, or NAC resulted in persistent pERK in the cytoplasmic extracts (Figure 6B). Ketanserin, the 5-HT receptor blocker, repressed pERK in both nuclear and cytoplasmic fractions.

Immunofluorescent staining of hPA-SMC provided further evidence that a signal from the 5-HT receptor causes phosphorylation of ERK1/2 and that SERT, MAO-A, and ROS-mediated events result in nuclear translocation of pERK (Figure 6C). Clear punctuate nuclear localization of pERK1/2 was observed following stimulation with 5-HT compared with unstimulated control cells. Inhibition of SERT and MAO-A (fluoxetine and iproniazid, respectively) reduced the nuclear translocation of pERK1/2, with enhanced cytoplasmic pERK1/2 evident. Little nuclear pERK1/2 was observed in cells treated with either the 5-HT receptor antagonist ketanserin (Figure 6C) or with PD98059 (data not shown).

Phosphorylation of GATA-4 Is Required for 5-HT–Induced S100A4/Mts1 Expression
Serotonin-induced expression of cyclin D2 in PA-SMC has been previously attributed to pERK-mediated phosphorylation of the transcription factor GATA-4, and analysis of the promoter region of the S100A4/Mts1 gene revealed a GATA-4 binding site. We, therefore, used siRNA-targeting
GATA-4 and achieved a 90% knock down in GATA-4 mRNA (data not shown) and pGATA-4 protein (Figure 7A). We subsequently showed that this level of reduction in GATA-4 prevented 5-HT–induced S100A4/Mts1 expression in cell lysates (Figure 7B). 5-HT–induced phosphorylation of GATA-4 was blocked with ketanserin, as well as with fluoxetine, iproniazid, and the inhibitor of pERK1/2, PD98059 (Figure 7C).

**Discussion**

There is an increase in a SERT polymorphism in PAH patients that results in SERT overexpression, and 5-HT receptor and SERT knockout transgenic mice are equally protected against the development of PAH. This study shows how a 5-HT receptor and SERT cooperatively interact to regulate S100A4/Mts1, a gene induced in clinical PAH. The use of selective 5-HT receptor blockers identified the 5-HT\textsubscript{1B} receptor as involved in the regulation of S100A4/Mts1, and this receptor has been implicated in PAH. S100A4/Mts1, secreted into the media following 5-HT stimulation, can facilitate both the proliferation and migration of hPA-SMC in a manner dependent on the receptor, RAGE. Heightened stimulation of S100A4/Mts1 occurs when the level of SERT is increased in hPA-SMC cultures and in a transgenic mouse. Although 5-HT receptor activity is required for phosphorylation of ERK, nuclear transport of phosphorylated ERK depends on SERT, MAO-A activity, and ROS production in these hPA-SMC. Nuclear transloc-
tion of pERK is necessary for phosphorylation of GATA-4, a transcription factor required for expression of S100A4/Mts1, in response to 5-HT. A diagram of this proposed model is shown in Figure 8.

The mitotic and vasconstrictive roles of 5-HT in PA-SMC have been well documented,1,5,6,26,30 and recent work has highlighted the regulation of genes specific to cell cycle progression12 but not those selectively identified with disease. Although previous studies have shown that the 5-HT pathway can regulate other members of the S100 family,14 we now present an intriguing link between 5-HT and PVD through the induction of S100A4/Mts1 gene expression. We recognize that the concentration of 5-HT that produced a significant increase in S100A4/Mts1 was higher than circulating levels, but local concentrations could also be higher when released from platelets, neuropithelial cells, and mast cells. In addition, the availability of SERT would limit the intracellular accumulation of 5-HT to within physiological levels. Previous studies have shown that a small subset of transgenic mice that overexpress S100A4/Mts1 have PVD15 and that increased expression of S100A4/Mts1 is localized to the neointima in advanced PVD lesions.15 We did not detect S100A4/Mts1 expression in human PA endothelial cells in those studies, but its ability to stimulate proliferation and migration of PA endothelial cells17,18 may also be relevant to the pathology of PVD.31

Expression of RAGE is increased in cancer,32 as well as in atherosclerosis,33 restenosis,34 and diabetic vasculopathy.35 Our studies show that RAGE is necessary for S100A4/Mts1-mediated effects in hPA-SMC and are in keeping with RAGE being the receptor for this ligand in these cells. Other studies by our group, in which RAGE coimmunoprecipitates with S100A4/Mts1 in hPA-SMC lysates, reinforce this concept (data not shown).

Because heightened expression of S100A4/Mts1 in response to 5-HT is observed when SERT is overexpressed, it would be interesting to investigate whether enhanced S100A4/Mts1 expression is linked to patients with the increased incidence of the SERT polymorphism associated with heightened serotonin transport.

It has previously been shown in bovine PA-SMC that 5-HT transport via SERT results in the production of ROS including both superoxide36 and H2O2.37 Recent studies in cardiac myocytes have suggested that intracellular 5-HT metabolism by MAO-A is necessary for the generation of ROS.38 Other studies show that stimulation of cells with 5-HT can induce ROS via NADPH oxidase.39 We established that phosphorylation of ERK1/2 is required for 5-HT–mediated S100A4/Mts1 production in hPA-SMC and is specifically related to the activity of the 5-HT1B receptor. The signal necessary for nuclear translocation of pERK comes via SERT, MAO-A activity, and ROS40 production. These observations are in keeping with studies by Greene et al23 and Grewal et al24 but differ from those of Fanburg and coworkers,12,41–43 who proposed that ERK phosphorylation occurs via SERT-mediated ROS production. These discrepancies may be explained by differences in expression of the 5-HT receptor subtypes among species and cell type (reviewed by Hoyer et al44), as documented by Lee et al.36
RhoA and its downstream target ROCK control the nuclear localization of serum response factor and facilitate the nuclear translocation of pERK1/2 in bovine PA-SMC in a manner dependent on ROS. How ROS mediate nuclear translocation of pERK1/2 remains to be determined. It is possible that ROS modify a protein such as Sef (similar expression to fgf genes) or apolipoprotein D that block pERK1/2 nuclear translocation.

Phosphorylation of GATA-4 following nuclear translocation of pERK1/2 was required for 5-HT induction of S100A4/Mts1 expression. GATA-4 is an important transcription factor in cardiac development, hypertrophic responses, and in the formation of coronary arteries, but the role of cardiac myocyte expression of S100A4/Mts1 in these processes has not been investigated. It is also possible that other stimuli, such as endothelin-1 or α1-adrenergic agonists, that lead to the phosphorylation of GATA-4 at serine 105 via the MEK/ERK pathway may regulate S100A4/Mts1.

Mutations in bone morphogenetic protein receptor II have been linked to a heightened risk of developing PVD in patients with IPAH, but only 20% of affected family members succumb to the disease. Although loss of bone morphogenetic protein receptor II does not further induce 5-HT–mediated S100A4/Mts1 production (our unpublished data), it may enhance the cellular effects of S100A4/Mts1. Our results, in showing the interdependency of events mediated by the 5-HT1B receptor and by SERT, suggest that inhibition of either pathway may be sufficient to block the development of experimental PAH and PVD.

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Figure 8. Schema describing cooperative interaction between 5-HT1B receptor and SERT in mediating S100A4/Mt1 expression and cellular sequelae. 5-HT–induced expression of S100A4/Mt1 requires interdependent signals from the 5-HT1B receptor and SERT. The 5-HT1B receptor mediates the phosphorylation of ERK in response to 5-HT, whereas 5-HT transport, via SERT, MAO-A, activity, and production of free radicals (ROS), allow the translocation of pERK to the nucleus. Once in the nucleus, pERK phosphorylates GATA-4, which leads to S100A4/Mt1 expression. S100A4/Mt1 is secreted from the cell, where it then interacts with RAGE to mediate proliferation and migration of PA-SMC.


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Supplemental Materials and Methods:

**Cell Culture:** Human PA-SMC were purchased from Cascade Biologics (Portland, OR, USA) and maintained in Medium 231 supplemented with Smooth Muscle Growth Supplement (SMGS), and with 100U/ml penicillin G, 100µg/ml Streptomycin sulfate, and 0.25µg/ml Amphotericin B (PSA Solution) (all Cascade Biologics). Cells were received at passage 3 and used between passages 5 and 9.

**Serotonin (5-HT related Reagents):** Serotonin (5-Hydroxytryptamine, 5-HT) the creatinine sulfate complex (Sigma H7752), kentanserin, fluoxetine, iproniazid and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The ERK 1/2 inhibitor PD98059 was purchased from Calbiochem (San Diego, CA, USA). Selective 5-HT receptor antagonists were purchased from Sigma (SB204741 selective for 5-HT$_{2A}$ and SB224289 selective for 5-HT$_{1B}$). Recombinant S100A4/Mts1 and rabbit anti-S100A4/Mts1 polyclonal antibody were provided by Dr. N. Ambartsumian. Soluble RAGE and rabbit polyclonal anti-RAGE antibody were provided by Dr. A-M Schmidt.

**Lentivirus Propagation:** Lentivirus was propagated in 293FT packaging cells (Invitrogen) according to the manufacturer’s instructions. The viral supernatant was collected 48h following transfection of the packaging cells, filtered and stored at ~80° C until required. The viral supernatant was used in a 1:1 dilution in complete M231 media, and added to the cells overnight.

**Western Immunoblots:** Cell lysates were prepared by adding boiling lysis buffer (10mM Tris.HCl, 1mM Sodium orthovanadate, 1% SDS (all Sigma-Aldrich) and 1X protease inhibitor
cocktail (Roche, Indianapolis, IN, USA) to the cells, incubating for 5min prior to scraping into a 1.5ml microcentrifuge tube and boiling for 10min prior to centrifugation. The supernatants were transferred to fresh microcentrifuge tubes and protein concentration measured using the Lowry based DC Protein Assay (Bio-Rad, Hercules, CA, USA). Nuclear and cytoplasmic extracts were prepared using Nuclear and Cytoplasmic Extraction Reagents (NE-PER, Pierce Biotechnology, Rockford, IL, USA).

Twenty μg of each sample were loaded on a 4-12% Bis-Tris NuPage gel and run under reducing conditions in MES running buffer (Invitrogen) prior to transfer to a nitrocellulose membrane (Invitrogen). Transfer was confirmed with Ponceau S (Sigma) staining and the membrane was then blocked for 1h in 5% non-fat milk at room temperature. The blots were incubated with rabbit anti-S100A4/Mts1 antibody (1:2500) (N. Ambartsumian) and mouse anti-tubulin antibody (1:5000) (Sigma) for 2h at room temperature unless stated. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (1:5000) (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) were incubated for 1h at room temperature prior to performing an enhanced chemiluminescence (ECL, Amersham Biosciences) reaction and exposure to autoradiographic film (Amersham Biosciences).

**Cell counting (Coulter counter method):** Cells were seeded at 2.5x10⁴ cells per well of a 24-well plate in 500μl of growth medium and allowed to adhere overnight. The medium was removed and the cells washed 3 times with D-PBS prior to the addition of DMEM containing 0.2% FBS (SF) and incubated at 37°C, 5% CO₂ for 48h prior to stimulation (treatments and concentrations stated in the paper) for 48h. The medium was then removed, cells washed and
trypsinized in 500µl of Trypsin/EDTA (Cascade Biologics). The trypsinized cells were added to 9.5ml of Isoton (BD Biosciences), and cell counts were calculated from the average of 3 measurements of duplicate wells on the coulter counter.

**Design of oligonucleotides for RNA-interference transfection:** Two siRNA oligonucleotides were designed, one targeting the SERT DNA sequence AAGAACTCCTGGAACACTGGC (Forward Sequence GAACUCCUGGAACACUGG CTT, Reverse Sequence GCCAGUGUUCCAGGAGUUCTT) and a scrambled sequence (Forward Sequence ACAACUCGUGCAAUACCAGGUU Reverse Sequence CCGGUAUUGCAGACGUUG UUUCTT). siRNA oligos for GATA-4 (SMARTpool), luciferase (control siRNA duplex) and human cyclophilin B (siCONTROL) were purchased from Dharmacon (Lafayette, CO, USA).

**Immunofluorescence detection:** Immunofluorescent detection was performed using goat anti-mouse Alexa Fluor® 594nm secondary antibody (Molecular Probes, Eugene, OR, USA) and mounted using SlowFade® anti-fade with DAPI (Molecular Probes). Images were acquired on a Zeiss LSM 510 two-photon confocal laser scanning microscope. Confocal micrographs were processed with Openlab 3.1.4 and Volocity 3.0 software (Improvision, Coventry, UK).