Serotonin Receptor Agonist 5-Nonyloxytryptamine Alters the Kinetics of Reovirus Cell Entry

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ABSTRACT

Mammalian orthoreoviruses (reoviruses) are nonenveloped double-stranded RNA viruses that infect most mammalian species, including humans. Reovirus binds to cell surface glycans, junctional adhesion molecule A (JAM-A), and the Nogo-1 receptor (depending on the cell type) and enters cells by receptor-mediated endocytosis. Within the endocytic compartment, reovirus undergoes stepwise disassembly, which is followed by release of the transcriptionally active viral core into the cytoplasm. In a small-molecule screen to identify host mediators of reovirus infection, we found that treatment of cells with 5-nonyloxytryptamine (5-NT), a prototype serotonin receptor agonist, diminished reovirus cytotoxicity. 5-NT also blocked reovirus infection. In contrast, treatment of cells with methiothepin mesylate, a serotonin antagonist, enhanced infection by reovirus. 5-NT did not alter cell surface expression of JAM-A or attachment of reovirus to cells. However, 5-NT altered the distribution of early endosomes with a concomitant impairment of reovirus transit to late endosomes and a delay in reovirus disassembly. Consistent with an inhibition of viral disassembly, 5-NT treatment did not alter infection by *in vitro*-generated infectious subviral particles, which bind to JAM-A but bypass a requirement for proteolytic uncoating in endosomes to infect cells. We also found that treatment of cells with 5-NT decreased the infectivity of alphavirus chikungunya virus and coronavirus mouse hepatitis virus. These data suggest that serotonin receptor signaling influences cellular activities that regulate entry of diverse virus families and provides a new, potentially broad-spectrum target for antiviral drug development.

IMPORTANCE

Identification of well-characterized small molecules that modulate viral infection can accelerate development of antiviral therapeutics while also providing new tools to increase our understanding of the cellular processes that underlie virus-mediated cell injury. We conducted a small-molecule screen to identify compounds capable of inhibiting cytotoxicity caused by reovirus, a prototype double-stranded RNA virus. We found that 5-nonyloxytryptamine (5-NT) impairs reovirus infection by altering viral transport during cell entry. Remarkably, 5-NT also inhibits infection by an alphavirus and a coronavirus. The antiviral properties of 5-NT suggest that serotonin receptor signaling is an important regulator of infection by diverse virus families and illuminate a potential new drug target.

As obligate intracellular pathogens, viruses exploit broadly conserved cellular processes to internalize, replicate, assemble, and release to infect adjacent cells and tissues. To counteract viral infection, hosts have evolved a variety of innate and adaptive immune mechanisms that antagonize these viral replication steps. Understanding host components that regulate the outcome of viral infection can uncover previously overlooked cellular pathways that might serve as new targets for antiviral therapeutics and enhance design of viral vectors and vaccines.

Nonfusogenic mammalian orthoreoviruses (called reoviruses here) are nonenveloped, double-stranded RNA viruses that are formed from two concentric protein shells (1). Reoviruses infect most mammalian species, and although most humans are exposed to reovirus during childhood, infection seldom results in disease (1, 2). Reovirus infects primary and transformed cells in culture, although infection of transformed cells is more efficient (3–5). Infection of host cells by reovirus results in cell death via caspase-dependent and caspase-independent pathways (6–8). Because of its enhanced replication in transformed cells and its efficient cell killing capacity, reovirus is currently being tested in clinical trials for efficacy as an oncolytic agent against a variety of human cancers (9, 10).

Reovirus attaches to host cells via interactions with cell surface glycans (11, 12), junctional adhesion molecule A (JAM-A) (13–15), and the Nogo-1 receptor (NgR1) (16), depending on the cell type. In JAM-A-expressing cells, reovirus is internalized in a β 1 integrin-dependent manner via receptor-mediated endocytosis (17). Following internalization, reovirus activates Src kinase (18) and traverses through Rab5-positive early endosomes and Rab7- and Rab9-positive late endosomes (19, 20) in a process dependent on functional microtubules (21). In the acidic environment of late endosomes, virions undergo stepwise proteolytic disassembly cat-

Received 18 March 2015 Accepted 17 June 2015

Accepted manuscript posted online 24 June 2015

Citation Mainou BA, Ashbrook AW, Smith EC, Dorset DC, Denison MR, Dermody TS. 2015. Serotonin receptor agonist 5-nonyloxytryptamine alters the kinetics of reovirus cell entry. *J Virol* 89:8701–8712. doi:10.1128/JVI.00739-15.

Editor: D. S. Lyles

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doi:10.1128/JVI.00739-15

alyzed by cysteine cathepsin proteases to form infectious subviral particles (ISVPs) (22–24). During ISVP formation, outer capsid protein $\sigma 3$ is removed, and outer capsid protein $\mu 1$ is cleaved, releasing fragments that mediate endosomal membrane penetration and delivery of the transcriptionally active viral core into the cytoplasm (23–25). Host factors that trigger internalization and intracellular transport of reovirus virions are not completely understood.

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter present in invertebrates and vertebrates (26, 27). In mammals, the majority of serotonin is found within the intestinal lumen, where enterochromaffin cells release 5-HT in response to a broad range of stimuli, which include cholera toxin, rotavirus, and *Salmonella enterica* serotype Typhimurium (28–30). 5-HT initiates signals by engagement of 5-HT receptors (5-HTRs), which influence a variety of cellular functions, including cell survival and proliferation through NF- κ B signaling in cardiomyocytes, inflammation via production of interleukin 1 β (IL-1 β), IL-6, and IL-8, leukocyte migration, and phagocytosis (31–35). There are at least 15 5-HTRs in humans that are grouped within seven families based on structure and signaling properties (36, 37). 5-HTRs are expressed in many cell types, including airway epithelial cells, renal epithelial cells, neurons, peripheral blood mononuclear cells, and dendritic cells (31–33, 38–40). The affinity of 5-HT for 5-HTRs can be altered by the addition of unbranched alkyl groups to the ligand (41, 42). 5-Nonyloxytryptamine (5-NT) has a substitution at indole position 5, which enhances its affinity for some 5-HTRs in the 5-HTR1 and 5-HTR2 families while also binding to 5-HTRs in other families, albeit with lower affinity (42). 5-HTR2 facilitates cell entry of JC polyomavirus, a nonenveloped DNA virus, and treatment of cells with 5-HT impairs JC polyomavirus infection (43–45). Despite the many properties of 5-HT, it is not known whether 5-HT-dependent signaling affects the replication of RNA viruses.

In this study, we show that 5-NT inhibits reovirus infection by impeding cell entry. Treatment of cells with 5-NT did not affect reovirus attachment or internalization but instead delayed the intracellular transport of incoming virions by altering the distribution of early endosomes with a concomitant decrease in disassembly kinetics. The cell entry impairment induced by 5-NT resulted in decreased infectivity and impaired cell killing by reovirus. Infectivity of chikungunya virus (CHIKV) and mouse hepatitis virus (MHV), two unrelated RNA viruses, was also diminished by 5-NT. These findings suggest that signaling initiated by 5-NT leads to antiviral activities against diverse RNA viruses.

MATERIALS AND METHODS

Cells, viruses, chemical inhibitors, and antibodies. Spinner-adapted murine L929 cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (JMEM) (Lonza) supplemented to contain 5% fetal bovine serum (FBS) (Life Technologies) and 2 mM L-glutamine (Life Technologies). CCL2 HeLa cells (obtained from Carolyn Coyne, University of Pittsburgh) and HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented to contain 10% FBS, MEM nonessential amino acid solution (Sigma), and 0.11 mg sodium pyruvate (Sigma) per ml. U-2 OS cells (gift from Anne Kenworthy, Vanderbilt University) were grown in McCoy's 5A medium (Life Technologies) supplemented to contain 10% FBS and 2 mM L-glutamine. Vero cells (ATCC CCL-81) and baby hamster kidney cells (BHK-21; ATCC CCL-10) were grown in alpha-minimal essential medium (α MEM; Life Technologies) supplemented to

contain either 5% FBS or 10% FBS and 10% tryptose phosphate (Sigma), respectively. Baby hamster kidney cells expressing the MHV receptor (BHK-MHVR) were grown in DMEM supplemented to contain 10% FBS and 10 mM HEPES (46, 47). Delayed brain tumor 9 (DBT-9) cells were grown in DMEM supplemented to contain 10% FBS and 10 mM HEPES. All cells were also supplemented to contain 100 U of penicillin per ml, 100 μ g of streptomycin per ml (Life Technologies), and 0.25 mg of amphotericin B per ml (Sigma).

Reovirus strain type 3 SA+ (T3SA+) working stocks were prepared by plaque purification and passage using L929 cells (48). Purified virions were prepared using second-passage L929 cell lysate stocks. Virus was purified from infected-cell lysates by Freon or Vertrel XF (DuPont) extraction and CsCl gradient centrifugation as described previously (49). The band corresponding to the density of reovirus particles (1.36 g/cm³) was collected and dialyzed exhaustively against virion storage buffer (150 mM NaCl, 15 mM MgCl₂, 10 mM Tris-HCl [pH 7.4]). Reovirus particle concentration was determined from the equivalence of 1 unit of optical density at 260 nm to 2.1×10^{12} particles (50). Viral titers were determined by plaque assay using L929 cells (48). ISVPs were generated by treating particles with α -chymotrypsin (Sigma) as described previously (18). Reovirus virions were labeled with succinimidyl ester Alexa Fluor 546 (A546) or pHrodo SE (pHrodo) (Life Technologies) as described previously (19, 21).

CHIKV strain SL15649 virus stocks were prepared using full-length virus infectious clones as described previously (21, 51). Plasmids containing virus cDNAs were linearized by digestion with NotI-HF (NEB). Capped, full-length RNA transcripts were generated *in vitro* using mMessage mMachine SP6 transcription kits (Ambion) and introduced into BHK-21 cells by electroporation using a Gene Pulser electroporator (Bio-Rad). Culture supernatants were harvested 24 h after electroporation and clarified by centrifugation. Virus stocks were purified by ultracentrifugation of clarified supernatants through a 20% sucrose cushion in TNE buffer (50 mM Tris-HCl [pH 7.2], 0.1 M NaCl, and 1 mM EDTA) at $115,000 \times g$. Virus pellets were resuspended in virus dilution buffer (RPMI 1640 medium containing 20 mM HEPES [Gibco] supplemented to contain 1% FBS) and stored at -80°C . Virus titers were determined by plaque assay using Vero cells.

MHV-A59 replicase reporter virus (MHV-FFL2) was prepared as described previously (52). RNA transcripts from MHV cDNA were generated *in vitro* using mMessage mMachine T7 transcription kits. Transcripts were electroporated into BHK-MHVR cells. Transfected cells were seeded onto a layer of DBT-9 cells and incubated at 37°C for 30 to 90 h. Virus viability was confirmed by syncytium formation.

Ammonium chloride (NH₄Cl) (Gibco) was resuspended in water. E64-d, 5-nonyloxytryptamine oxalate (5-NT) (Tocris), and methiothepin mesylate (MM) (Sigma) were resuspended in dimethyl sulfoxide (DMSO). The immunoglobulin G (IgG) fraction of a rabbit antiserum raised against reovirus strains T1L or T3D was purified as described previously (11). Reovirus $\sigma 3$ -specific monoclonal antibody 10C1 (53), α -tubulin monoclonal antibody (DM1A), early endosome antigen 1 (EEA1) monoclonal antibody (C45B10) (Cell Signaling Technologies), and lysosome-associated membrane protein 1 monoclonal antibody (LAMP1) (H4A3; Abcam) were used for indirect immunofluorescence experiments. JAM-A-specific monoclonal antibody (J10.4; provided by Charles Parkos, University of Michigan) was used for flow cytometry experiments. CHIKV-specific antiserum (ATCC) was used for infectivity assays. Alexa Fluor-conjugated antibodies (Life Technologies) were used as secondary antibodies.

Biosafety. All studies using viable CHIKV SL15649 were conducted in a certified biological safety level 3 facility in biological safety cabinets with protocols approved by the Vanderbilt University Department of Environment, Health, and Safety and the Vanderbilt Institutional Safety Committee.

Cell viability assay. HeLa S3 cells were incubated with Opti-MEM-I (OMEM-I; Life Technologies) medium containing DMSO, E64-d,

NH₄Cl, or 5-NT at 37°C for 1 h and adsorbed with T3SA+ at a multiplicity of infection (MOI) of 200 PFU/cell in the presence of DMSO, E64-d, NH₄Cl, or 5-NT in OMEM-I medium at 37°C for 48 h. Cell viability was quantified using a Cell Titer-Glo assay (Promega) and a Synergy H1 plate reader (Biotek).

Serotonin receptor RT-PCR. RNA was isolated from CCL2 HeLa, HeLa S3, and U-2 OS cells using an RNeasy minikit (Qiagen). RNA (50 ng) was converted to cDNA by reverse transcription-PCR (RT-PCR) using a OneStep RT-PCR kit (Qiagen) and the 5-HTR-specific primers previously described (39) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific sense (5'-CCCATCACCCTCTCCAG-3') and antisense (5'-ATGACCTTGCCCACAGCC-3') primers. Following RT-PCR, aliquots of the reaction products were resolved by electrophoresis in 1% agarose gels (Life Technologies).

Quantification of reovirus infectivity. Reovirus infectivity was assessed by indirect immunofluorescence (54). The cells were incubated with complete medium containing DMSO, E64-d, NH₄Cl, MM, or 5-NT at 37°C for 1 h, adsorbed with reovirus virions or ISVPs at room temperature for 1 h, and incubated with complete medium containing DMSO, E64-d, NH₄Cl, MM, or 5-NT at 37°C for 20 h. The cells were fixed and stained with reovirus-specific antiserum and goat anti-rabbit IRDye 800 (LI-COR), DRAQ5 (Cell Signaling), and Sapphire700 (LI-COR). Immunofluorescence was detected using a LI-COR Odyssey infrared imaging system (LI-COR), and infectivity was quantified using the In-Cell Western feature of the Odyssey software suite.

Flow cytometric analysis of cell surface JAM-A expression, reovirus attachment, and reovirus exposure to acidified organelles. CCL2 HeLa cells were treated with DMSO, 5-NT, or NH₄Cl in complete medium at 37°C for 1 h and adsorbed with A546-labeled reovirus at an MOI of 5×10^3 particles/cell at room temperature for 1 h. The inoculum was removed, and cells were incubated with complete medium containing DMSO, 5-NT, or NH₄Cl for various intervals. The cells were detached with Cellstripper (Cellgro) at 37°C for 15 min, quenched with fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% FBS), and stained with JAM-A-specific antibody and reovirus-specific polyclonal antiserum in FACS buffer at 4°C for 30 min. The cells were washed with FACS buffer, stained with Alexa Fluor-conjugated antibodies in FACS buffer at 4°C for 30 min, and fixed in PBS with 1% electron microscopy (EM)-grade paraformaldehyde (FACS Fix; Electron Microscopy Sciences). To calculate percent extracellular virus, the mean fluorescence intensity (MFI) obtained following staining with reovirus-specific polyclonal antiserum was divided by the MFI following adsorption of A546-conjugated reovirus virions for each sample.

Flow cytometric analysis of reovirus acidification was performed as described previously (19). The cells were treated with DMSO, 5-NT, or NH₄Cl in complete medium at 37°C for 1 h and adsorbed with pHrodo-labeled reovirus at an MOI of 5×10^3 particles/cell, incubated in complete medium containing DMSO, 5-NT, or NH₄Cl for various intervals, and fixed in FACS Fix.

Cell staining was assessed using a BD LSRII or LSRFortessa flow cytometer and quantified using FlowJo software.

Quantification of reovirus disassembly kinetics. CCL2 HeLa cells were treated with DMSO or 5-NT in complete medium at 37°C for 1 h, adsorbed with reovirus at an MOI of 10 PFU/cell at room temperature for 1 h, and incubated in complete medium with DMSO or 5-NT for various intervals. Whole-cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted using reovirus-specific polyclonal antiserum. The intensity of immunoblot bands was quantified by densitometric analysis using Odyssey software.

Confocal microscopy of reovirus-infected cells and cytoskeleton markers. CCL2 HeLa cells were incubated with complete medium containing DMSO or 5-NT at 37°C for 1 h. The cells were adsorbed with A546-labeled reovirus at an MOI of 10^4 particles/cell and either fixed with 5% formalin or incubated in complete medium containing DMSO or 5-NT at 37°C for various intervals, followed by fixation with 5% formalin.

The cells were quenched with 0.1 M glycine, washed with PBS, and stained with reovirus $\sigma 3$ -, EEA1-, or LAMP1-specific antibodies using permeabilizing conditions (PBS-BGT, which is PBS supplemented with 0.5% bovine serum albumin [BSA], 0.1% glycine, and 0.05% Tween 20), followed by Alexa Fluor 488 (A488)-labeled IgG, and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Coverslips were placed on slides using Aqua-Poly/Mount mounting medium (Poly-sciences, Inc.).

To visualize actin and tubulin, CCL2 HeLa cells were incubated with DMSO or 5-NT at 37°C for 1 h, fixed with 5% formalin, and quenched with 0.1 M glycine (actin) or ice-cold methanol (tubulin). The cells were washed with PBS, stained with A546-conjugated phalloidin (Life Technologies) or α -tubulin-specific antibody, followed by A488-labeled IgG, and counterstained with DAPI in PBS-BGT. Coverslips were placed on slides using Aqua-Poly/Mount mounting medium.

The images were captured using a Zeiss LSM 510 Meta laser-scanning confocal microscope using a 63 $\times$ Plan-Apochromat oil objective with a numerical aperture (NA) of 1.40. Pinhole size was identical for all fluors. The images were normalized for pixel intensity, brightness, and contrast. Single sections of 0.39- μ m thickness from a Z-stack are presented. Colocalization was quantified using the Profile function of LSM Image software (Zeiss) (18, 19, 21).

Cholera B toxin and transferrin internalization assays. CCL2 HeLa cells were incubated with complete medium containing DMSO or 5-NT at 37°C for 1 h, incubated with 5 μ g/ml of A488-conjugated cholera toxin B (CTB) (Life Technologies) or 5 μ g/ml of A488-conjugated transferrin (Tfn) (Life Technologies) in Live-Cell imaging solution (Life Technologies) for 30 min. The inoculum was removed, cells were incubated in acid wash (0.2 M acetic acid, 0.5 M NaCl) for 1 min, washed three times with PBS, detached with Cellstripper, and fixed in FACS Fix. Intracellular CTB and Tfn was assessed using a BD LSRII or LSRFortessa flow cytometer and quantified using FlowJo software.

CHIKV infectivity assay. U-2 OS cells were incubated with DMSO or 5-NT in complete medium at 37°C for 1 h and adsorbed with CHKV strain SL15649 at an MOI of 5 PFU/cell at 37°C for 1 h. The inoculum was removed, and the cells were incubated with complete medium containing DMSO or 5-NT at 37°C for 5 h. The cells were fixed with ice-cold 100% methanol and incubated with CHKV-specific polyclonal antiserum, A488-labeled IgG, and DAPI. U-2 OS cells were visualized using an ImageXpress Micro XL imaging system (Molecular Devices). Total and CHIKV-infected cells in four fields of view containing at least 100 cells per field were quantified using MetaXpress software (Molecular Devices). Percent infectivity was determined by enumerating the number of CHIKV-positive cells normalized to the total number of cells per field.

MHV infectivity assay. DBT-9 cells were incubated with DMSO or 5-NT in complete medium at 37°C for 1 h, adsorbed with MHV-FFL2 at an MOI of 1 PFU/cell at 37°C for 30 min, and incubated with DMSO or 5-NT in complete medium at 37°C for 10 h. MHV-FFL2 infectivity was assessed using a luciferase assay kit (Promega), and cell viability was assessed using Cell Titer-Glo in parallel wells using a Veritas luminometer (Turner BioSystems). Percent infectivity was calculated by normalizing luciferase units from MHV-FFL2-infected cells to light units from Cell Titer-Glo.

Statistical analysis. Mean values for at least triplicate experiments were compared using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test (Graph Pad Prism). *P* values of <0.05 were considered statistically significant.

RESULTS

Serotonin receptor agonist 5-nonyloxytryptamine impairs reovirus-induced cell death. In a previous study (21), we identified 11 compounds in a small-molecule screen for host factors required for reovirus-mediated cytotoxicity, including the serotonin receptor agonist 5-NT. To confirm these screening results, HeLa S3 cells, which are susceptible to reovirus-induced cell death

FIG 1 Serotonin agonist 5-nonyloxytryptamine (5-NT) impairs reovirus-mediated cytotoxicity. HeLa S3 cells were incubated with DMSO, increasing concentrations of 5-NT, 4 μM E64-d, or 20 mM NH₄Cl for 1 h, adsorbed with reovirus T3SA+ at an MOI of 200 PFU/cell, and incubated for 48 h. Cell viability was quantified using an ATP-dependent luminescence assay. Results are expressed as mean cell viability plus standard error of the mean (error bars) as determined by luminescence for five independent experiments. Values that are significantly different ($P < 0.05$) from the value for the control (DMSO) by one-way ANOVA with Dunnett's multiple-comparison test are indicated (**).

and were used in the original screen (21), were incubated with vehicle control (DMSO), increasing concentrations of 5-NT, or 4 μM cysteine protease inhibitor E64-d or 20 mM NH₄Cl as controls for 1 h, adsorbed with cytopathic reovirus strain T3SA+, incubated for 48 h in the presence of DMSO or inhibitors, and assessed for viability (Fig. 1). Treatment of cells with either DMSO, 0.1 μM 5-NT, or 1 μM 5-NT had no detectable effect on reovirus-mediated cell death. In contrast, cells treated with 5 μM or 10 μM 5-NT significantly impaired virus-induced cytotoxicity, albeit to a lesser extent than either E64-d or NH₄Cl. These data corroborate the screening results and show that 5-NT impairs reovirus-mediated cell killing.

5-HT receptors are expressed in HeLa cells and U-2 OS cells. 5-NT engages 5-HTRs on the cell surface (36). To determine whether CCL2 HeLa, HeLa S3, and U-2 OS cells, which were the cell lines chosen for this study, express 5-HTRs, we used RT-PCR to probe for expression of 10 different 5-HTRs (Fig. 2). CCL2 HeLa, HeLa S3, and U-2 OS cells displayed similar 5-HTR mRNA profiles. In these cells, mRNA was detected for all 5-HTRs tested

FIG 2 Serotonin receptors are expressed in HeLa cells and U-2 OS cells. RNA was extracted from CCL2 HeLa cells, HeLa S3 cells, and U-2 OS cells and subjected to RT-PCR for members of the serotonin receptor (5-HTR) family and GAPDH (G) as a control. The expected electrophoretic migration positions of PCR amplicons are indicated by asterisks.

FIG 3 Engagement of serotonin receptors modulates reovirus infection. (A) CCL2 HeLa cells were incubated with DMSO, increasing concentrations of 5-NT, or 4 μM E64-d for 1 h, adsorbed with reovirus T3SA+ at an MOI of 2 PFU/cell, and incubated in the presence of compounds for 20 h. The cells were scored for infection by indirect immunofluorescence. (B) CCL2 HeLa cells were incubated with DMSO, increasing concentrations of methiothepin mesylate (MM), or 4 μM E64-d for 1 h, adsorbed with reovirus T3SA+ at an MOI of 5 PFU/cell, and incubated in the presence of compounds for 20 h. The cells were scored for infection by indirect immunofluorescence. Results are expressed as percent infectivity (MFI compared to the DMSO value, normalized to background fluorescence) for triplicate experiments. Values are means plus standard errors of the means (error bars). **, $P < 0.05$ in comparison to DMSO values by one-way ANOVA with Dunnett's multiple-comparison test.

except for 5-HTR2B and 5-HTR7. Also, 5-HTR6 was detected in HeLa S3 cells and U-2 OS cells, but not in CCL2 HeLa cells. These findings indicate that CCL2 HeLa, HeLa S3, and U-2 OS cells express serotonin receptor mRNA.

Engagement of 5-HTRs regulates reovirus infectivity. To determine whether small molecules that agonize or antagonize 5-HTRs affect reovirus infectivity, CCL2 HeLa cells were treated with DMSO, increasing concentrations of 5-NT (Fig. 3A) or 5-HTR antagonist methiothepin mesylate (MM) (Fig. 3B), or 4 μM E64-d for 1 h, adsorbed with reovirus, incubated for 20 h in the presence of inhibitors, and scored for infection by indirect immunofluorescence. Concordant with the inhibition of reovirus-mediated cell death by 5-NT, treatment of cells with 10 μM 5-NT or 4 μM E64-d diminished reovirus infection, while treatment of cells with DMSO, 0.1 μM 5-NT, or 1 μM 5-NT did not. In contrast, treatment of cells with MM at concentrations of 5 μM or 10 μM enhanced reovirus infection relative to DMSO-treated cells. These results indicate that drugs that agonize or antagonize 5-HTRs have opposing effects on reovirus infectivity.

5-NT impairs reovirus infection at early times of infection. To determine the temporal window in which 5-NT inhibits reovirus infection, CCL2 HeLa cells were treated with DMSO, 4 μM E64-d, or 10 μM 5-NT at 15-min intervals beginning 60 min prior to adsorption (−60) or 60 min after adsorption (+60), incubated for 20 h, and scored for infection by indirect immunofluorescence

FIG 4 Serotonin agonist 5-NT diminishes reovirus infectivity at an early step in infection. (A) CCL2 HeLa cells were incubated with DMSO or 4 μ M E64-d for 1 h before adsorption or 10 μ M 5-NT at 15-min intervals prior to adsorption (–15 to –60) or 60 min after adsorption (+60). Cells were adsorbed with reovirus T3SA+ at an MOI of 2 PFU/cell and incubated in the presence of inhibitors for 20 h. Cells were scored for infection by indirect immunofluorescence. Results are expressed as percent infectivity (MFI compared to the DMSO value, normalized to background fluorescence) for quadruplicate samples. Values are means plus standard deviations (error bars). (B) CCL2 HeLa cells were incubated with DMSO, increasing concentrations of 5-NT, or 4 μ M E64-d for 1 h, adsorbed with reovirus T3SA+ virions at an MOI of 1 PFU/cell or ISVPs at an MOI of 0.1 PFU/cell, and incubated in the presence of inhibitors for 20 h. Cells were scored for infection by indirect immunofluorescence. Results are expressed as percent infectivity (MFI compared to the DMSO value, normalized to background fluorescence) for quadruplicate samples. Values are means plus standard deviations (error bars).

(Fig. 4A). Maximal impairment of reovirus infectivity was achieved when 5-NT was added to cells 60 min prior to adsorption (60% decrease), with gradual loss of inhibition when drug was administered 45 min (43% decrease), 30 min (42% decrease), or 15 min (22% decrease) before adsorption. Addition of 5-NT 60 min after adsorption resulted in negligible inhibition (5% decrease). These data suggest that 5-NT must be added to cells for at least 60 min prior to adsorption to substantially impair reovirus infection.

Reovirus ISVPs, which can be generated *in vitro* by protease treatment of virions (23, 24), bind to JAM-A and enter cells at or near the cell surface, bypassing a requirement for cathepsin proteolysis of the viral capsid in acidified endosomes (13, 20, 24, 56). To determine whether 5-NT treatment blocks infection following adsorption with ISVPs, CCL2 HeLa cells were treated with DMSO, increasing concentrations of 5-NT, or 4 μ M E64-d, adsorbed with reovirus virions or ISVPs, and scored for infection by indirect immunofluorescence after 20 h of incubation (Fig. 4B). Treatment of cells with the higher concentrations of 5-NT or 4 μ M E64-d decreased infection by virions. In contrast, treatment of cells with 5-NT or E64-d did not significantly diminish infection by ISVPs. These data suggest that 5-NT impairs reovirus infection at a point in the replication cycle after attachment to cells but before formation of ISVPs. Importantly, since treatment of cells with 10 μ M 5-NT does not alter infection by ISVPs, the inhibitory effect of 5-NT on infection by virions is not due to cytotoxicity of the compound.

Reovirus attachment and internalization are not affected by 5-NT. To determine whether 5-NT alters cell surface expression of reovirus receptor JAM-A or virus binding to cells, CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with Alexa Fluor 546-labeled reovirus, and assessed for cell surface JAM-A expression at 0, 1, and 2 h after adsorption (Fig. 5A) or reovirus binding at 0 h (Fig. 5B). 5-NT treatment did not significantly diminish the cell surface expression of JAM-A during the interval tested and did not alter reovirus attachment to cells. These data, in addition to the finding that 5-NT does not impair infection by ISVPs, which must bind JAM-A to initiate infection, indicate that 5-NT does not compromise reovirus attachment to target cells.

To determine whether 5-NT alters reovirus internalization, CCL2 HeLa cells were incubated with DMSO or 10 μ M 5-NT, adsorbed with Alexa Fluor 546-labeled reovirus, and incubated for 0, 1, or 2 h with DMSO or 10 μ M 5-NT. Extracellular virus was detected by staining cells with reovirus-specific antiserum using nonpermeabilizing conditions (Fig. 5C). At all times tested, reovirus internalization was comparable in cells treated with DMSO or 5-NT, with only a slight increase in the quantity of extracellular virus at 2 h in 5-NT-treated cells compared with DMSO-treated cells. These results suggest that 5-NT treatment does not impede internalization of reovirus.

5-NT impairs reovirus disassembly kinetics. Reovirus must be delivered to acidified endosomes to efficiently infect cells (19, 20, 57). To assess whether 5-NT alters reovirus access to acidified intracellular compartments, CCL2 HeLa cells were incubated with DMSO, 10 μ M 5-NT, or 20 mM NH_4Cl , adsorbed with reovirus conjugated with the pH-sensitive dye, pHrodo, incubated for 0, 1, or 2 h, and monitored for MFI as a proxy for virus delivery to acidified endosomes (Fig. 5D). In contrast to DMSO, 5-NT delayed the kinetics of reovirus transport to acidified endocytic organelles, with a small decrease observed at 1 h and a more pronounced defect at 2 h postadsorption. As a positive control, treatment of cells with 20 mM NH_4Cl strongly impaired reovirus access to acidified endosomes. These data indicate that 5-NT impairs reovirus transport to the acidified endocytic compartments used to undergo disassembly during cell entry. The stronger block to reovirus acidification observed with 20 mM NH_4Cl compared with 5-NT parallels the greater impairment of reovirus infection observed following NH_4Cl treatment.

FIG 5 Treatment with 5-NT delays reovirus access to acidic compartments. (A) CCL2 HeLa cells were incubated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with A546-labeled reovirus T3SA+ at an MOI of 5×10^3 particles/cell, and incubated with DMSO or 5-NT. (A to C) Cells were stained with JAM-A-specific antibody at the times shown (A), assessed for levels of cell surface reovirus at 0 h (B), or stained with reovirus-specific antiserum under nonpermeabilizing conditions at the times shown (C). MFI was assessed by flow cytometry. Results are expressed as MFI for JAM-A expression (A), reovirus binding (B), and as a ratio of extracellular virus to total virus (C) for triplicate samples. Values are means plus standard deviations (error bars). (D) CCL2 HeLa cells were incubated with DMSO, 10 μ M 5-NT, or 20 mM NH₄Cl for 1 h, adsorbed with pHrodo-labeled reovirus T3SA+ at an MOI of 5×10^3 particles/cell, and incubated with DMSO or 5-NT for the times shown. MFI was assessed by flow cytometry. Results are expressed as MFI of pHrodo-labeled reovirus for triplicate samples for each time point. Each symbol represents the value for one sample. The horizontal lines depict the means for samples at that time point.

To determine whether the kinetics of reovirus disassembly are affected by 5-NT, CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT, adsorbed with reovirus, and incubated for 0, 1, or 2 h. Whole-cell lysates were resolved by SDS-PAGE and immunoblotted using reovirus-specific antiserum (Fig. 6A). Reovirus disassembly is characterized by cathepsin-mediated proteolysis of the virion, yielding the δ fragment of viral capsid protein μ 1 (23–25). In DMSO-treated cells, the δ fragment was apparent by the 1-h time point. However, in 5-NT-treated cells, the appearance of the δ fragment was delayed, with significant levels not detected until 2 h after adsorption. Densitometric analysis of three independent experiments shows that 5-NT treatment delays reovirus disassembly kinetics by about 1 h (Fig. 6B).

To visualize the kinetics of reovirus virion-to-ISVP conversion, CCL2 HeLa cells were incubated with DMSO or 10 μ M 5-NT, adsorbed with Alexa Fluor 546-labeled reovirus, and stained with a monoclonal antibody specific for reovirus σ 3 outer capsid protein at 0, 60, and 120 min after adsorption (Fig. 7A). During reovirus disassembly, the σ 3 protein is removed from incoming virions by cathepsin-mediated proteolysis (23). In these experiments, virions, which contain intact σ 3, are recognized by the σ 3-specific antibody and appear yellow. In contrast, ISVPs, which lack σ 3, are not recognized by the σ 3-specific antibody and appear red. At 0 min, virions comprised more than 80% of particles observed in cells treated with either DMSO or 5-NT (Fig. 7B). At 60 and 120 min after adsorption of DMSO-treated cells, virions represented less than 57% of particles detected. In contrast, more

FIG 6 Kinetics of reovirus disassembly are prolonged in 5-NT-treated cells. (A) CCL2 HeLa cells were incubated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with reovirus T3SA+ at an MOI of 10 PFU/cell, and incubated with DMSO or 5-NT for the times shown. Cells were lysed, resolved by SDS-PAGE, and immunoblotted using reovirus-specific antiserum. The positions of viral proteins are labeled to the left of the gel. The δ cleavage fragment is indicated by a black arrowhead to the right of the gel. (B) Densitometric analysis of the δ cleavage fragment of reovirus μ 1 protein from immunoblots of triplicate experiments. Values are means plus standard errors of the means (error bars).

FIG 7 Intracellular transport of reovirus is altered in 5-NT-treated cells. (A) CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with A546-labeled reovirus T3SA+ at an MOI of 10^4 particles/cell, and incubated with DMSO or 5-NT for the times shown. The cells were stained for reovirus outer capsid protein $\sigma 3$ (green) and imaged by confocal microscopy. A single section from a Z-stack is shown. Insets depict enlarged areas from the boxed regions. Bars, 10 μ m. (B) Quantification of spectral overlap of virions (yellow) and ISVPs (red). Data are expressed as the percentage of virions within a cell ($n = 12$ cells per time point per condition). Each symbol represents the percentage of virions within a cell. The horizontal lines depict the means for cells at that time point.

than 77% of particles detected in 5-NT-treated cells were virions at both 60 and 120 min after adsorption. In DMSO-treated cells, ISVPs could be readily detected in the cell interior, especially at 120 min. In 5-NT-treated cells, ISVPs were only occasionally seen; instead, virions were observed to aggregate in a perinuclear area in 5-NT-treated cells, which was not seen following DMSO treatment. Together, these results suggest that 5-NT treatment alters the transport of internalized reovirus virions to acidified endosomes with a concomitant delay in virion disassembly that leads to impaired infectivity.

5-NT does not affect the cytoskeleton or internalization of cholera toxin B or transferrin. To determine whether 5-NT treatment of cells alters the cytoskeleton, CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT, stained with A546-labeled phalloidin to visualize actin or an α -tubulin-specific antibody to visualize microtubules, and imaged by confocal microscopy (Fig. 8A). Treatment of cells with 5-NT did not significantly affect actin or tubulin distribution in cells, suggesting that the effects of 5-NT on reovirus infection are not due to impairment of the cytoskeleton.

To determine whether 5-NT impairs the uptake of ligands that

use clathrin-dependent or clathrin-independent uptake mechanisms, CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT, incubated with the B subunit of cholera toxin (CTB), which is internalized primarily via clathrin-independent endocytosis (58, 59), or with transferrin (Tfn), which is internalized via clathrin-dependent endocytosis (60–62), and assessed for ligand uptake by flow cytometry. Treatment of cells with 5-NT did not alter internalization of either CTB (Fig. 8B) or Tfn (Fig. 8C). These data suggest that 5-NT does not inhibit the internalization of cargoes that require either clathrin-dependent or clathrin-independent uptake mechanisms.

Reovirus endocytic transport kinetics are delayed by 5-NT. To establish whether 5-NT affects reovirus transport to early and late endosomes, CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with A546-labeled reovirus, and incubated for 0, 30, 60, and 120 min in the presence of DMSO or 5-NT. The cells were stained with an antibody specific for early endosome antigen 1 (EEA1), which labels early endosomes (Fig. 9A), or lysosome-associated membrane protein 1 (LAMP1), which labels late endosomes (Fig. 9C), and imaged by confocal microscopy. In DMSO-treated cells, EEA1-positive endosomes

FIG 8 Treatment with 5-NT does not alter cytoskeletal protein organization or decrease internalization of cholera toxin B or transferrin. (A) CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h, stained for actin (red), α -tubulin (green), or nuclei (blue), and imaged by confocal microscopy. A single section from a Z-stack is shown. Bars, 10 μ m. (B and C) CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h and incubated with A488-labeled (B) cholera toxin B (CTB) or (C) transferrin (Tfn) for 30 min. Internalization of CTB and Tfn was assessed by flow cytometry. Results are expressed as MFI of internalized CTB or Tfn for triplicate samples. Values are means plus standard deviations (error bars).

were distributed throughout the cytosol. Reovirus distribution to this compartment peaked at 30 min postinfection (Fig. 9B). In 5-NT-treated cells, few EEA1-positive endosomes were observed at 0 min (Fig. 9A), but by 30 min, abundant EEA1-positive endosomes were detected. Reovirus was found in fewer EEA1-positive endosomes in 5-NT-treated cells at 0 and 30 min relative to control-treated cells but was observed in slightly more of these organelles at 60 min. Although 5-NT treatment did not significantly alter the overall distribution of LAMP1-positive late endosomes, reovirus access to LAMP1-positive endosomes was greater in DMSO-treated cells than in 5-NT-treated cells, with the largest difference observed at 60 min after adsorption (Fig. 9D). These data suggest that 5-NT impairs reovirus infectivity by temporarily dispersing EEA1-positive early endosomes, which delays the transport of reovirus through early and late endosomes.

CHIKV and MHV infection is inhibited by 5-NT. To determine whether 5-NT treatment blocks infection by other RNA viruses, we determined the effect of 5-NT on infection by CHIKV

(Fig. 10A) or MHV (Fig. 10B). U-2 OS cells (CHIKV) or DBT-9 cells (MHV) were treated with increasing concentrations of 5-NT, adsorbed with either CHIKV or MHV, and assessed for infectivity by indirect immunofluorescence (CHIKV) or luciferase expression (MHV). 5-NT treatment impaired infection of both CHIKV and MHV, with significant inhibition of both viruses at concentrations of 5 μ M and 10 μ M. Interestingly, infection by CHIKV and MHV was impaired at lower 5-NT concentrations than those required to impair reovirus (compare Fig. 3 and 10). These data indicate that 5-NT has antiviral properties that extend to a diverse group of RNA viruses.

DISCUSSION

In this study, we show that 5-NT, a drug identified in a small-molecule screen for host factors required for reovirus-mediated cytotoxicity (21), has antiviral properties against reovirus and two unrelated RNA viruses, CHIKV and MHV. 5-NT impaired the establishment of reovirus infection and diminished reovirus-me-

FIG 9 Reovirus endocytic transport kinetics are delayed by 5-NT treatment. CCL2 HeLa cells were treated with DMSO or 10 μ M 5-NT for 1 h, adsorbed with A546-labeled reovirus T3SA+ at an MOI of 10^4 particles/cell, and incubated with DMSO or 5-NT for the times shown. (A and C) Cells were stained for early endosomal antigen 1 (EEA1) (green) (A) or lysosome-associated membrane protein 1 (LAMP1) (green) (C) and nuclei (blue) (A and C) and imaged by confocal microscopy. A single section from a Z-stack is shown. Bars, 10 μ m. (B and D) Quantification of spectral overlap of virions and EEA1-positive compartments (B) or LAMP1-positive compartments (D). Data are expressed as the percentage of virions that display spectral overlap with EEA1 or LAMP1 at the times shown ($n = 8$ cells per time point per condition). Values are means $\pm$ standard deviations (error bars).

diated cell killing. Conversely, the serotonin antagonist methiothepin mesylate increased reovirus infection. Treatment of cells with 5-NT 60 min prior to viral adsorption resulted in maximal inhibition; addition of the drug after viral adsorption had little effect. Concordant with 5-NT blocking an early step in the reovirus replication cycle, 5-NT did not alter infection by *in vitro*-generated ISVPs. 5-NT also did not diminish reovirus attachment or internalization into cells. Instead, 5-NT affected the cellular distribution of EEA1-positive early endosomes with a concordant impairment of viral transport to acidified organelles, resulting in a significant delay in viral disassembly kinetics. Taken together, we show that 5-NT impedes reovirus infection by blocking a cell entry step and displays anti-infective activity against diverse RNA viruses.

The effects of 5-NT and other 5-HT ligands on target cells are mediated by signaling events following 5-HT₁ ligation, with the specific receptor engaged dictating the precise biological outcome (30, 63). 5-NT can bind various members of the 5-HT₁ family, with preferential binding for 5-HT_{1A} and 5-HT_{1B} subfamilies (42). Following ligation of 5-HT₁, ATP is converted to the secondary messenger, cyclic AMP (cAMP), intracellular calcium stores are released, and transcription factor cAMP response element binding protein (CREB) is activated (30). CREB activation occurs following phosphorylation at serine 133 in re-

sponse to stress signals, including infection by enterovirus 71, severe acute respiratory syndrome (SARS) coronavirus, Sendai virus, and vesicular stomatitis virus (64–66). After reovirus infection, we did not detect significant changes in CREB activity, as assessed by a CREB-specific reporter assay or CREB phosphorylation at serine 133 in the presence or absence of 5-NT (data not shown). Moreover, forskolin, a potent CREB activator, did not affect reovirus infection despite inducing robust CREB transcriptional stimulation (data not shown). These findings suggest that the effect of 5-NT on reovirus infection is not attributable to the transcriptional activities of CREB.

In some cell types, 5-HT induces production of gamma interferon (IFN- γ) (67), suggesting that 5-NT can affect expression of genes regulated by signal transducer and activator of transcription (STAT) factors. However, we did not detect a change (either increase or decrease) in IFN- γ activation sequence (GAS)- or interferon-stimulated response element (ISRE)-mediated transcriptional activity following 5-NT treatment in the cells selected for this study (data not shown), suggesting that the broad antiviral effects of 5-NT are not due to induction of IFN signaling. As such, it appears that the antiviral effects of 5-NT are not attributable to canonical 5-HT₁ signaling pathways, although a role for cAMP or cAMP sensors in modulating 5-NT antiviral effects has not been conclusively excluded.

FIG 10 Infection by CHIKV and MHV is inhibited by 5-NT treatment. (A) U-2 OS cells were treated with DMSO or increasing concentrations of 5-NT for 1 h, adsorbed with CHIKV at an MOI of 5 PFU/cell, and incubated with DMSO or 5-NT for 6 h. The cells were stained with CHIKV-specific antiserum and DAPI to detect nuclei. Infection was quantified by indirect immunofluorescence. Results are expressed as percent infected cells normalized to the DMSO value for triplicate experiments. Values are means plus standard errors of the means (error bars). (B) DBT-9 cells were treated with DMSO or increasing concentrations of 5-NT for 1 h, adsorbed with MHV-FFL at an MOI of 1 PFU/cell, and incubated with DMSO or 5-NT for 10 h. Infectivity was quantified by monitoring luciferase activity (measured in relative light units [RLU]) normalized to cell viability. Results are expressed as infectivity normalized to the DMSO value for triplicate experiments. Values are means plus standard errors of the means (error bars). **, $P < 0.05$ in comparison to DMSO value by one-way ANOVA with Dunnett's multiple-comparison test.

The inhibitory effects of 5-NT on reovirus infection are likely due to the disruption of early endosomes following 5-NT treatment. The effect of 5-NT on early endosomes was transient, observed at 0 min of adsorption (60 min after drug treatment) but not at 30 min. This finding is concordant with a delay, rather than complete impairment, of reovirus entry kinetics. 5-HTRs are internalized rapidly following ligand engagement and distribute to early endosomes (68, 69). It is possible that 5-NT-mediated signaling induces rapid uptake of 5-HTRs, which upon traversing to early endosomes, leads to the temporary dispersal of this compartment. It is also possible that the 5-NT-mediated effects on early endosomes are the by-product of a signaling cascade following 5-HTR ligation that results in the impairment of early endosome homeostasis. The precise mechanism by which 5-NT disrupts early endosomes remains to be elucidated.

We find it noteworthy that 5-NT impairs reovirus infection by inhibiting cell entry, adding to a growing list of small molecules that block this step in the reovirus replication cycle. Of the 11 compounds identified in our screen for cellular mediators of reovirus cytotoxicity (21), 6 target cell entry, including 5-NT. To date,

small molecules that inhibit clathrin-dependent uptake (chlorpromazine (62), microtubule dynamics (vinca alkaloids) (21), Src kinase (PP2) (18), vacuolar ATPase (bafilomycin A1) (70), and cathepsins (E64) (22) have been shown to inhibit reovirus cell entry. That so many compounds have been identified that block reovirus cell entry steps may reflect enhanced understanding of this aspect of reovirus replication. Alternatively, drugs that inhibit host processes required for viral entry may be better tolerated by cells than those that compromise other key steps in viral infection, such as transcription, translation, or genome replication. Moreover, inhibition of viral entry may dampen activation of innate immune responses that compromise cell viability, which would facilitate identification of entry inhibitors in cytotoxicity screens. As reovirus entry is not completely understood, we think it likely that additional entry-inhibiting compounds will be identified.

One of the most remarkable findings in our study is that 5-NT diminishes infectivity of the unrelated RNA viruses, CHIKV and MHV, in addition to reovirus. These viruses display marked differences in tissue tropism, disease manifestations, and transmission pathways. Yet, CHIKV, MHV, and reovirus share common mechanisms of cell entry. CHIKV and reovirus use glycans to attach to cells (12, 71–74), and CHIKV, MHV, and reovirus enter at least some cell types via clathrin-dependent endocytosis (62, 75–77). All three viruses are transported to early, Rab5-marked endosomes during cell entry (19, 20, 76, 77), and some strains of MHV, including the one used in this study (76), and reovirus (19, 20, 23, 24) require access to late endosomes and cathepsin-mediated proteolysis to complete the cell entry steps. CHIKV differs from MHV and reovirus in the requirement for transport to late endosomes and cathepsin activity (77). As such, 5-NT likely affects the infectivity of CHIKV, MHV, and reovirus by disrupting transport of incoming virions to early endosomes, an entry step common to all three viruses. Alternatively, 5-NT could impair the function of endosomal components responsible for regulating endosomal pH, such as the vacuolar ATPase. Data gathered in this study provide evidence that it is possible to impair viruses with diverse biological profiles using small molecules that do not compromise cell viability and point to serotonin signaling as a new target for development of broadly active antiviral therapeutics.

ACKNOWLEDGMENTS

We thank Kristen Ogden, Andrea Pruijssers, Laurie Silva, and Danica Sutherland for careful reviews of the manuscript and members of the Dermody laboratory for useful suggestions during the course of this study. Flow cytometry experiments were performed in the Vanderbilt Cytometry Shared Resource. Confocal microscopy experiments were conducted in the Vanderbilt Cell Imaging Shared Resource.

This work was supported by Public Health Service awards T32 HL07751 (B.A.M. and A.W.A.), F32 AI0801082 (B.A.M.), T32 AI095202 and F32 AI108102 (E.C.S.), U19 AI109680 (M.R.D.), and R01 AI032539 (T.S.D.) and the Elizabeth B. Lamb Center for Pediatric Research. Additional support was provided by Public Health Service awards P30 CA68485 for the Vanderbilt-Ingram Cancer Center and P60 DK20593 for the Vanderbilt Diabetes Research and Training Center. JAM-A antibody was purified with support from the Vanderbilt Institute for Clinical and Translational Research (VICTR), which is supported by CTSA award UL1 TR000445 from the National Center for Advancing Translational Sciences.

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