Host sphingomyelin increases West Nile virus infection in vivo

Miguel A. Martín-Acebes,*,† Enrique Gabandé-Rodríguez,1,8 Ana M. García-Cabrero,2,** Marina P. Sánchez,3,9 María Dolores Ledesma,5 Francisco Sobrino,3,4,§ and Juan-Carlos Saiz3,4,†

Departments of Virology and Microbiology* and Molecular Neurobiology,5 Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Madrid 28049, Spain; Department of Biotechnology,1 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid 28040, Spain; and Laboratory of Neurology,2,** Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid 28040, Spain

Abstract Flaviviruses, such as the dengue virus and the West Nile virus (WNV), are arthropod-borne viruses that represent a global health problem. The flavivirus lifecycle is intimately connected to cellular lipids. Among the lipids co-opted by flaviviruses, we have focused on SM, an important component of cellular membranes particularly enriched in the nervous system. After infection with the neurotropic WNV, mice deficient in acid sphingomyelinase (ASM), which accumulate high levels of SM in their tissues, displayed exacerbated infection. In addition, WNV multiplication was enhanced in cells from human patients with Niemann-Pick type A, a disease caused by a deficiency of ASM activity resulting in SM accumulation. Furthermore, the addition of SM to cultured cells also increased WNV infection, whereas treatment with pharmacological inhibitors of SM synthesis reduced WNV infection. Confocal microscopy analyses confirmed the association of SM with viral replication sites within infected cells. Our results unveil that SM metabolism regulates flavivirus infection in vivo and propose SM as a suitable target for antiviral design against WNV.—Martin-Acebes, M. A., E. Gabandé-Rodríguez, A. M. García-Cabrero, M. P. Sánchez, M. D. Ledesma, F. Sobrino, and J-C. Saiz. Host sphingomyelin increases West Nile virus infection in vivo. J. Lipid Res. 2016. 57: 422–432.

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The flaviviruses comprise a group of arthropod-borne viruses that include important human and animal pathogens responsible for life-threatening diseases, such as dengue, yellow fever, Japanese encephalitis, or West Nile fever (1). The flavivirus, West Nile virus (WNV), is a neurotropic enveloped plus-strand RNA virus transmitted through the bite of mosquitoes. This virus is responsible for recurrent outbreaks of febrile illness and meningoencephalitis worldwide, accounting for hundreds of human deaths every year (2). The WNV lifecycle is intimately associated to cellular lipids, and RNA replication and virion biogenesis are coupled into highly remodeled intracellular membranes (3, 4). In a parallel manner to that described for other flaviviruses, both RNA replication and acquisition of lipid envelope are associated to specialized membranous structures derived from the endoplasmic reticulum (ER) (3–6). To generate this adequate environment for viral multiplication, WNV promotes the synthesis and accumulation of specific lipids (fatty acids, cholesterol, glycerophospholipids, and sphingolipids) within infected cells (5, 7–9). This selective manipulation of host cell lipid metabolism is not a unique feature of WNV infection, as it is also observed in cells infected with other flaviviruses (10–12).

Among the cellular lipids co-opted by WNV, sphingolipids merit special attention because they are particularly enriched in the nervous system, a major target tissue during WNV infection (13, 14). Sphingolipids derive from sphingosine, a long-chain amino alcohol that is acylated with a long-chain fatty acid to give ceramide, which

Abbreviations: ASM, acid sphingomyelinase; ASMko, ASM knock-out; BSL-3, biosafety level 3; CBMSO, Centro de Biología Molecular “Severo Ochoa”; dsRNA, double-strand RNA; ER, endoplasmic reticulum; INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria; MOI, multiplicity of infection; NPA, Niemann-Pick disease type A; PFU, plaque-forming unit; p.i., postinfection; SMS, SM synthase; WNV, West Nile virus; wt, wild-type.

*Present address of E. Gabandé-Rodríguez: Barts Cancer Institute, Centre for Cancer and Inflammation, Queen Mary University of London, Charterhouse Square, London E1M 6BQ, United Kingdom.
**Present address of A. M. García-Cabrero: Department of Immunology and Oncology and Protein Tools Unit, Centro Nacional de Biotecnología (CNB-CSIC), Madrid 28049, Spain.
§Present address of F. Sobrino and J. Carlos-Saiz are joint senior authors on this work.
‖To whom correspondence should be addressed, e-mail: fsobrino@cbm.csic.es (F.S.); jcsaiz@inia.es (J-C.S.)

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The levels of SM are tightly controlled by cellular sphingomyelinases (15). Consistently, defects in the activity of acid sphingomyelinase (ASM) result in the accumulation of SM, causing a sphingolipidosis termed Niemann-Pick disease type A (NPA) (20). Because ASM is a ubiquitous enzyme, SM accumulates in all kinds of cells in NPA patients leading to peripheral symptoms, especially evident in enlarged liver and spleen. However, the most affected organ is the brain, resulting in severe cognitive deficits, neurodegeneration, and early death. Mice lacking ASM [ASM knockout mice (ASMko)] reproduce NPA and accumulate SM, but not ceramide, in their tissues (21–23). Accumulation of SM in the ASMko mouse brain cells is progressive, reaching a 5-fold increase compared with wild-type (wt) mice (20–22). This increase is similar to that reported in the cerebral cortex of NPA patients (24). ASMko mice and primary cell cultures from NPA patients have provided useful models to study the role of SM in viral infection (25, 26). Using these models, we here show, for the first time, that SM levels modulate WNV infection in vitro and in vivo, thus identifying this sphingolipid as a key cellular factor for WNV replication.

**MATERIALS AND METHODS**

**Cells and viruses**

All virus manipulations were performed in our biosafety level 3 (BSL-3) facilities. The origin and passage history of WNV strain NY99 has been described (27, 28). Vero 76 cells, clone E6 (ATTC CRL-1586), were used for amplification and titration of viruses. NY99 has been described (27, 28). Vero 76 cells, clone E6 (ATTC CRL-1586) were used for amplification and titration of viruses. All virus manipulations were performed in our biosafety level 3 (BSL-3) facilities. The origin and passage history of WNV strain NY99 has been described (27, 28). Vero 76 cells, clone E6 (ATTC CRL-1586), were used for amplification and titration of viruses.

**Infections and virus titrations**

For infections in liquid medium, the viral inoculum was incubated with cell monolayers for 1 h, and then the inoculum was removed and fresh medium containing 1% FBS was added. This time point was considered 1 h postinfection (p.i.). Virus titrations were performed by standard plaque assay on Vero cells (28). The multiplicity of infection (MOI) used in each experiment was expressed as the number of plaque-forming units (PFUs) per cell and is indicated in the corresponding figure legend.

**Mice**

Age- and sex-matched 5-month-old wt (C57BL/6) or ASMko (21) mice were used. A breeding colony was established from a couple of ASM heterozygous C57BL/6 mice kindly donated by Prof. E. H. Schuchman (Mount Sinai School of Medicine, New York) and genotyped as described (29). Mice were intraperitoneally inoculated with 10⁵ PFU per mouse of WNV and monitored daily for signs of infection up to 20 days p.i. Animals were kept with ad libitum access to food and water and those exhibiting clear signs of disease were anesthetized and euthanized, as were all surviving mice, at the end of the experiment. All animals were handled in strict accordance with the guidelines of the European Community 86/609/CEE at the BSL-3 animal facilities of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and the CBMSO. The protocols were approved by the Committee on Ethics of Animal Experimentation of INIA (permit number 2015-044E and PROEX 05/14) and the Animal Welfare Committee of Centro de Biología Molecular “Severo Ochoa” (CBMSO) (permit numbers CEEA-CBMSO-221149 and PROEX 034/15).

**Hippocampal slice cultures**

Organotypic slice cultures of the hippocampus were prepared from 5-month-old wt or ASMko mice, as previously described (30). Six slices (400 μm thick each) were placed per insert and cultured for 24 h at 37°C in a 5% CO₂ atmosphere prior to infection. Each slice was individually infected with 10⁵ PFU of WNV. Slices were harvested 24 h p.i. and homogenized in PBS for quantitative RT-PCR analysis.

**Cell treatments**

SM (Santa Cruz Biotechnology, Dallas, TX) was dissolved in ethanol and added to the cells 24 h before infection (29). D609 (Sigma, St. Louis, MO), SPK-601 (LMV-601; Eurodiagnostico, Madrid, Spain), and MS-209 (dofequidar fumarate; Sigma) were dissolved in DMSO and added to infected cultures 1 h p.i. Control cells were treated in parallel with the same amount of drug vehicle.

**SM quantification**

Biochemical analysis of SM in brain extracts or cultured cells containing the same amount of protein was performed using an enzymatic fluorescence assay. Briefly, lipid extracts were dried in the presence of detergent (Thesit), and SM was subsequently converted into choline by means of sphingomyelinase and alkaline phosphatase, and coupled to the production of fluorescence with choline oxidase, peroxidase, and homovanillic acid, as modified from Hojiyati and Jiang (31).

**Immunohistochemistry**

Right brain hemispheres from four randomly selected mice per condition were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Serial sagittal sections (3 μm thick)
were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was inactivated by incubation in 1.5% 
H$_2$O$_2$ in methanol for 20 min. Sections were placed in blocking buffer (1% BSA, 5% FBS, 2% Triton X-100 in PBS) for 1 h and 
incubated overnight at 4°C with an anti-WNV E glycoprotein 
(3.67G) mouse monoclonal antibody (Millipore, Temecula, CA) 
in blocking buffer. Subsequently, sections were incubated with 
biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) 
and stained using the Elite Vectastain ABC kit (Vector). Immunoo-
reactivity was developed with diaminobenzidine (Dako, Glostrup, 
Denmark), which yields a brownish precipitate, and sections 
were counterstained with hematoxylin. Contiguous brain sections 
were processed in parallel without primary antibody in order to 
determine nonspecific staining. Images were acquired using a 
Leica DM LB microscope equipped with a HCX PL 40×/0.75 objective and a digital camera, Leica DC 100.

**Immunofluorescence and confocal microscopy**

Immunofluorescence was performed as described (5). Plasma 
membrane staining with lysenin, which specifically binds to SM, 
was accomplished as described (23). For intracellular SM detec-
tion, lysenin staining was performed in a similar manner, except 
that cells were permeabilized with 50 μg/ml digitonin after fixa-
tion (32). Lysenin (Peptanova, Sandhausen, Germany) was 
detected using a specific rabbit antiserum (Peptanova). Double-
strand RNA (dsRNA) and WNV E protein were detected using 
mouse monoclonal antibodies J2 (English and Scientific Consult-
ing, Hungary) and 3.67G, respectively. Actin microfilaments were 
visualized with TRITC-labeled phalloidin (Sigma). The ER was 
visualized by transfection with plasmid IgLD1Kdel (33) encoding 
an ER-targeted red fluorescent protein (5). Appropriate second-
ary antibodies labeled with Alexa Fluor 488, 594, or 647 were 
from Invitrogen. For plasma membrane lysenin staining, cells 
were observed using an Axioskop (Zeiss, Oberkochen, Germany) 
epifluorescence microscope with a Plan-Neofluar Ph3 40×/1.3 oil 
immersion objective. Images were acquired with a monochrome 
Coolscan FX camera (Roper Scientific, Tucson, AZ) and fluores-
cence was quantified using ImageJ software (http://imagej.nih.
gov/ij/). In the case of confocal microscopy analyses, cells were 
observed using a Leica TCS SPE confocal laser scanning micro-
scope using an HCX PL APO 63×/1.4 oil immersion objective. 
Optical slice thickness for all confocal images displayed was of 
1 airy unit.

**Quantification of viral RNA**

For quantification of cell-associated viral RNA, infected cell 
monolayers were washed, supernatants were replaced by fresh 
medium, and the cells were subjected to three freeze-thaw cycles. 
In the case of animal samples or organotypic slice cultures, tis-
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ASMko mice that exhibited a 2.7-fold increase in their brain SM content relative to wt mice were used (218 ± 19 nmol/mg and 81 ± 5 nmol/mg of protein, respectively) (Fig. 1A). To determine whether the alteration of SM levels could modulate WNV infection in vivo, we first infected ASMko mice (21) with a highly neurovirulent WNV NY99 strain (28) derived from the virus causing the outbreak of encephalitis in the Northeastern United States in 1999 (37). ASMko mice were significantly more susceptible to WNV-induced lethality than wt mice when challenged with 10^4 PFU by intraperitoneal inoculation (Fig. 1B). Quantitative RT-PCR revealed a significantly higher viral load in the brains of dead ASMko mice than in those of dead wt mice (Fig. 1C), indicating that WNV replicated to higher levels in ASMko mice. To test this hypothesis, the viral load in several neural and peripheral tissues was analyzed in randomly chosen animals at 6 days p.i. The values found were

![Fig. 2.](image-url)

The localization of WNV antigen is revealed by the presence of brownish precipitates. Sections were counterstained with hematoxylin. Bar, 50 μm. B: Quantification of WNV antigen in brain sections of mice infected with WNV at 6 days p.i. The number of WNV antigen-positive cells, as immunostained with 3.67G antibody, was determined in four mice per condition. At least three different fields per mouse were counted. C: Analysis of WNV replication in organotypic hippocampal slice cultures. Hippocampal slice cultures (six slices per insert) from wt or ASMko mice were infected with WNV (10^5 PFU per slice) and the amount of viral RNA in tissue slices was determined by real-time RT-PCR at 24 h p.i. ∗P < 0.05; ∗∗P < 0.005.

**RESULTS**

ASMko mice are more susceptible to WNV-induced lethality and display enhanced virus replication compared to wt mice

SM accumulation is an age-related process in ASMko mice that, close to the end of their life (7 months of age), exhibits a 5-fold higher level of SM in brain, while cholesterol and ceramide content remains unchanged with respect to wt mice (22, 23, 36). In our experiments, 5-month-old ASMko mice that exhibited a 2.7-fold increase in their brain SM content relative to wt mice were used (218 ± 19 nmol/mg and 81 ± 5 nmol/mg of protein, respectively) (Fig. 1A). To determine whether the alteration of SM levels could modulate WNV infection in vivo, we first infected ASMko mice (21) with a highly neurovirulent WNV NY99 strain (28) derived from the virus causing the outbreak of encephalitis in the Northeastern United States in 1999 (37). ASMko mice were significantly more susceptible to WNV-induced lethality than wt mice when challenged with 10^4 PFU by intraperitoneal inoculation (Fig. 1B). Quantitative RT-PCR revealed a significantly higher viral load in the brains of dead ASMko mice than in those of dead wt mice (Fig. 1C), indicating that WNV replicated to higher levels in ASMko mice. To test this hypothesis, the viral load in several neural and peripheral tissues was analyzed in randomly chosen animals at 6 days p.i. The values found were...
WNV infection is enhanced in fibroblasts from NPA

significantly higher (by several orders of magnitude) in the brain of ASMko than in wt mice (Fig. 1D). In addition, the amount of viral RNA in peripheral tissues (liver, spleen, kidney, and heart) of ASMko mice was also higher than in wt mice (Fig. 1D). These results confirm that WNV infection is exacerbated in ASMko mice.

Brain tissues from ASMko mice display enhanced WNV infection both in vivo and ex vivo

WNV-infected cells were analyzed by immunohistochemistry in brain sections from wt and ASMko mice at 6 days p.i. using a monoclonal antibody that recognizes the WNV E glycoprotein. Our analysis was focused on the cortex and hippocampus, because both areas are important targets for WNV infection (38, 39). Cells showing cytoplasmic positive staining for viral antigen were found in the prefrontal cortex, as well as in the dentate gyrus and in areas CA1 and CA3 of the hippocampus from both wt and ASMko infected mice (Fig. 2A). Quantification of WNV antigen-positive cells revealed that ASMko mice showed significantly more infected cells than wt mice in all the regions analyzed (Fig. 2B). To investigate whether this increase of WNV infection in neural tissues of ASMko mice was also observed ex vivo, organotypic hippocampal slice cultures were produced from noninfected wt and ASMko mice. Slice cultures were infected with WNV and the amount of viral RNA in the tissue was analyzed 24 h p.i. by quantitative RT-PCR. A significant increase (about 3-fold) in the content of viral RNA was observed in hippocampal slices derived from ASMko mice as compared with those from wt mice (Fig. 2C). Overall, these results confirm that the replication of WNV is enhanced in brain tissues of ASMko mice.

WNV infection is enhanced in fibroblasts from NPA patients

Together with brain cells, which are particularly affected by WNV, dermal fibroblasts are also a relevant type of cell for WNV infection, because they are one of the first exposed to the virus-infected mosquitoes (40). For this reason, and to extend our analyses to the human scenario, we tested the vulnerability to infection of skin fibroblasts derived from NPA patients, which also showed SM accumulation (26, 29). Quantification of SM confirmed that NPA fibroblasts displayed significantly higher levels of SM than control fibroblasts (52.05 ± 1.26 nmol/mg and 13.09 ± 0.56 nmol/mg of protein, respectively) (Fig. 3A). In addition, because SM is enriched in the plasma membrane, the levels of SM at this cellular site were also analyzed in both control and NPA fibroblasts by lysenin staining of nonpermeabilized cells (29). A significant 3-fold increase in lysenin staining was noticed in NPA fibroblasts when compared with control fibroblasts (Fig. 3B, C), which further confirmed the accumulation of SM in cells derived from NPA patients. To test the vulnerability to infection by WNV of skin fibroblasts derived from NPA patients, two NPA fibroblast lines (GM13205 and GM16195) derived from different NPA patients and two control fibroblast lines derived from unaffected individuals (AG07310 and AG07471) were infected with WNV. Virus replication was analyzed by means of immunofluorescence and confocal microscopy 24 h p.i. Cells showing cytoplasmic cumuli of dsRNA intermediates, which are markers of WNV replication (3, 5), as well as cells positive for WNV E glycoprotein, were observed in infected cultures (Fig. 4A), supporting the ability of WNV to replicate in these fibroblast lines. No positive signal for either dsRNA or E protein was observed in uninfected cultures included as negative controls, thus confirming the specificity of the staining (Fig. 4A). The amount of infectious virus released into the culture medium by NPA and control fibroblasts was measured at 24 and 48 h p.i. Maximum viral production was observed at 24 h p.i., which is consistent with previous reports showing that multiplication of WNV in normal human dermal fibroblast cultures peaks about 24 h p.i. (40). Remarkably, titration of supernatants from infected cultures indicated that NPA fibroblasts produced significantly more infectious virus than control fibroblasts at both 24 and 48 h p.i. (Fig. 4B). This observation was confirmed when the amount of genome-containing units in the culture medium was analyzed by quantitative RT-PCR 24 h p.i. (Fig. 4C). Furthermore, a statistically significant increase in the amount of cell-associated viral RNA in NPA fibroblasts was noticed when compared with control fibroblasts 24 h p.i. (Fig. 4D). These results support that WNV replication is increased in NPA patient fibroblasts in comparison to those from unaffected individuals.

SM promotes WNV infection

The results obtained so far in ASMko mouse brains and in fibroblasts from NPA patients, which contain high levels
of SM (22, 26, 29, 36), suggest that the accumulation of SM could result in increased WNV multiplication. To directly test this, we first added 40 μM SM to NPA or control fibroblasts. Enzymatic quantification of SM content confirmed that addition of the lipid significantly increased SM levels in control fibroblasts by 1.3-fold (16.93 ± 0.39 nmol/mg and 12.69 ± 0.32 nmol/mg of protein in treated and non-treated control fibroblasts, respectively) (Fig. 5A). Treatment with SM did not significantly increase SM levels in NPA fibroblasts (Fig. 5A), likely due to the already very high SM levels in these cells. Similar results were obtained when SM was detected by lysenin staining (Fig. 5B), confirming that addition of exogenous SM successfully increased this lipid content in control fibroblasts. To test the effects of SM increase in WNV infectivity, control and NPA fibroblasts were treated with the lipid and then infected with WNV. The titration of culture supernatants from infected fibroblasts revealed that the addition of SM significantly increased WNV production in control fibroblasts when compared with those cultures treated with vehicle alone (Fig. 5C). Moreover, the production of WNV in control line AG07471 was increased by the addition of SM, reaching the level observed in NPA fibroblasts. Consistent with the unchanged SM levels in NPA fibroblasts upon addition of the lipid, the amount of virus in these cells, which was already high compared with control fibroblasts, did not increase after SM addition (Fig. 5C). Again, this lack of effect of addition of SM could be due to the high levels of SM already existing in NPA fibroblasts. In fact, no significant increase on SM levels was noticed upon addition of exogenous SM to NPA fibroblasts (Fig. 5A, B). To further analyze this point and to test whether high SM levels had the same effect in other cell lines, we added SM to Vero cells, which are a widely used model for WNV infection (3, 5). The addition of SM significantly increased SM content of Vero cells (5.95 ± 1.23 nmol/mg and 12.44 ± 2.34 nmol/mg of protein in control and treated cells, respectively) (Fig. 5D), which correlated with enhanced viral production (Fig. 5E). These results further support the positive role of high SM levels on the infection of WNV.
Effect of SM on WNV infection. A: Quantification of SM content in Vero cells treated with AG07310 and AG07471 were infected with WNV (MOI, 1 PFU/cell) and virus yield was determined by plaque assay at 24 h p.i. B: Estimation of relative SM content in the plasma membrane of fibroblasts indicated in (A) by lysenin staining of nonpermeabilized cells after 24 h of treatment with 40 μM SM or not (Veh, vehicle). C: Primary human skin fibroblasts derived from two different control individuals (AG07310 and AG07471) and two distinct NPA patients (GM13205 and GM16195) were treated with 40 μM SM or not treated, and infected with WNV (MOI, 1 PFU/cell). Virus yield in the culture medium was determined by plaque assay at 24 h p.i. D: Quantification of SM content in Vero cells treated with 40 μM SM or not treated for 24 h. E: Vero cells treated or not treated with 40 μM SM were infected with WNV (MOI, 1 PFU per cell) and virus yield was determined by plaque assay at 24 h p.i. *P<0.05; **P<0.005.

**Fig. 5.** Effect of SM on WNV infection. A: Quantification of SM content in primary human skin fibroblasts from a control individual (AG07310) or from a NPA patient (GM13205) after 24 h of treatment with 40 μM SM or not (Veh, vehicle). B: Estimation of relative SM content in the plasma membrane of fibroblasts indicated in (A) by lysenin staining of nonpermeabilized cells after 24 h of treatment with 40 μM SM or no treatment. C: Primary human skin fibroblasts derived from two different control individuals (AG07310 and AG07471) and two distinct NPA patients (GM13205 and GM16195) were treated with 40 μM SM or not treated, and infected with WNV (MOI, 1 PFU/cell). Virus yield in the culture medium was determined by plaque assay at 24 h p.i. D: Quantification of SM content in Vero cells treated with 40 μM SM or not treated for 24 h. E: Vero cells treated or not treated with 40 μM SM were infected with WNV (MOI, 1 PFU per cell) and virus yield was determined by plaque assay at 24 h p.i. *P<0.05; **P<0.005.

**SM and WNV colocalize in ER membranes within infected cells**

When the distribution of intracellular SM within infected Vero cells was evaluated by lysenin staining of permeabilized cells (41), colocalization between dsRNA and SM was observed in cytoplasmic foci (Fig. 6A). This sustained the localization of this lipid at WNV replication complex. Considering that lysenin recognizes SM clusters rather than monomeric SM (42), these results indicate SM clustering within the membranes where WNV replicates, thus reinforcing the idea of membrane remodeling during WNV infection (7). Because WNV replication takes place on specialized membranes derived from the ER (see Introduction), Vero cells were transfected with plasmid IgLdR1Kdel that encodes an ER-targeted red fluorescent protein (33) to allow visualization of this organelle. Transfected cells were infected with WNV and both SM and dsRNA were labeled by immunofluorescence (Fig. 6B). Confocal microscopy analyses revealed colocalization among dsRNA, SM, and the ER markers in discrete cytoplasmic foci, which confirms the association of SM to the membranes derived from the ER where WNV replication takes place.

**Inhibition of SM biosynthesis reduces WNV infection**

Considering that our observations were consistent with the involvement of SM on WNV infection, we explored whether the inhibition of the synthesis of this lipid could provide not only further evidence for SM contribution to WNV infection, but also a feasible druggable target for antiviral strategies. SM is produced through the transfer of a phosphocholine headgroup from phosphatidylcholine to ceramide in a reaction catalyzed by SM synthases (SMs) (15). Thus, we investigated the effect in WNV infection of SMS inhibitors D609, SPK-601 (an enantiomeric pure isomer of D609), and MS-209 (structurally unrelated to D609 and SPK-601) (43, 44). Unfortunately, the low amount of endogenous SM displayed by Vero cells impaired the experimental confirmation of the reduction of SM levels (using the enzymatic assay) as a result of the treatment with the inhibitors. Nevertheless, SM levels would be expected to decrease in cells treated with these inhibitors, as shown previously (45–47). Indeed, the three inhibitors significantly reduced, by several orders of magnitude, the production of infectious particles in WNV-infected Vero cells (Fig. 7A). These data are consistent with the idea that whereas an increase in SM content increases virus multiplication, a decrease in SM could reduce virus infection. Interestingly, even when the three drugs exerted a reduction in the amount of genome-containing units released to the culture medium of infected cells (Fig. 7B), the extent of these inhibitions was lower than that of infectivity (compare Fig. 7A, B). A possible explanation for this discrepancy is that treatment with SMS inhibitors increased the production of noninfectious genome-containing WNV particles. Considering that SM could play a structural role in WNV particles (8), the expected reduction on SM content due to SMS inhibition could result in a reduced availability of SM for viral envelopment, thus causing a decrease in the infectious viral particles produced. Similar effects have been observed in other viral models using other drugs that also block lipid synthesis (48–50). The amount of cell-associated viral RNA was also measured in cells treated with the inhibitors (Fig. 7C). Within the concentration range tested, D609 and MS-209 induced a significant dose-dependent reduction of WNV RNA accumulation inside cells that indicated a reduction in viral replication. On the contrary, no significant differences were observed in cells treated with SPK-601, an isomer of D609. In contrast to SPK-601, D609 consists on a mixture of diastereomers (44), so the differences between both inhibitors could rely on the inhibitory properties of diastereomers present in D609.
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RNA replication and virus biogenesis (51, 52). Among the lipids co-opted by viruses for this process, sphingolipids are of growing interest (17, 18). In fact, an upregulation of sphingolipid synthesis occurs in cells infected with WNV (8) and other related viruses (12, 19). However, to our knowledge, the relevance of sphingolipids in flavivirus pathogenesis in vivo remains unexplored. Our results showed that WNV infection was exacerbated in ASMko mice displaying both increased viral replication and enhanced lethality. Histological analyses confirmed that the viral content is increased in the brain of ASMko mice, which is but absent in SPK-601. The analysis of the cellular ATP content in drug-treated cells confirmed that these drugs inhibited WNV at concentrations that exerted negligible cytotoxicity (Fig. 7D), pointing to SMS as a feasible druggable antiviral target against WNV.

**DISCUSSION**

Plus-strand RNA viruses rearrange intracellular membranes to generate specialized organelle-like structures for RNA replication and virus biogenesis (51, 52). Among the lipids co-opted by viruses for this process, sphingolipids are of growing interest (17, 18). In fact, an upregulation of sphingolipid synthesis occurs in cells infected with WNV (8) and other related viruses (12, 19). However, to our knowledge, the relevance of sphingolipids in flavivirus pathogenesis in vivo remains unexplored. Our results showed that WNV infection was exacerbated in ASMko mice displaying both increased viral replication and enhanced lethality. Histological analyses confirmed that the viral content is increased in the brain of ASMko mice, which is but absent in SPK-601. The analysis of the cellular ATP content in drug-treated cells confirmed that these drugs inhibited WNV at concentrations that exerted negligible cytotoxicity (Fig. 7D), pointing to SMS as a feasible druggable antiviral target against WNV.
lack of ASM activity leading to the accumulation of SM promotes WNV infection.

To further investigate the role of SM in WNV infection, we reproduced the SM accumulation induced by the lack of ASM activity by adding exogenous SM to cell cultures (23, 29). The quantification of SM content confirmed that treatment with SM was enough to increase cellular SM levels. In these experiments, we noticed that the addition of SM increased WNV multiplication. Thus, we propose that SM positively regulates WNV infection.

In fact, the results shown in the present report indicate that a higher content of cellular SM (ASMko mice, NPA fibroblasts, or addition of exogenous SM) promoted WNV infection. This effect of SM on WNV infection is also consistent with the recently reported positive effect of SM on hepatitis C virus replication (19, 54).

On the other hand, treatment with pharmacological inhibitors of SM synthesis, which would be expected to decrease cellular SM levels, resulted in an inhibition of infectious virus production. Interestingly, the extent of the inhibition of infectious particle production was higher than that of the inhibition of RNA replication, suggesting that production of infectious particles could be more sensitive to SM depletion than the replication of the viral genome. These results also support a functional role of SM on the infectivity of WNV particles, which is consistent with our previous observation of an enrichment of this lipid in the viral envelope of WNV (8).

Moreover, intracellular SM detected by lysenin staining was found associated to dsRNA and also to an ER marker by confocal analyses of WNV-infected cells. These results support previous data pointing to the clustering and localization of this lipid within the replication complexes of particularly enriched in SM (22, 23, 36). Furthermore, the increased ability of WNV to replicate in neural tissues from ASMko mice was also confirmed ex vivo using organotypic hippocampal slice cultures. It has to be considered that neurons, which constitute a major target for WNV replication in the nervous system (53), are specially enriched in SM (22), which may also reinforce the relevance of this lipid’s levels in WNV infection. Although increased levels of other lipids, such as cholesterol or ceramide, can also promote WNV multiplication in cultured cells (7, 9), this possibility was excluded in ASMko mice because neither cholesterol nor ceramide are enriched in their brains (36). Considering all these factors, the results observed for ASMko mice provide solid evidence of the involvement of the SM pathway in WNV infection and in vivo pathogenesis.

Dermal fibroblasts are one of the first cell types supposed to be exposed to WNV during a blood meal by an infected mosquito and, hence, represent a relevant model for WNV infection (40). Therefore, to investigate the effect of the alteration of lipid metabolism on WNV lifecycle, we analyzed the infection in fibroblasts from NPA patients. The quantification of SM confirmed that, as expected, NPA fibroblasts displayed increased levels of SM compared to control fibroblasts. Furthermore, lysenin staining showed that this accumulation of SM was also patent in nonlysosomal membranes such as the plasma membrane, which is consistent with previous data showing that the deficiency of ASM causes an accumulation of SM in both lysosomal and nonlysosomal membranes (23). Remarkably, our results confirmed that WNV multiplication, including RNA replication and infectious virus production, was increased in NPA fibroblasts, which again support that the lack of ASM activity leading to the accumulation of SM promotes WNV infection.

To further investigate the role of SM in WNV infection, we reproduced the SM accumulation induced by the lack of ASM activity by adding exogenous SM to cell cultures (23, 29). The quantification of SM content confirmed that treatment with SM was enough to increase cellular SM levels. In these experiments, we noticed that the addition of SM increased WNV multiplication. Thus, we propose that SM positively regulates WNV infection. In fact, the results shown in the present report indicate that a higher content of cellular SM (ASMko mice, NPA fibroblasts, or addition of exogenous SM) promoted WNV infection. This effect of SM on WNV infection is also consistent with the recently reported positive effect of SM on hepatitis C virus replication (19, 54).

On the other hand, treatment with pharmacological inhibitors of SM synthesis, which would be expected to decrease cellular SM levels, resulted in an inhibition of infectious virus production. Interestingly, the extent of the inhibition of infectious particle production was higher than that of the inhibition of RNA replication, suggesting that production of infectious particles could be more sensitive to SM depletion than the replication of the viral genome. These results also support a functional role of SM on the infectivity of WNV particles, which is consistent with our previous observation of an enrichment of this lipid in the viral envelope of WNV (8).
related flaviviruses (12). Flavivirus replication complexes, including those of WNV, are developed in highly modified membranes derived from the ER (3–6), and both RNA replication and particle biogenesis are coupled in these membranous structures (4). Along this line, because both the flaviviral envelope and the replication complex are enriched in SM (8, 12), we propose that SM could positively contribute to both RNA replication and particle biogenesis during WNV infection.

In summary, we have provided evidence showing that sphingolipid metabolism, and especially SM, can modulate WNV infection both in vitro and in vivo, thus making SM a key factor in the WNV lifecycle. Because sphingolipid metabolism is currently considered as a suitable target for antiviral development (55–57), our data support the potential of modulating SM levels as an antiviral strategy to combat WNV.

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