



HOKKAIDO UNIVERSITY

Title	Autophagy inhibits viral genome replication and gene expression stages in West Nile virus infection
Author(s)	Kobayashi, Shintaro; Orba, Yasuko; Yamaguchi, Hiroki et al.
Citation	Virus Research, 191, 83-91 https://doi.org/10.1016/j.virusres.2014.07.016
Issue Date	2014-10-13
Doc URL	https://hdl.handle.net/2115/57541
Type	journal article
File Information	West Nile virus Autophagy 14.pdf



Autophagy Inhibits Viral Genome Replication and Gene Expression Stages in West Nile Virus Infection

**Shintaro Kobayashi¹, Yasuko Orba¹, Hiroki Yamaguchi¹, Kenta Takahashi^{2†}, Michihito Sasaki¹, Rie Hasebe³,
Takashi Kimura¹, Hirofumi Sawa^{1, 4*}**

¹Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan

²Department of Cancer Pathology, Hokkaido University Graduate School of Medicine, N15, W7, Kita-Ku, Sapporo 060-8638, Japan

³Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, N18, W9, Kita-ku, Sapporo 060-0818, Japan.

⁴Global Institution for Collaborative Research and Education (GI-CORE), Hokkaido University

[†]Present address: Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-Ku, Tokyo 162-8640, Japan

***To whom correspondence should be addressed:** Hirofumi Sawa, Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan. Tel: +81-11-706-5185; Fax: +81-11-706-7370; E-mail: h-sawa@czc.hokudai.ac.jp

Abstract

Autophagy is a lysosomal degradation pathway that is implicated in many viral infections. However, its role in West Nile virus (WNV) infection remains controversial. In the present study, we examined the relationship between WNV infection and autophagy in infected cells. We demonstrated that LC3-II expression, a molecular marker for autophagosomal membranes, was enhanced in WNV-infected cells 6 h post-infection. LC3-II expression was further enhanced in WNV-inoculated cells when treated with a lysosomal protease inhibitor. Meanwhile, WNV replication in cells lacking *Atg5*, an essential factor for autophagy, was increased compared with replication in wild-type cells. In addition, WNV replication was inhibited in cells lacking *Atg5* when they were transfected with an ATG5 expression plasmid. These results suggest an antiviral role for autophagy in WNV-infected cells. We also examined which viral replication stages were affected by autophagy by using a Tat-beclin 1 peptide to induce autophagy and pseudo-infectious WNV reporter virus particles (WNV-RVPs) that monitor viral genome replication and gene expression stages via GFP expression. We found that autophagy induction in HeLa cells by Tat-beclin 1 peptide 3 h after WNV inoculation inhibited viral replication, and GFP expression was significantly inhibited in wild-type cells when compared with cells lacking *Atg5*. Taken together, these results suggest that autophagy is induced by WNV infection, and that this induction inhibits WNV replication at the viral genome replication and gene expression stages.

Keywords

Autophagy; Flaviviruses; Host defense; Viral replication

1. Introduction

West Nile virus (WNV) is a single-stranded, positive-sense RNA virus in the *Flaviviridae* family that causes West Nile encephalitis and death in humans and horses (Kobayashi et al., 2012; Suthar et al., 2013). The viral genome encodes a single polyprotein that is processed by host and viral proteases into three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Samuel and Diamond, 2006). To initiate the WNV replication life cycle, WNV attaches to host cell receptor(s). Thereafter, WNV is endocytosed, fused with the endosomal membrane, and the viral genome is delivered into the cytoplasm (Makino et al., 2014; Medigeschi et al., 2008). Viral RNA and protein synthesis occurs in association with endoplasmic reticulum-associated membranes (Fernandez-Garcia et al., 2009). In flavivirus infection, these stages take place as early as 3 h post-infection (hpi) (Lindnbach et al., 2007). Synthesized structural proteins and viral RNA are assembled and transported through the host secretory pathway, and infectious virions are released from the infected cell by exocytosis (Samuel and Diamond, 2006). WNV replication appears dependent on the availability of host factors to interact with viral proteins, but the interaction between host factors and viral proteins is not fully understood.

Macroautophagy (herein referred to as autophagy) is a highly conserved, intracellular degradation system (Dong and Levine, 2013). Cytoplasmic components are sequestered by autophagosomes and degraded upon fusion with lysosomes to maintain cellular homeostasis (Morishita et al., 2013). Genetic studies in yeast have identified a set of autophagy-related genes (*Atg*) genes that are required for autophagy (Mizushima et al., 2011). For instance, *Atg5* and *Atg12* are required for autophagosome formation (Mizushima et al., 1998; Mizushima et al., 2001). The presence of ATG5 and its proper conjugation with ATG12 are essential for elongation of the autophagic isolation membrane (Mizushima et al., 1998; Mizushima et al., 2001). In mammalian cells, autophagy is involved in starvation, quality control of intracellular proteins and organelles, and elimination of intracellular microbes including viruses (Dong and Levine, 2013; Shoji-Kawata and Levine, 2009).

The autophagic pathway can play an antiviral role by restricting the replication of some viruses, such as vesicular stomatitis virus and Chikungunya virus (Joubert et al., 2012; Shelly et al., 2009). Autophagic antiviral function may be summarized as the following: (1) digestion of intracytoplasmic viral components (virophagy); and (2) activation of innate and adaptive immunity by presentation of viral molecules (Dong and Levine, 2013). In contrast, some viruses such as poliovirus, dengue virus, and hepatitis C virus, have evolved mechanisms to escape host autophagy

1 and use components of the autophagic machinery for replication (Dreux et al., 2009; Heaton and Randall, 2010;
2 Jackson et al., 2005).

3 The role of autophagy in WNV infection is less clear, as conflicting results have been reported (Beatman et al.,
4 2012; Shoji-Kawata et al., 2013; Vandergaast and Fredericksen, 2012). No difference in WNV replication in mouse
5 embryonic fibroblasts (MEFs) lacking *Atg5* has been observed at a multiplicity of infection (MOI) of 3 or 0.1
6 (Beatman et al., 2012; Vandergaast and Fredericksen, 2012). However, the autophagy-inducing peptide, Tat-beclin 1,
7 decreases WNV replication and reduces mortality in mice infected with WNV (Shoji-Kawata et al., 2013).

8 In the present study, we examined LC3-II expression, a marker for autophagosomal membranes, in
9 WNV-infected cells. The effects of autophagy on WNV replication in *Atg5*^{-/-} MEFs were evaluated to better
10 understand the role of autophagy in WNV infection. Furthermore, we examined the WNV viral replication stages
11 that are affected by autophagy using Tat-beclin1 and pseudo-infectious WNV reporter virus particles (WNV-RVPs)
12 able to infect susceptible cells without producing progeny virions (Pierson et al., 2006), to address how autophagy is
13 involved in WNV replication cycle.

2. Materials and Methods

2.1 Cells and viruses

Human neuroblastoma cells (SK-N-SH cells) and Vero E6 cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma, St. Louis, MO). HEK-293T cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS. *Atg5^{+/+}* and *Atg5^{-/-}* MEFs (Kuma et al., 2004) were provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT, Japan. HeLa cells were obtained from the Health Science Research Resource Bank (Osaka, Japan). *Atg5^{+/+}* MEFs, *Atg5^{-/-}* MEFs, and HeLa cells were grown in DMEM supplemented with 10% FBS, penicillin, streptomycin, and 2 mM L-glutamine. The WNV 6-LP strain, previously established by plaque purification of the WNV NY99-6922 strain isolated from mosquitoes in 1999 (Shirato et al., 2004a; Shirato et al., 2004b), was provided by Dr. I. Takashima (Hokkaido University, Japan). Indicated cell line cultures were infected with WNV at specified MOI. After 1 h at 37 °C, the inoculum was replaced with culture medium and cells were incubated at 37 °C. Cells and culture supernatants were harvested at indicated time points. All experiments with WNV were performed at the Biosafety Level-3 facility at Hokkaido University in accordance with institutional guidelines. *Atg5^{+/+}* and *Atg5^{-/-}* MEFs were infected with Herpes Simplex virus type-1 (F strain) at MOI = 0.01. After 1 h at 37 °C, the inoculum was replaced with culture medium and the cells were incubated at 37 °C. Cells and culture supernatants were harvested at the indicated time points.

2.2 Antibodies, plasmids, and reagents

Mouse anti-LC3, rabbit anti-LC3, and rabbit anti-ATG5 polyclonal antibodies were purchased from MBL (Nagoya, Japan). A rabbit anti-LC3 monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). The anti-JEV serum that crossreacted with the structural proteins of WNV was prepared from rabbits immunized by 2 intravenous inoculations of JEV (JaGAr-01 strain) (2×10^9 plaque-forming units / rabbit) (Kimura et al., 1994; Kobayashi et al., 2012). The mouse anti-actin monoclonal antibody was purchased from Millipore (Billerica, MA). For the expression of murine ATG5 in *Atg5^{-/-}* MEFs, pCI-neo-mApg5 (plasmid 22956), and pCI-neo-mApg5 (K130R) (plasmid 22957) were obtained from Addgene (Cambridge, MA) (Mizushima et al., 2001). To make WNV-RVP, WNV structural genes were cloned into pCXS_N, which was generated from

pCMV-myc (Clontech, Mountain View, CA) by replacing the sequence of the myc tag and multicloning site with restriction enzyme sites *XhoI*, *SalI*, and *NotI* (Kobayashi et al., 2013). The resultant plasmid was designated pCXS-N-CME (Hasebe et al., 2010). The WNV replicon cDNA construct, pWNVrep-GFP, was generously provided by Dr. Robert W. Doms (Pierson et al., 2006). E64d and pepstatin A, the lysosomal protease inhibitor, were purchased from the Peptide Institute (Osaka, Japan). The Tat-beclin1 peptide (YGRKKRRQRRRGGTNVFNATFEIWHDGEFGT) and the Tat-scrambled peptide (YGRKKRRQRRRGVGNDFFINHETTGFATEW) were synthesized and purified to 95% by Sigma as described previously (Shoji-Kawata et al., 2013). Peptides were dissolved in phosphate buffered saline (PBS) (20 mM) and stored at -80 °C. For peptide treatment, cells were washed with PBS and treated with peptides (10 μM) dissolved in OPTI-MEM (Life Technologies, Rockville, MD) acidified with 0.15% (v/v) 6N HCl.

2.3 Immunocytochemistry

To observe viral antigen positive cells, SK-N-SH cells were seeded at 200,000 cells per well in a 24-well dish, in which each well contained a 12-mm cover glass, and the cells were then WNV inoculated (MOI = 0.01) for 6 h. The WNV infected and mock-infected cells were fixed with 4% paraformaldehyde for 10 min, before washing with PBS. The cells were permeabilized in 100 μg / ml digitonin for 15 min, blocked with 1% bovine serum albumin (BSA)-PBS, and then stained with mouse anti-LC3 monoclonal antibody and anti-JEV rabbit serum in 1% BSA-PBS for 3 h at room temperature. The immune complexes were visualized by incubating with either Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies). The cells were then observed using a Zeiss 780 LSM confocal microscope (Jena, Germany). Immunopositive foci were confirmed by observing each cover glass. The number of punctate structures of LC3 per cell was counted from different microscopic fields.

2.4 Immunoblotting

For immunoblotting, cells were harvested at indicated time points after WNV infection and transfection, lysed in TNE buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100] supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Cell lysates were fractionated by SDS-PAGE, and separated proteins were transferred to a PVDF filter (Millipore). The filter was incubated with each antibody, and the immune complexes

1 were detected with HRP-conjugated secondary antibodies (Biosource International, Camarillo, CA) and
2 Immobilon Western HRP Substrate (Millipore). The chemiluminescence signals were visualized with the
3 VersaDoc 5000MP (Bio-Rad, Hercules, CA), and obtained images were analyzed using Quantity One
4 software (Bio-Rad).

6 **2.5 RT-PCR**

7 For RNA extraction, cells were collected 3 h after WNV infection. Total RNA was isolated using Trizol (Life
8 Technologies) and a PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's protocol. RT-PCR
9 was performed with a SuperScript III One-Step RT-PCR with Platinum Taq kit (Life Technologies). For detection of
10 WNV RNA, primer pairs for WNV were 5'-AAGTTGAGTAGACGGTGCTG-3' and
11 5'-AGACGGTTCTGAGGGCTTAC-3'. Primers corresponding to glyceraldehyde-3-phosphate dehydrogenase
12 (GAPDH) were used as a control (5'-TTCGTCATGGGTGTGAACCA-3' and
13 5'-GGTCATGAGTCCTTCCACGATAC-3').

15 **2.6 Plaque assay**

16 Diluted culture supernatants from infected cells were inoculated into monolayers of Vero E6 cells. After 1 h of
17 incubation at 37 °C with rocking, the inoculums were removed, and overlay medium (MEM containing 5%
18 FBS and 1.25% methyl cellulose) was added and incubated for 3 days. Plaques were visualized with a 1%
19 crystal violet solution in 70% ethanol.

21 **2.7 Transfection of wild type and mutant ATG5**

22 Transfection of the control, wild-type ATG5 expression plasmid, and the mutant ATG5 expression plasmid into
23 *Atg5*^{+/+} or *Atg5*^{-/-} MEFs was performed using TransIT 2020 (Mirus, Madison, WI) according to the
24 manufacturer's instructions. For immunoblotting, cells were harvested at 24 h post-transfection (hpt).
25 Transfected *Atg5*^{-/-} MEFs were infected with WNV (MOI = 0.01) at 24 hpt, and culture supernatants were
26 harvested at 48 hpi and relative viral titers were measured by plaque assay.

28 **2.8 WNV-RVPs preparation**

29 HEK-293T cells were cultured in 100-mm dishes and were transfected with 6 µg pWNIIrep-GFP and 18 µg

pCXS-N-CME using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies). After 48 h, supernatants were collected and cell debris was removed with 0.45 µm pore size filters. Supernatants containing WNV-RVPs were purified with a sucrose gradient [8 ml 10% sucrose in 50 mM Tris-HCl (pH 8.0), 8 ml 25% sucrose, and 35.7% Meglumine Iotalamate (Daiichi-Sankyo, Tokyo, Japan) in 50 mM Tris-HCl (pH 8.0)] by ultracentrifugation in a Beckman SW32Ti rotor at 4 °C for 2.5 h at 76,800 g. The samples were fractionated from the bottom into 14 fractions of 1.5 ml each. HA titers for fractions 5 to 10 were determined and high HA titer fractions were stored at -80 °C. Each fraction was subjected to a serial 2-fold dilution in PBS containing 0.2% BSA (pH 8.0) and mixed with pigeon red blood cells in 0.2 M PBS. The HA assay was performed at pH 6.5 and incubated for 1 h at 37 °C, followed by microscopic observation.

2.9 Infection of *Atg5*^{+/+} and *Atg5*^{-/-} MEFs with WNV-RVPs

Atg5^{+/+} and *Atg5*^{-/-} MEFs were inoculated with WNV-RVPs (2⁸ HA/200 µl) for 24 h. GFP signals were observed with an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) and images were processed using DP Manager software (Olympus). Cells were washed twice with PBS and were dissociated with 0.25% trypsin. WNV-RVP-infected cells were counted by measuring GFP signals with a FACS Canto system (BD Biosciences, San Diego, CA).

2.10 Quantitative RT-PCR

For RNA extraction, cells were collected at indicated time points after WNV infection. Total RNA was extracted using Trizol (Life Technologies) and a PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's protocol. Quantitative RT-PCR was performed with Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent Technologies, Palo Alto, CA) with the CFX96 real-time PCR detection system (BioRad). The WNV primer and probe sequences used were as follows: WNV forward, 5'-AAGTTGAGTAGACGGTGCTG-3'; WNV reverse, 5'-AGACGGTTCTGAGGGCTTAC-3'; and WNV probe, FAM-5'-GCTCAACCCCAGGAGGACTGG-3'-BHQ. Endogenous control primers and probes for mouse actin were purchased from Applied Biosystems (Foster City, CA).

3. Results

3.1 Autophagy is induced by WNV infection

To determine whether WNV infection regulates autophagy, we examined the lipidated, autophagosome-associated form of LC3 (LC3-II). When the autophagy occurs, LC3-I is conjugated with phosphatidylethanolamine (PE), converting into the autophagic form, LC3-II (Mizushima et al., 2011). During autophagy, LC3-II is recruited to autophagosomal membranes and is monitored by visualizing punctate structures in the cytoplasm (Klionsky et al., 2012). SK-N-SH cells were infected WNV (MOI = 0.01) for 6 h, and punctate structures of LC3 were analyzed by confocal microscopy. The punctate structures of LC3 were increased in WNV antigen positive cells compared to mock-infected cells or viral antigen negative cells (Fig. 1a and 1b), suggesting that WNV infection induces autophagosome formation. We also examined the levels of LC3-II in SK-N-SH cells infected with WNV (MOI = 0.01) by immunoblotting. At 6 hpi, LC3-II levels in WNV-infected SK-N-SH cells were enhanced compared with those seen in mock-infected cells (Fig. 1c, lanes 5 and 6), whereas at 1 and 3 hpi the LC3-II levels were similar in the mock-infected and the WNV-infected cells (Fig. 1c, lanes 1 to 4). Because LC3-II is subject to lysosomal degradation during autophagy, it was unclear whether increased LC3-II levels indicated that WNV infection enhanced autophagy flux or inhibits lysosomal degradation. To precisely interpret the increased expression of LC3-II by WNV infection, we measured LC3-II levels in both mock-infected and WNV-infected SK-N-SH cells treated with E64d and pepstatin A, which inhibit lysosomal proteases and interfere with autolysosomal digestion. In this assay, increasing LC3-II expression with inhibitors indicated that WNV enhanced autophagy flux, whereas no change of LC3-II expression levels in the presence of inhibitors indicated inhibition of autophagosomal degradation by WNV infection. LC3-II levels were increased in inhibitor-treated SK-N-SH cells (Fig. 1d, lanes 2 and 4). At 6 hpi, increased LC3-II levels were observed in WNV-infected cells compared with those of mock-infected cells (Fig. 1d, lanes 1 and 3) and in inhibitor-treated WNV-infected cells compared with non-treated WNV-infected cells (Fig. 1d, lanes 3 and 4). WNV infection was confirmed by RT-PCR by amplifying the WNV genome (Fig. 1d). Densitometry analysis of the LC3-II band, corrected for actin, also showed that LC3-II levels were significantly increased in WNV-infected cells compared with mock-infected cells and in inhibitor-treated WNV-infected cells compared with inhibitor-treated mock-infected cells (Fig. 1e). In addition, inhibitor-treated WNV infected cells had significantly higher levels of LC3-II compared with non-treated WNV-infected cells (Fig. 1e). Together, these results suggest that autophagy was enhanced in WNV-infected cells.

3.2 Autophagy inhibits WNV replication

ATG5 conjugates to the ubiquitin-like molecule ATG12, and *Atg5* deficient cells are unable to undergo autophagy (Mizushima et al., 2001). *Atg5*-deficient cells are frequently used to analyze the relationship between viral infection and autophagy (Alexander et al., 2007; Gannagé et al., 2009; Orvedahl et al., 2007; Orvedahl et al., 2010; Zhao et al., 2007). To examine the effect of autophagy on WNV replication, we used *Atg5*^{+/+} and *Atg5*^{-/-} MEFs (Kuma et al., 2004). Cells were infected with WNV and analyzed for expression levels of LC3-II and viral production. There was no significant difference in viral titers of the culture supernatants between *Atg5*^{+/+} and *Atg5*^{-/-} MEFs at 12 and 24 hpi when a high infectious dose was used (MOI = 1) (Supplemental Fig. S1). At a low infectious dose (MOI = 0.01), however, the viral titer was significantly higher in *Atg5*^{-/-} MEFs when compared with *Atg5*^{+/+} MEFs at 24 and 48 hpi (Fig. 2a). There was no difference in viral titer at 72 hpi. These results indicate that autophagy inhibits WNV replication when low amounts of WNV are used to inoculate MEFs. To address the specificity of autophagy's effect upon viral replication we also infected cells with HSV-1 at MOI = 0.01 and analyzed viral production. HSV-1 has previously been shown not to increase the viral infectivity of *Atg5*^{-/-} MEFs (Alexander et al., 2007; Dong and Levine, 2013). Our results were consistent with the previous report (Alexander et al., 2007): the viral titer of the culture supernatants in *Atg5*^{-/-} MEFs was comparable to that of *Atg5*^{+/+} MEFs at 24 and 48 hpi (Supplemental Fig. S2). To further confirm the specificity of the relationship between autophagy and WNV replication, we complemented *Atg5*^{-/-} MEFs with wild type ATG5 (WT) or mutant ATG5 (K130R), a protein that is incapable of conjugating with ATG12 and is unable to form autophagosomes (Mizushima et al., 2001). These complemented *Atg5*^{-/-} MEFs were infected with WNV at MOI = 0.01. In MEFs expressing WT, conjugation of ATG5 to ATG12 and LC3 conversion (LC3-I to LC3-II) were observed (Fig. 2b, lane 3), although conjugation and conversion were weaker than observed for *Atg5*^{+/+} MEFs (Fig. 2b, lanes 1 and 3). The K130R mutant did not conjugate with ATG12 and was incapable of LC3 conversion, while expression of ATG5 was observed (Fig. 2b, lane 4). At 48 hpi, the viral titer in the culture supernatants was significantly lower in the *Atg5*^{-/-} MEFs transiently expressing WT ATG5 compared with the viral titer in MEFs transfected with a control plasmid (Fig. 2c). In contrast, the viral titer in the culture supernatants of MEFs transiently expressing the ATG5 K130R mutant was similar to that of control MEFs (Fig. 2c). Together, these results suggest that autophagy plays an antiviral role in WNV replication.

3.3 Autophagy inhibits WNV genome replication and gene expression

We next examined which stages in the replication cycle of WNV are affected by autophagy. Because Tat-beclin 1, an autophagy-inducing peptide, is known to inhibit WNV replication (Shoji-Kawata et al., 2013), we used this peptide to induce autophagy. We also used a Tat-scrambled peptide as a control. We initially examined the kinetics of autophagy induction by Tat-beclin 1 peptide in HeLa cells (Fig. 3a), as well as the recovery times after removing the peptides by washing with PBS (Fig. 3b). LC3-II expression was increased 1 h after Tat-beclin 1 peptide treatment (Fig. 3a). These results indicate that 1 h incubation with the Tat-beclin1 peptide was sufficient to induce autophagy in HeLa cells. We next examined LC3-II expression levels 1 and 3 h after the removal of the peptides by washing the HeLa cells with PBS (Fig. 3b). At 1 h after washing with PBS, LC3-II expression was not so different between in Tat-beclin 1 peptide-treated cells, compared with that in Tat-scrambled peptide-treated cells (Fig. 3b). By 3 h after washing with PBS, the LC3-II expression levels were similar in Tat-beclin 1 peptide-treated cells and the Tat-scrambled-treated cells (Fig. 3b). These results indicate that the induction of autophagy through Tat-beclin 1 peptide treatment of HeLa cells had recovered by 3 h after the removal of the peptide. To examine whether autophagy affects WNV during the early or late stages of infection, HeLa cells were treated with Tat-scrambled or Tat-beclin 1 peptide at pre- (Fig. 3c) or post-infection (Fig. 3d) for 3 h, the viral titers of the culture supernatants were quantified using a plaque assay (Fig. 3c and 3d). To remove the peptides and the viruses which were not attached to HeLa cells, the cells were washed using PBS at 1 hpi and 3 h after the peptide incubation. Because it is difficult to measure viral titer in the supernatants of WNV-infected HeLa cells at low MOI (≤ 0.1) using a plaque assay, we applied WNV at a MOI of 1 in this study. Tat-beclin 1 peptide treatment prior to WNV infection did not change the viral titer (Fig. 3c). In contrast, Tat-beclin 1 peptide treatment resulted in a significant reduction in the viral titer when the peptide was added at 3 hpi (Fig. 3d). These results suggested that autophagy inhibits the WNV infection at 3–6 hpi, and not at the entry stage.

WNV-RVPs were used to examine whether autophagy affected WNV genome replication, gene expression, or later stages. WNV-RVPs have replicon RNA that can replicate in the cytoplasm and express the WNV nonstructural proteins, and GFP that is expressed instead of structural proteins (Pierson et al., 2006). Because of lack of structural proteins in the replicon RNA, WNV-RVPs are unable to produce progeny particles. However, genome replication and gene expression stages may be monitored via GFP expression. After *Atg5^{+/+}* and *Atg5^{-/-}* MEFs were inoculated with WNV-RVPs, GFP expression was analyzed by fluorescent microscopy and quantified by flow cytometry. A large number of GFP-positive cells were observed in *Atg5^{-/-}* MEFs, whereas few GFP-positive cells were observed in *Atg5^{+/+}* MEFs (Fig. 4a). In addition, the ratio of GFP-positive cells in *Atg5^{-/-}* MEFs was significantly higher than that

1 in *Atg5^{+/-}* MEFs (Fig. 4b). These results suggest that autophagy inhibits WNV genome replication and gene
2 expression stages prior to viral assembly.

3 To confirm that autophagy affected WNV genome replication and gene expression, we measured the amount of
4 viral genome present in *Atg5^{+/-}* MEFs and *Atg5^{-/-}* MEFs infected with WNV at 3 or 6 hpi. The rate of genome
5 replication at 3 and 6 hpi was measured using quantitative RT-PCR. We found that the relative amount of WNV
6 genome in *Atg5^{-/-}* MEFs was significantly higher than that in *Atg5^{+/-}* MEFs (Fig. 4c). This result provides convincing
7 evidence that WNV genome replication is suppressed by autophagy.

4. Discussion

In the present study, we demonstrate that autophagy is induced in WNV-infected cells and WNV replication is inhibited at the genome replication and gene expression stages. Our results provide evidence for a protective role of autophagy in antiviral host defense against WNV.

It has previously been reported that autophagy deficiency does not significantly increase WNV viral titers (Beatman et al., 2012; Vandergaast and Fredericksen, 2012). However, the amount of inoculated WNV used in these studies was relatively higher (MOI = 3 or 0.1) than in our experiment (MOI = 0.01) (Beatman et al., 2012; Vandergaast and Fredericksen, 2012). Similarly, higher amounts of WNV (MOI = 1) resulted in little difference between the viral titers in the supernatants from *Atg5^{+/+}* MEFs and *Atg5^{-/-}* MEFs (Supplemental Fig. S1), whereas a lower MOI (MOI = 0.01) resulted in a significantly higher WNV titer in *Atg5^{-/-}* MEFs supernatants when compared with WNV-infected *Atg5^{+/+}* MEF supernatants (Fig. 2b). These data suggest that the inhibitory effect of autophagy on WNV replication depends on a balance between the efficacy of autophagy induction in WNV infected cells and the amount of WNV infected. In the case of a high MOI of inoculated WNV, the replication rate of WNV exceeds the inhibitory effect of the autophagy induced by the WNV infection; WNV therefore seems to be independent on autophagy for viral replication. Moreover, our result that the induction of autophagy using a Tat-beclin1 peptide inhibited viral replication in HeLa cells (Fig. 3d) is consistent with a previous report that this autophagy-inducing peptide reduces mortality of neonatal mice infected with WNV (Shoji-Kawata et al., 2013). Although this study demonstrates an antiviral role for autophagy in *in vitro* WNV infection, we hypothesize that autophagy may potentiate prevention of WNV replication *in vivo*.

There was no difference in supernatant viral titers between *Atg5^{+/+}* MEFs and *Atg5^{-/-}* MEFs at 72 hpi, although a significant difference was observed at 24 and 48 hpi (Fig. 2b). In addition, cytopathic effects (CPE) induced by WNV infection were observed in *Atg5^{-/-}* MEFs earlier than *Atg5^{+/+}* MEFs (data not shown). It is possible that CPE caused by WNV in *Atg5^{-/-}* MEFs at 72 hpi affect the viral titer in culture supernatants taken from these cells.

It is unknown whether the antiviral effects of autophagy are restricted to the capture of newly synthesized viral proteins and viral genomes of WNV or whether WNV can also be targeted to autophagosomes during cellular entry. Our results showed that WNV infection increases LC3-II expression at 6 hpi, whereas an increase in LC3-II expression was not observed at 1 hpi (Fig. 1c). Increased LC3-II expression was due to an increase in autophagy activity, but not a block in downstream factors after LC3-II turnover. These results suggest that autophagy was

1 induced at the late stage of WNV infection rather than the early stage. For some viruses, viral replication induces
2 autophagy. In Sindbis virus (SIN) infection, replication-competent SIN induces autophagy in mammalian cells, while
3 UV-inactivated SIN does not (Orvedahl et al., 2010). Thus, newly synthesized viral genomes and/or viral proteins
4 appear necessary for autophagy induction in SIN-infected cells (Orvedahl et al., 2010). A similar mechanism may
5 exist in WNV-infected cells because autophagy was not induced at 1 and 3 hpi. Instead, autophagy was induced at 6
6 hpi when WNV genome replication, gene expression, and protein synthesis occurs.

7 The mechanisms in which WNV replication is inhibited by autophagy are unknown. During viral infection,
8 autophagy stimulates the innate immune system and consequent production of Type I interferon (IFN) (Iwasaki,
9 2007; Lee et al., 2007). Type I IFN inhibits WNV genome replication and gene expression stages (Szretter et al.,
10 2011). To elucidate these inhibitory mechanisms, studies clarifying the relationship between autophagy and innate
11 immunity in WNV infection are in progress.

5. Conclusion

In conclusion, this study provides important information on the significance of autophagy in WNV replication in mammalian cells. Although further investigations are required to elucidate the mechanisms underlying autophagic inhibition of WNV replication, we conclude that induction of the autophagosomal pathway has potential for controlling WNV infection.

6. Acknowledgements

We thank Dr. Takashima for providing the WNV NY99 6-LP strain; Dr. Mizushima for the *Atg5*^{+/+} and *Atg5*^{-/-} MEFs (obtained through RIKEN) and for the pCI-neo-mApg5 and pCI-neo-mApg5 (K130R) plasmids (obtained through Addgene); Dr. Doms for the WNV replicon cDNA construct pWNIrep-GFP.

This study was supported in part by JSPS KAKENHI Grant Number 24880002 and in part by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT); the Ministry of Health, Labour and Welfare of Japan; and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), as well as by the Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan, for Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University.

7. References

- Alexander, D.E., Ward, S.L., Mizushima, N., Levine, B., Leib, D.A., 2007. Analysis of the role of autophagy in replication of herpes simplex virus in cell culture. *J Virol* 81(22), 12128-12134.
- Beatman, E., Oyer, R., Shives, K.D., Hedman, K., Brault, A.C., Tyler, K.L., Beckham, J.D., 2012. West Nile virus growth is independent of autophagy activation. *Virology* 433(1), 262-272.
- Dong, X., Levine, B., 2013. Autophagy and viruses: adversaries or allies? *J Innate Immun* 5(5), 480-493.
- Dreux, M., Gastaminza, P., Wieland, S.F., Chisari, F.V., 2009. The autophagy machinery is required to initiate hepatitis C virus replication. *Proc Natl Acad Sci U S A* 106(33), 14046-14051.
- Fernandez-Garcia, M.D., Mazzon, M., Jacobs, M., Amara, A., 2009. Pathogenesis of flavivirus infections: using and abusing the host cell. *Cell Host Microbe* 5(4), 318-328.
- Gannagé, M., Dormann, D., Albrecht, R., Dengjel, J., Torossi, T., Rämer, P.C., Lee, M., Strowig, T., Arrey, F., Conenello, G., Pypaert, M., Andersen, J., García-Sastre, A., Münz, C., 2009. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. *Cell Host Microbe* 6(4), 367-380.
- Hasebe, R., Suzuki, T., Makino, Y., Igarashi, M., Yamanouchi, S., Maeda, A., Horiuchi, M., Sawa, H., Kimura, T., 2010. Transcellular transport of West Nile virus-like particles across human endothelial cells depends on residues 156 and 159 of envelope protein. *BMC Microbiol* 10, 165.
- Heaton, N.S., Randall, G., 2010. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe* 8(5), 422-432.
- Iwasaki, A., 2007. Role of autophagy in innate viral recognition. *Autophagy* 3(4), 354-356.
- Jackson, W.T., Giddings, T.H., Taylor, M.P., Mulinyawe, S., Rabinovitch, M., Kopito, R.R., Kirkegaard, K., 2005. Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol* 3(5), e156.
- Joubert, P.E., Werneke, S.W., de la Calle, C., Guivel-Benhassine, F., Giodini, A., Peduto, L., Levine, B., Schwartz, O., Lenschow, D.J., Albert, M.L., 2012. Chikungunya virus-induced autophagy delays caspase-dependent cell death. *J Exp Med* 209(5), 1029-1047.
- Kimura, T., Kimura-Kuroda, J., Nagashima, K., Yasui, K., 1994. Analysis of virus-cell binding characteristics on the determination of Japanese encephalitis virus susceptibility. *Arch Virol* 139(3-4), 239-251.
- Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., Ahn, H.J., Ait-Mohamed, O., Ait-Si-Ali, S., Akematsu, T., Akira, S., Al-Younes, H.M., Al-Zeer, M.A., Albert, M.L., Albin, R.L., Alegre-Abarategui, J., Aleo, M.F., Alirezaei, M., Almasan, A., Almonte-Becerril, M., Amano, A., Amaravadi, R., Amarnath, S., Amer, A.O., Andrieu-Abadie, N., Anantharam, V., Ann, D.K., Anoopkumar-Dukie, S., Aoki, H., Apostolova, N., Arancia, G., Aris, J.P., Asanuma, K., Asare, N.Y., Ashida, H., Askanas, V., Askew, D.S., Auberger, P., Baba, M., Backues, S.K., Baehrecke, E.H., Bahr, B.A., Bai, X.Y., Bailly, Y., Baiocchi, R., Baldini, G., Balduini, W., Ballabio, A., Bamber, B.A., Bampton, E.T., Bánhegyi, G., Bartholomew, C.R., Bassham, D.C., Bast, R.C.,

1 Batoko, H., Bay, B.H., Beau, I., Béchet, D.M., Begley, T.J., Behl, C., Behrends, C., Bekri, S., Bellaire, B.,
 2 Bendall, L.J., Benetti, L., Berliocchi, L., Bernardi, H., Bernassola, F., Besteiro, S., Bhatia-Kissova, I., Bi, X.,
 3 Biard-Piechaczyk, M., Blum, J.S., Boise, L.H., Bonaldo, P., Boone, D.L., Bornhauser, B.C., Bortoluci, K.R.,
 4 Bossis, I., Bost, F., Bourquin, J.P., Boya, P., Boyer-Guittaut, M., Bozhkov, P.V., Brady, N.R., Brancolini, C.,
 5 Brech, A., Brenman, J.E., Brennand, A., Bresnick, E.H., Brest, P., Bridges, D., Bristol, M.L., Brookes, P.S.,
 6 Brown, E.J., Brumell, J.H., et al., 2012. Guidelines for the use and interpretation of assays for monitoring
 7 autophagy. *Autophagy* 8(4), 445-544.
 8 Kobayashi, S., Orba, Y., Yamaguchi, H., Kimura, T., Sawa, H., 2012. Accumulation of ubiquitinated proteins is
 9 related to West Nile virus-induced neuronal apoptosis. *Neuropathology* 32(4), 398-405.
 10 Kobayashi, S., Suzuki, T., Igarashi, M., Orba, Y., Ohtake, N., Nagakawa, K., Niikura, K., Kimura, T., Kasamatsu, H.,
 11 Sawa, H., 2013. Cysteine Residues in the Major Capsid Protein, Vp1, of the JC Virus Are Important for
 12 Protein Stability and Oligomer Formation. *PLoS One* 8(10), e76668.
 13 Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhiisa, T., Mizushima,
 14 N., 2004. The role of autophagy during the early neonatal starvation period. *Nature* 432(7020), 1032-1036.
 15 Lee, H.K., Lund, J.M., Ramanathan, B., Mizushima, N., Iwasaki, A., 2007. Autophagy-dependent viral recognition
 16 by plasmacytoid dendritic cells. *Science* 315(5817), 1398-1401.
 17 Lindnbach, B.D., Thiel, H.J., Rice, C.M., 2007. *Flaviviridae: the viruses and their replication*. in *Fields Virology, 5 th*
 18 *Edition* (Knipe, D. M., and Howley, P. M. eds.). Lippincott Williams & Wilkins, Philadelphia. pp 1101-1152,
 19 pp. 1101-1152.
 20 Makino, Y., Suzuki, T., Hasebe, R., Kimura, T., Maeda, A., Takahashi, H., Sawa, H., 2014. Establishment of tracking
 21 system for West Nile virus entry and evidence of microtubule involvement in particle transport. *J Virol*
 22 *Methods* 195, 250-257.
 23 Medigeshi, G.R., Hirsch, A.J., Streblow, D.N., Nikolich-Zugich, J., Nelson, J.A., 2008. West Nile virus entry requires
 24 cholesterol-rich membrane microdomains and is independent of alphavbeta3 integrin. *J Virol* 82(11),
 25 5212-5219.
 26 Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., Ohsumi, Y.,
 27 1998. A protein conjugation system essential for autophagy. *Nature* 395(6700), 395-398.
 28 Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhiisa, T., Ohsumi, Y.,
 29 Yoshimori, T., 2001. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem
 30 cells. *J Cell Biol* 152(4), 657-668.
 31 Mizushima, N., Yoshimori, T., Ohsumi, Y., 2011. The role of Atg proteins in autophagosome formation. *Annu Rev*
 32 *Cell Dev Biol* 27, 107-132.
 33 Morishita, H., Eguchi, S., Kimura, H., Sasaki, J., Sakamaki, Y., Robinson, M.L., Sasaki, T., Mizushima, N., 2013.
 34 Deletion of autophagy-related 5 (Atg5) and Pik3c3 genes in the lens causes cataract independent of
 35 programmed organelle degradation. *J Biol Chem* 288(16), 11436-11447.
 36 Orvedahl, A., Alexander, D., Tallóczy, Z., Sun, Q., Wei, Y., Zhang, W., Burns, D., Leib, D.A., Levine, B., 2007.

1 HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 1(1),
2 23-35.

3 Orvedahl, A., MacPherson, S., Sumpter, R., Tallóczy, Z., Zou, Z., Levine, B., 2010. Autophagy protects against
4 Sindbis virus infection of the central nervous system. *Cell Host Microbe* 7(2), 115-127.

5 Pierson, T.C., Sánchez, M.D., Puffer, B.A., Ahmed, A.A., Geiss, B.J., Valentine, L.E., Altamura, L.A., Diamond,
6 M.S., Doms, R.W., 2006. A rapid and quantitative assay for measuring antibody-mediated neutralization of
7 West Nile virus infection. *Virology* 346(1), 53-65.

8 Samuel, M.A., Diamond, M.S., 2006. Pathogenesis of West Nile Virus infection: a balance between virulence, innate
9 and adaptive immunity, and viral evasion. *J Virol* 80(19), 9349-9360.

10 Shelly, S., Lukinova, N., Bambina, S., Berman, A., Cherry, S., 2009. Autophagy is an essential component of
11 *Drosophila* immunity against vesicular stomatitis virus. *Immunity* 30(4), 588-598.

12 Shirato, K., Kimura, T., Mizutani, T., Kariwa, H., Takashima, I., 2004a. Different chemokine expression in lethal and
13 non-lethal murine West Nile virus infection. *J Med Virol* 74(3), 507-513.

14 Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueki, T., Kariwa, H., Takashima, I., 2004b. Viral envelope protein
15 glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus.
16 *J Gen Virol* 85(Pt 12), 3637-3645.

17 Shoji-Kawata, S., Levine, B., 2009. Autophagy, antiviral immunity, and viral countermeasures. *Biochim Biophys*
18 *Acta* 1793(9), 1478-1484.

19 Shoji-Kawata, S., Sumpter, R., Leveno, M., Campbell, G.R., Zou, Z., Kinch, L., Wilkins, A.D., Sun, Q., Pallauf, K.,
20 MacDuff, D., Huerta, C., Virgin, H.W., Helms, J.B., Eerland, R., Tooze, S.A., Xavier, R., Lenschow, D.J.,
21 Yamamoto, A., King, D., Lichtarge, O., Grishin, N.V., Spector, S.A., Kaloyanova, D.V., Levine, B., 2013.
22 Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* 494(7436), 201-206.

23 Suthar, M.S., Diamond, M.S., Gale, M., 2013. West Nile virus infection and immunity. *Nat Rev Microbiol* 11(2),
24 115-128.

25 Szretter, K.J., Brien, J.D., Thackray, L.B., Virgin, H.W., Cresswell, P., Diamond, M.S., 2011. The interferon-inducible
26 gene viperin restricts West Nile virus pathogenesis. *J Virol* 85(22), 11557-11566.

27 Vandergaast, R., Fredericksen, B.L., 2012. West Nile virus (WNV) replication is independent of autophagy in
28 mammalian cells. *PLoS One* 7(9), e45800.

29 Zhao, Z., Thackray, L.B., Miller, B.C., Lynn, T.M., Becker, M.M., Ward, E., Mizushima, N., Denison, M.R., Virgin
30 Iv, H.W., 2007. Coronavirus replication does not require the autophagy gene ATG5. *Autophagy* 3(6),
31 581-585.

Figure Legends

Fig. 1. WNV infection induces autophagy in SK-N-SH cells. (a) SK-N-SH cells were mock-infected (Mock) or infected with WNV (MOI = 0.01). Cells were harvested at 6 hpi and double stained with LC3 (*green*) and WNV antigen (*red*). Cell nuclei were counterstained with DAPI (*blue*). Scale bars: 10 μ m. (b) The number of punctate structures of LC3 per cell was depicted. Error bars represent standard deviation (SD) from mean. Statistical significance was assessed using the Student's *t*-test, and is indicated by asterisks (** p <0.01). (c) SK-N-SH cells were mock-infected (-) or infected with WNV (MOI = 0.01). Cells were harvested at indicated time points and analyzed by immunoblotting for LC3 and actin. (d) SK-N-SH cells were mock-infected or infected with WNV (MOI = 0.01) in the presence of a lysosomal protease inhibitors E64d (2 μ M), and pepstatin A (2 μ M), or DMSO as the solvent control. Cells were harvested at 6 h post-infection (hpi) and analyzed by immunoblotting for LC3 and actin, or by RT-PCR to detect the WNV genome and GAPDH. (e) The results of (d) were quantified by Quantity One software. The bar graph represents relative LC3-II band densities that were normalized for actin band densities. Data represent mean values $\pm$ SD of three independent experiments. Statistical significance was assessed using the Student's *t*-test, and is indicated by asterisks (* p <0.05 and ** p <0.01).

Fig. 2. Autophagy deficiency increases WNV replication. (a) *Atg5*^{+/+} and *Atg5*^{-/-} MEFs were infected with WNV (MOI = 0.01). Culture supernatants were harvested at indicated time points and viral titers determined by plaque assay. Data represent mean values $\pm$ SD of three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (* p <0.05 and ** p <0.01). (b) Cell lysates from *Atg5*^{+/+} MEFs transfected with the control plasmid and *Atg5*^{-/-} MEFs transfected with the control plasmid, the wild-type ATG5 expression plasmid (WT), or the mutant ATG5 expression plasmid (K130R) were analyzed by immunoblotting for ATG5, LC3, and actin. These cells were prepared 24 h after transfection. (c) *Atg5*^{-/-} MEFs were transfected with the control plasmid, WT, or K130R. After 24 h, transfected *Atg5*^{-/-} MEFs were infected with WNV (MOI = 0.01), culture supernatants were harvested at 48 hpi, and relative viral titers were measured by plaque assay. Data represent mean values $\pm$ SD of three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (** p < 0.01).

Fig. 3. Autophagy prevents late stage WNV infection. (a) Schematic diagram of experiment (upper panel). HeLa

cells were incubated for 1 or 3 h with Tat-scrambled (T-S, 10 μ M) or Tat-beclin 1 (T-B, 10 μ M) peptide. Cells were harvested at indicated time points and analyzed by immunoblotting for LC3 and actin (lower panel). (b) Schematic diagram of experiment (upper panel). HeLa cells were incubated for 3 h with T-S (10 μ M) or T-B (10 μ M), washed extensively with PBS, and incubated in culture medium for 1 or 3 h. Cells were harvested at indicated time points and analyzed by immunoblotting for LC3 and actin (lower panel). (c) Schematic diagram of experiment (upper panel). HeLa cells were pre-treated for 3 h with T-S (10 μ M) or T-B (10 μ M), washed extensively with PBS, and infected with WNV (MOI = 1). Culture supernatants were harvested at 12 hpi and viral titers determined by plaque assay (lower panel). (d) Schematic diagram of the experiment (upper panel). HeLa cells were infected with WNV (MOI = 1). At 3 hpi, cells were incubated for 3 h with T-S (10 μ M) or T-B (10 μ M), washed extensively with PBS, and incubated in culture medium for 6 h. Culture supernatants were harvested at 12 hpi and viral titers determined by plaque assay (lower panel). Data represent mean values $\pm$ SD of three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (***p* < 0.01).

Fig. 4. Autophagy inhibits WNV genome replication and gene expression. (a) WNV-RVPs were inoculated into *Atg5*^{+/+} or *Atg5*^{-/-} MEFs. GFP signals were detected by fluorescent microscopy at 24 hpi. (b) GFP positivity was analyzed by flow cytometry. Data represent mean values $\pm$ SD of three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (***p* < 0.01). (c) *Atg5*^{+/+} or *Atg5*^{-/-} MEFs were infected with WNV (MOI = 1) and total RNA was extracted at indicated time points from WNV-infected cells. The viral genome was quantified by qRT-PCR. The bar graph demonstrates relative amounts of viral genome normalized with beta-actin. The relative amount of viral genome at 3 h was defined as 1. Data represent mean values $\pm$ SD of three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (***p* < 0.01).